

## ABSTRACT

PAUDEL, DIPTI. Elucidating the Secretome of Lung Spheroid Cells using LC/MS/MS. (Under the direction of Dr. Michael Goshe).

Lung diseases, such as idiopathic pulmonary fibrosis (IPF), are among the top five causes of mortality in the United States. Lung spheroid cells (LSC) are tissue-specific multicellular spheroids generated from human lung biopsies using a rapid and reproducible three-stage “adhesion-suspension-adhesion” culture process and have been established as an intrinsic source of therapeutic lung stem cells. Stem cells are thought to exert their therapeutic effects through secreted paracrine factors to recruit endogenous repair mechanisms. In this study, we developed a liquid chromatography-tandem mass spectrometry (LC/MS/MS) method to identify proteins secreted in conditioned media by LSC, derived from three donors, and provided a detailed analysis of secretome differences between three donors to identify potentially therapeutic factors.

Comprehensive LSC secretory proteome analysis by LC/MS/MS using an Orbitrap Elite revealed a total of 368 secreted proteins that were common in at least two out of three donors, out of which 103 were annotated as being extracellular. A minimum of 2 peptides per protein at a peptide and protein identity threshold of 95% within Scaffold was required for high confidence identification. The majority of LSC proteins identified were classified by Ingenuity Pathway Analysis software as being cytoplasmic, a finding that is consistent with other proteomic studies conducted with mesenchymal stem cells (MSC) and embryonic stem cells (ESC). Protein functional analysis of LSC revealed that majority of the extracellular proteins are involved in extracellular matrix remodeling, angiogenesis, and stem cell proliferation. For example, matrix metallopeptidases degrade the extracellular matrix to cause lasting structural and functional alteration in muscles. As such, they might be of importance in the reduction of fibrosis following IPF manifestation. In addition, platelet derived growth factor, vascular endothelial growth factor

(VEGF), and insulin like growth factor (ILGF) were identified and have been shown to be involved in the formation of new blood vessels along with regulation of cell growth and proliferation. Many of LSC secreted factors present in the conditioned media include well-known factors linked with MSCs, further demonstrating LSC stem cell like properties. Previously, infusion of VEGFA or IL6, candidate therapeutic factors secreted by MSC, did not completely replicate the effects of MSCs, suggesting there are combination of factors necessary for full substitution for MSC therapy, and may be the case for LSC as well. Further studies to characterize the secreted factors is needed for determination of specific therapeutic factors to develop for therapeutic applications and drug development.

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Elucidating the Secretome of Lung Spheroid Cells using LC/MS/MS

by  
Dipti Paudel

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North Carolina State University  
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requirements for the degree of  
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## **DEDICATION**

*To my family and friends for their care and support throughout the years. I wouldn't be half the person I am today without the*

## **BIOGRAPHY**

Dipti Paudel was born in Kathmandu, Nepal. She moved to the United States with her family when she was 11 years old. She attended University of North Carolina at Greensboro and obtained her bachelor's degree in Biochemistry in 2013. She decided to continue her education and attended North Carolina State University, after a short six-month break, to obtain her Masters in Biochemistry. Currently, she is working as a mass spectrometrists in an environmental health research lab.

## ACKNOWLEDGMENTS

Over the course of my lifetime, I have had so many wonderful people besides me supporting me. I would like to start by thanking my mom and dad for bringing me to this country, so I could have chance at a wonderful life and great education. I would like to thank my brothers and sisters for their support over the years, always lending a helping hand and putting up with my madness. A special shout out to my sister Anisha for being the most supportive person I know, and always being there for me while I carved my own path and made my own mistakes. Didz your love and encouragement over the years have lead me to make bold choices in life that have made me understand myself better. I would like to thank Mike Goshe for giving me the chance to work in his lab. I have learned so much in the past few years, knowledge that goes beyond what I learned in the class and the lab. Kevin, I am grateful for your patience in teaching me the ins and outs of mass spectrometry and giving me a chance to mess with the instrument and learn for myself. Thank you to Dr. Cheng and the entire Cheng lab, especially Uyen, for all the help, advice, and collaboration opportunity on this wonderful project. Thank you, my committee members Colleen and Bob, for the constant support over the years. Thank you to my lab mates and fellow graduate students Laura, Cyndell, Jeff, Sophia, Jon, Jackson, Dmitry, Sayan, Jigar, Eric, and Ryan for always being there to listen, help, and suffer together. You all made my graduate school experience extremely entertaining and memorable. Last but not the least, thank you to Tiayonna and Nancy for taking care of all the paperwork and putting up with my endless talking capabilities.

## TABLE OF CONTENTS

<b>1.1 Introduction of mass spectrometry .....</b>	<b>1</b>
1.1.1 Proteomics.....	2
1.1.2 Proteomic experiments.....	3
<b>1.2 Sample analysis using mass spectrometry .....</b>	<b>5</b>
1.2.1 Sample introduction .....	6
1.2.2 Electrospray Ionization .....	7
1.2.3 Orbitrap.....	8
<b>1.3 Tandem mass spectrometry and fragmentation techniques .....</b>	<b>9</b>
1.3.1 Collision-induced dissociation.....	10
1.3.2 Higher-energy collisional dissociation .....	11
1.3.3 Electron transfer dissociation.....	11
1.3.4 Data dependent vs. Data independent acquisition .....	11
<b>1.4 Label free quantitation .....</b>	<b>12</b>
<b>1.5 Data analysis tools.....</b>	<b>14</b>
1.5.1 Proteome discoverer.....	14
1.5.2 Mascot.....	14
1.5.3 Scaffold.....	15
1.5.4 Ingenuity Pathway Analysis .....	15
<b>1.6 Introduction to human lung.....</b>	<b>15</b>
1.5.1 Human lung cells.....	16
1.5.2 Idiopathic Pulmonary Fibrosis.....	18
<b>1.7 Stem cells and regenerative medicine.....</b>	<b>21</b>
1.6.1 Embryonic stem cells.....	22
1.6.2 Mesenchymal stem cells.....	23
1.6.3 Induced pluripotent stem cells.....	23
<b>1.8 Introduction to cell culture.....</b>	<b>23</b>
1.7.1 Three-dimensional cell culture .....	24
1.7.2 Lung spheroid cells.....	24
1.7.3 Regenerative potential of LSC .....	26
<b>1.9 Scope of my thesis research.....</b>	<b>29</b>
<b>2.1 Introduction.....</b>	<b>48</b>

<b>2.2</b>	<b>Methods and Materials</b> .....	<b>50</b>
2.2.1	Materials .....	50
2.2.2	Cell culture.....	51
2.2.3	Conditioned media preparation.....	51
2.2.4	Cell lysis.....	52
2.2.5	In-gel protein digestion.....	53
2.2.6	LC/MS/MS data acquisition .....	54
2.2.7	Data analysis .....	54
<b>2.3</b>	<b>Results</b> .....	<b>55</b>
2.3.1	Method development for LSC conditioned media preparation for LC/MS/MS acquisition.....	55
2.3.2	Secreted protein analysis with optimized sample preparation method.....	59
2.3.3	LSC donor variability .....	62
2.3.4	Protein functional analysis .....	64
2.3.5	Pathways characterization.....	71
2.3.6	Cytoplasmic protein comparison .....	75
<b>2.4</b>	<b>Discussion</b> .....	<b>77</b>
<b>2.5</b>	<b>Conclusion</b> .....	<b>83</b>
<b>2.6</b>	<b>References</b> .....	<b>85</b>

## LIST OF TABLES

<b>Table 1:</b> Proteins present in at least two out of three triplicate donor conditioned media annotated as being extracellular proteins by IPA. ....	66
<b>Table 2:</b> List of potentially LSC-exclusive extracellular proteins. Functions obtained from uniprot.org.....	71
<b>Table 3:</b> Potential key proteins secreted by LSC for reduction of lung tissue scarring and promoting repair.....	80

## LIST OF FIGURES

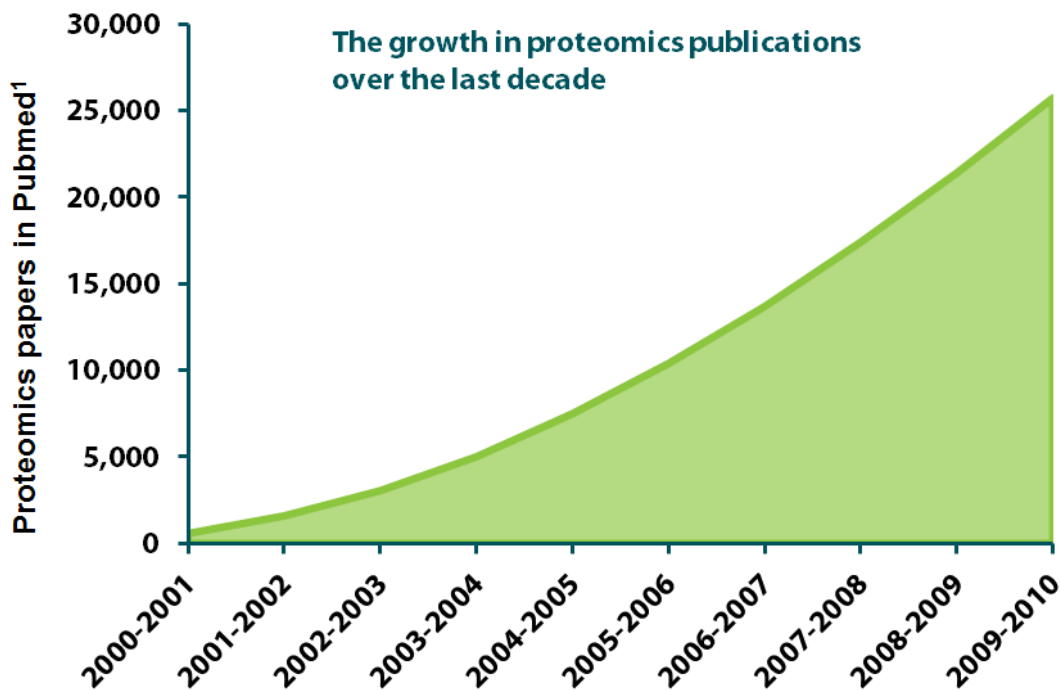
<b>Figure 1:</b> Trend of mass spectrometry use over the past few decades.....	2
<b>Figure 2:</b> A general “bottom-up” proteomic workflow. ....	3
<b>Figure 3:</b> Data generated during tandem mass spectrometry.....	5
<b>Figure 4:</b> The five steps for sample analysis using mass spectrometry .....	6
<b>Figure 5:</b> Schematic of the electrospray ionization. ....	8
<b>Figure 6:</b> Schematic of C-trap and orbitrap analyzer.....	9
<b>Figure 7:</b> Schematic of the orbitrap elite. Figure from ThermoFisher Scientific. ....	9
<b>Figure 8:</b> The Roepstroff-Fohlmann-Biemann nomenclature for peptide backbone.....	10
<b>Figure 9:</b> Label free quantification using area under the curve and spectral counting. ....	13
<b>Figure 10:</b> Schematic of human respiratory organs. ....	16
<b>Figure 11:</b> Lung stem cell niches after cellular injury. ....	18
<b>Figure 12:</b> Comparison of normal lung airways to IPF lung airways.....	19
<b>Figure 13:</b> Cellular remodeling in Idiopathic pulmonary fibrosis. ....	21
<b>Figure 14:</b> Schematic of stem cell differentiation.....	22
<b>Figure 15:</b> Formation of LSC.....	25
<b>Figure 16:</b> Microscopic images showing the generation of lung spheroid cells.....	26
<b>Figure 17:</b> Therapeutic benefits of human LSC in bleomycin induced pulmonary fibrotic mice.	27
<b>Figure 18:</b> Overview of the protein secretion pathway.....	28
<b>Figure 19:</b> Pathways for tissue regeneration using LSC.....	29
<b>Figure 20:</b> Lung Donor information. ....	56
<b>Figure 21:</b> Depletion of BSA from CM. ....	57
<b>Figure 22:</b> Protein precipitation using acetone. ....	58
<b>Figure 23:</b> Acetone precipitated conditioned media.....	59
<b>Figure 24:</b> Optimized protein workflow for conditioned media analysis.....	60
<b>Figure 25:</b> Analysis of conditioned media proteins derived from Donor 1, 2, and 3. ....	61
<b>Figure 26:</b> Subcellular localization of CM proteins.....	61
<b>Figure 27:</b> Protein relative abundances in the CM. ....	63
<b>Figure 28:</b> Mean vs Standard Deviation Plot for donor relative abundances.....	64
<b>Figure 29:</b> Protein functional analysis of extracellular sub-localized protein. ....	65
<b>Figure 30:</b> Abundances of matrix metalloproteinase proteins. ....	73
<b>Figure 31:</b> Proteins present in conditioned media (pink) affecting the matrix metalloproteinase pathway. ....	73
<b>Figure 32:</b> Abundances of stem cell growth factors across all donors. ....	74
<b>Figure 33:</b> SDS PAGE of LSC cytoplasmic fractions. ....	76
<b>Figure 34:</b> Subcellular localization of the proteins present in the cytoplasmic fraction of LSC.	77
<b>Figure 35:</b> Cytoplasmic proteins from cell lysis and secretome.....	77
<b>Figure 36:</b> Conditioned media treatment on fibrotic mice.....	79

## CHAPTER 1

### **Introduction to Mass Spectrometry-based Proteomic Approaches for Lung Regenerative Applications**

#### **1.1 Introduction of mass spectrometry**

Mass Spectrometry is a powerful technique for the detection of ionized analytes by measuring their “weight”, mass-to-charge ratio, in vacuum by electrostatic or magnetic fields. [1], [2]. Mass spectrometry is a versatile analytical technique and can not only be applied to chemistry and biochemistry, but also in food science, medical fields, pharmaceutical, forensics, environment, and many others. Mass spectrometers can be used to measure the mass of small molecules, proteins, and lipids [3]. Furthermore, they can be used to measure the mass of intact small molecules, proteins as well as determination of any post translational modifications using tandem mass spectrometry (MS/MS), where a specific precursor  $m/z$  is selected and fragmented through collision to obtain the fragmentation information. Several advances have occurred in the last few decades to optimize the mass spectrometer performance and different applications have made mass spectrometers one of the most powerful analytical instruments to date (Figure 1). The chapter will focus on proteomics, instrumentation, and their applications regarding proteomic analysis of stem cells secreted proteins.

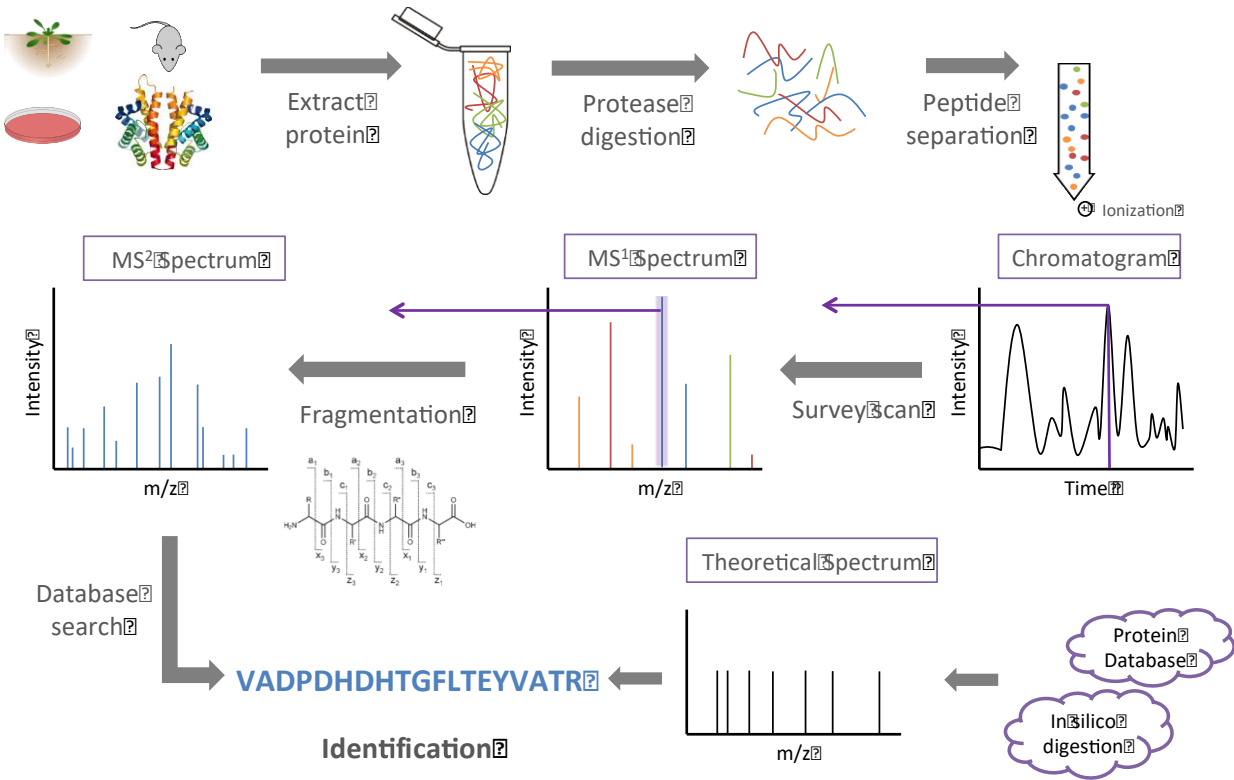


**Figure 1:** Trend of mass spectrometry use over the past few decades (PubMed).

### 1.1.1 Proteomics

Proteins are vital biomolecules in an organism that are formed from long chains of amino acids. There are 20 different amino acids that can be combined to make a protein. An organisms' proteome is the entire set of proteins that are produced or modified, and proteomics is the large-scale study of the proteome as they relate to cellular function. Proteome differs from cell to cell and is more varied than genomics and transcriptomics for the study of biological systems.

Proteins are subjected to wide variety of chemical modifications after translation, including phosphorylation, ubiquitination, methylation, and oxidation [4]. Mass spectrometry has been adopted as the choice for complex protein sample analysis as it allows for both *de novo* and targeted analysis of proteins isolated from various cells and/or tissues [5]–[8].



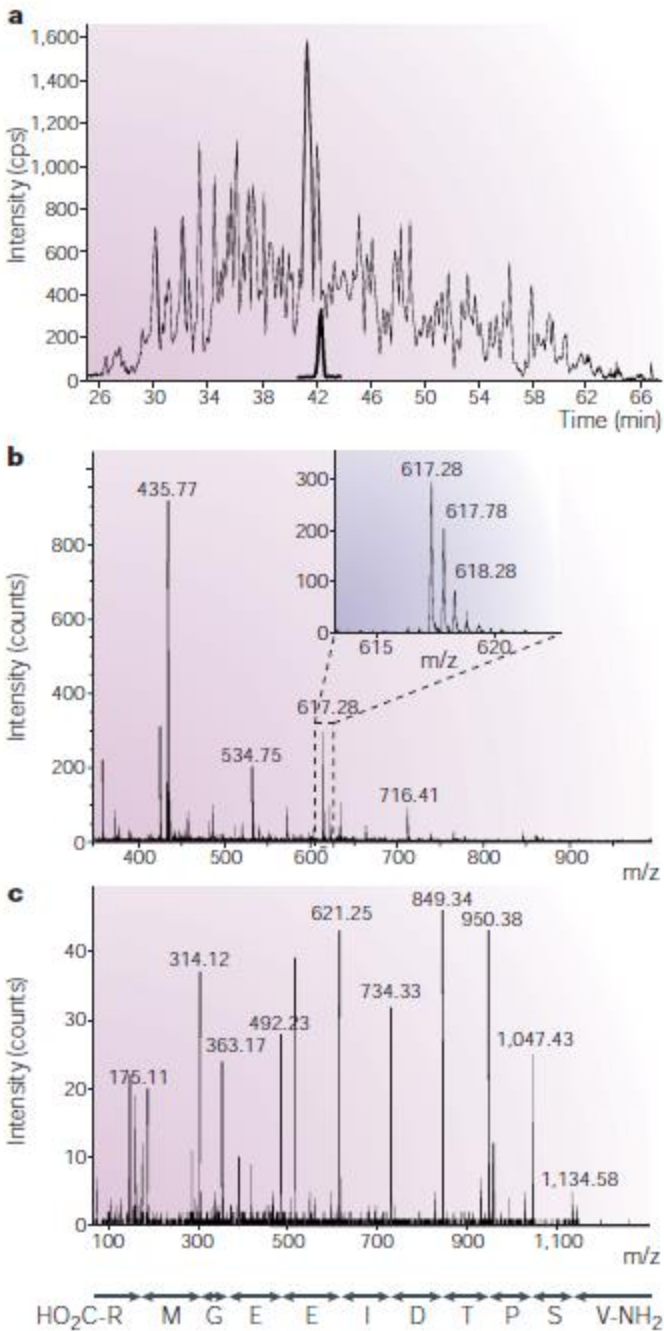
**Figure 2:** A general “bottom-up” proteomic workflow.

### 1.1.2 Proteomic experiments

Proteomics can be divided into two categories: discovery-based or targeted. The discovery-based method aims to identify unknown proteins in a sample and can be accomplished using either a “bottom-up” or “top-down” approach. A “bottom-up” approach of sample preparation is where the proteins are digested with a protease to convert the proteins into peptides [7]. Trypsin, the most commonly used protease, cleaves at a proteins lysine (K) and arginine (R) residues. Other proteases such as GlyC and AspN can also be used. Once digested, the samples can be processed by either performing some enrichment steps to identify phosphoproteins or fractionation to increase the number of identifications. The peptide mixture is separated on a linear gradient by reversed-phase liquid chromatography (RPLC). Once eluted, the peptides are introduced into the mass spectrometer by generating ions that enter the instrument. The initial MS scan ( $MS^1$ ) records the  $m/z$  values and relative intensity of the

precursor ions. For peptide sequencing, MS/MS is used, where precursor ions are fragmented to produce set of product ions to determine amino acid residue sequence of the peptide. While this sample preparation method is useful in identification of phosphorylation sites and modifications, it has its limitations as it can also produce an incomplete set of digested protein [7].

For a more “top-down” method, intact proteins are ionized and introduced into the mass spectrometer before fragmentation in the instrument to provide sequence information (Figure 3). This approach is used to characterize a single protein (or a mixture of proteins if a suitable LC method is established) to provide important structural information. One limitation of the top down approach is the requirement of a mass spectrometer with a high resolving power. Furthermore, fragmentation of the large proteins with LC is more analytically challenging and more energy is required for the fragmentation of the large molecules [7].



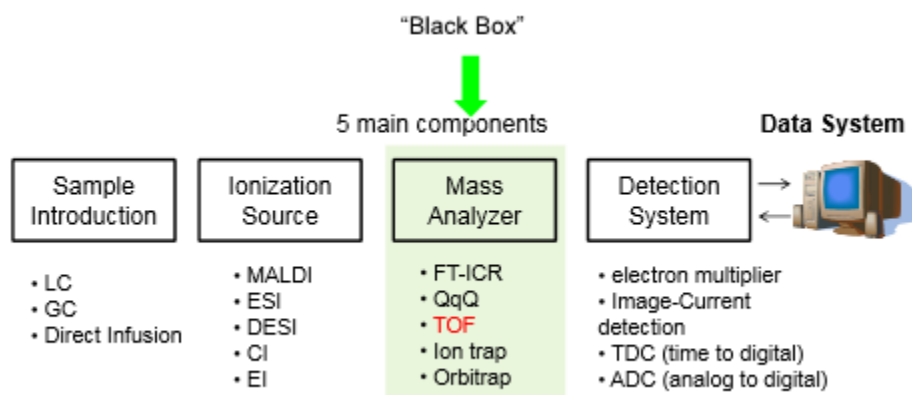
**Figure 3:** Data generated during tandem mass spectrometry

a) chromatogram, b) MS spectrum of precursor ion , c) MS/MS spectrum (product ion spectrum) of each selected precursor ion [7].

## 1.2 Sample analysis using mass spectrometry

A mass spectrometer contains an ion source, mass analyzer, and an ion detector. The components vary by the type of mass spectrometer and even the vendor that provides the

instrument. Ion sources are used to convert the analyte in liquid form into gas-phased ions, and a series of ion optics are used to generate electric fields to manipulate the generated ions through the system. Figure 4 depicts the different options for mass spectrometry analysis at the different stages of analysis. For my proteomic experiments, samples were introduced through LC, ionized using electrospray ionization, and analyzed with an Orbitrap using image current detection.



**Figure 4:** The five steps for sample analysis using mass spectrometry (Courtesy of Michael S. Bereman).

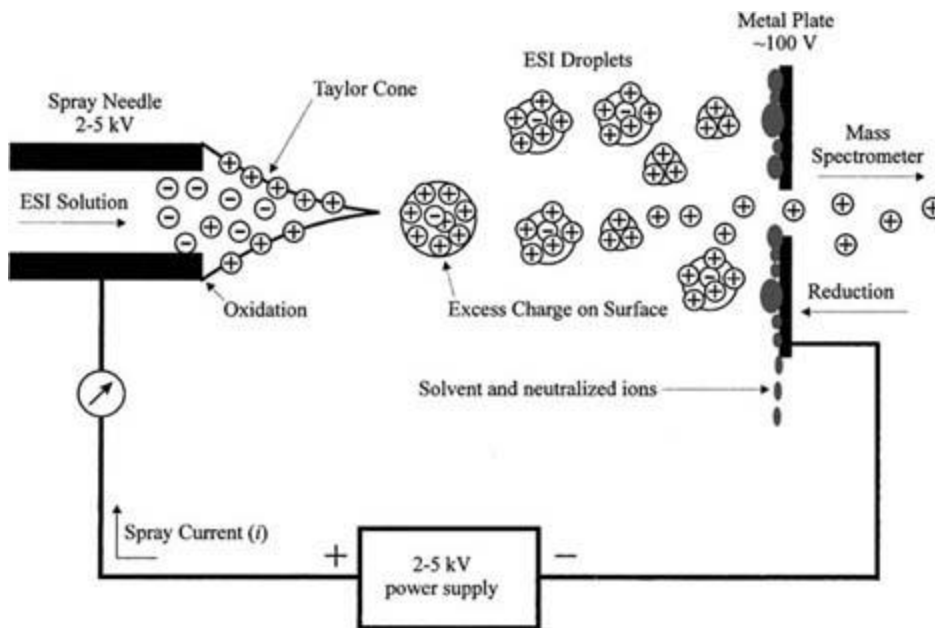
### 1.2.1 Sample introduction

Analytes are introduced to the mass spectrometer using liquid chromatography, gas chromatography, or direct infusion. Chromatography is separation principle where affinities between mobile and stationary phases result in the separation of analytes [9]. Reversed-phased nano high-performance liquid chromatography (RP-nHPLC) uses a hydrophobic stationary phase, such as a C18 particle. The molecules with hydrophobic functional groups have an increased affinity for the column packing and adsorb to the stationary phase, while the hydrophilic molecules have lower affinity and may preclude their retention. The hydrophobic molecules are eluted by changing (decreasing) the polarity of the mobile phase. The separation and resolution of the molecules is greatly affected by the length of the column, size of the stationary phase, and the mobile phases. Samples can be eluted using gradient elution, in which

the composition of the mobile phase is changed gradually for more efficient separation, or isocratic elution, where the mobile phase composition does not change throughout the run. For complex samples, such as discovery proteomic experiments, gradient elution allows for better separation and identification of the respective proteins [9].

### 1.2.2 Electrospray Ionization

Electrospray Ionization (ESI) is a common technique to ionize small molecules, large biomolecules, or peptides for mass spectrometric analysis. ESI is known as a soft ionization technique as it does not fragment the charged particles, rather turns it into smaller droplets [2], [10], [11]. A sample solution is sprayed through direct infusion, or from a LC column if coupled together, into an inlet with a strong electric field where the liquid sample is nebulized, forming a mist. Nitrogen gas is commonly used for desolvation. The gas helps evaporate the solvent from the ionized droplets and as they become smaller in size, the increase in electric field density causes the like charges to repel. When the droplet cannot support the surface tension, reaching the Rayleigh limit, the droplet divides into smaller droplets in a process known as coulombic explosion [12]–[17]. The resulting smaller gas-phase molecular ions from the ESI process are passed through to the instrument for detection with the mass analyzer. ESI allows for ions with multiple charges to enter the analyzer, reducing the mass-to-charge ratio, allowing mass spectrometry analysis of larger molecules (Figure 5) [2], [11], [18]. In 2002, John Fenn won the Noble Prize in Chemistry for his development of ESI for macromolecule analysis along with Koichi Tanaka and his work on another ionization technique MALDI (Matrix-Assisted Laser Desorption Ionization) for macromolecular analysis.

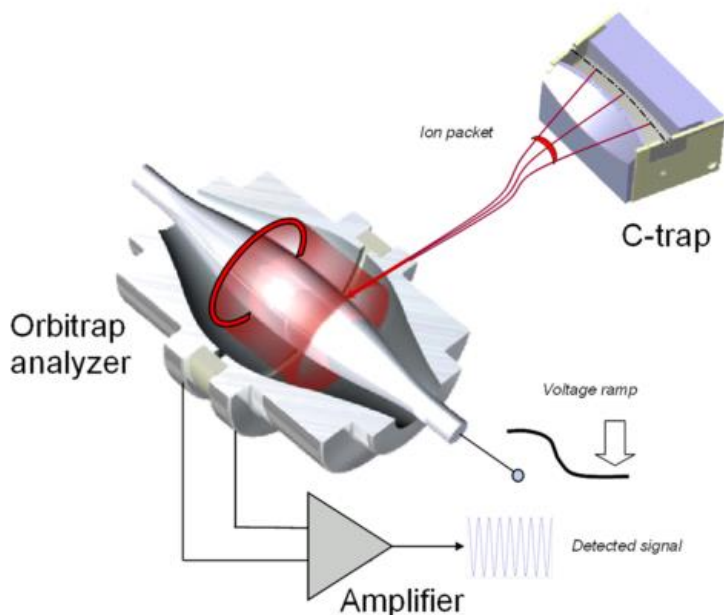


**Figure 5:** Schematic of the electrospray ionization.

The analyte solution is pumped through a needle and high voltage is applied at the inlet, forming a Taylor Cone because of the electric field in the counter electrode. The charged droplets formed evaporate as they move towards the inlet to enter the instrument and be analyzed using their mass to charge ratio[17].

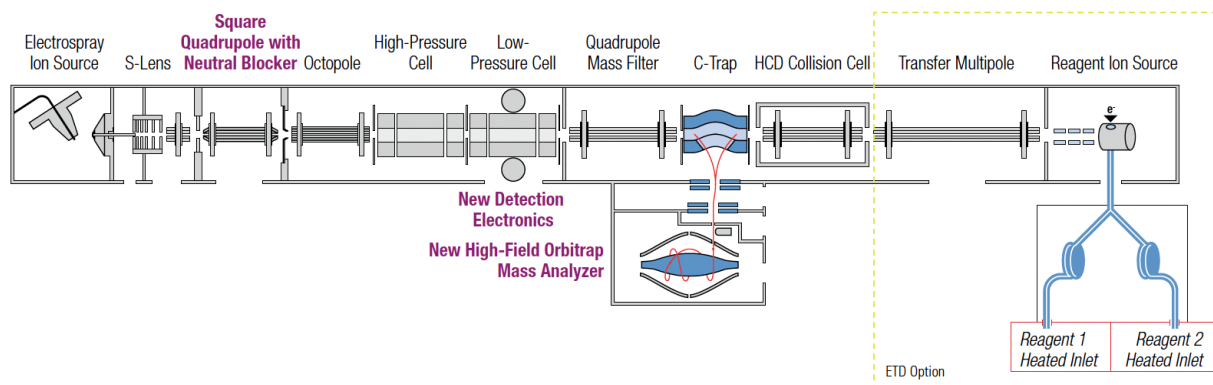
### 1.2.3 Orbitrap

The biological samples encountered in proteomics and metabolomics require a robust method for identification and quantification of these samples. Invented by Alexandar Makarov, the orbitrap is a kingdom trap mass analyzer that uses orbital trapping in an electrostatic field [19]–[21]. This palm sized analyzer contains a central electrode (cathode) that runs coaxially through an outer cylindrical electrode (anode) with flat end-cap electrodes (Figure 6,7) [6], [22]. A direct current (DC) voltage is applied between the electrodes for electrostatic potential distribution. The orbitrap contains a pulsed injection from external ion storage device known as the C-trap [23][21]. Ions injected in the orbitrap gets electrostatically trapped and rotate around the centrod electrode while oscillating along the horizontal axis to induce an image current signal. Image current signals are then converted to mass spectra by Fourier Transformation [6], [19], [21], [23], [24].



**Figure 6:** Schematic of C-trap and orbitrap analyzer.

Charged analytes are stored in the C-trap with RF (radio frequency). A high voltage pulse is applied to the trap and the analytes get ejected in a short packet that enter the orbitrap. The rf voltage is increased and the ions spread and oscillate around the central electrode to induce and amplify an image current [21].

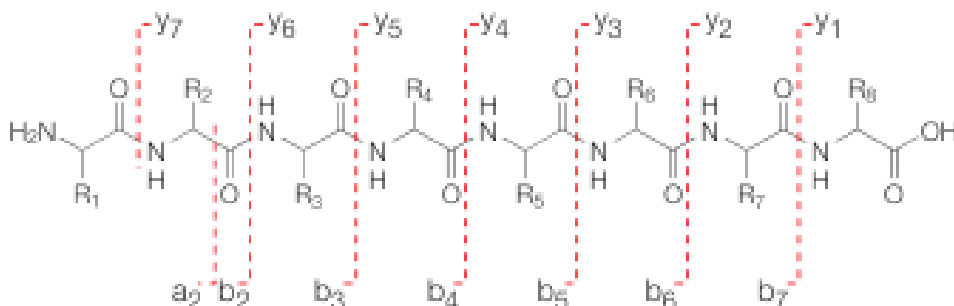


**Figure 7:** Schematic of the orbitrap elite. Figure from ThermoFisher Scientific.

### 1.3 Tandem mass spectrometry and fragmentation techniques

Tandem mass spectrometry, MS/MS or MS<sup>2</sup>, is the fragmentation, or molecular dissociation, is used in mass spectrometry to elucidate structural composition of molecules [25]. Once the initial scan (MS survey scan) of the peptides is performed the acquired data is used to produce the

chromatogram, (Figure 3a), the ions detected from the survey scan (Figure 3b), can be selected based on a measurable parameter (usually abundance) for further fragmentation (Figure 3c). Peptides can be dissociated into smaller ions as the collision typically breaks the most labile bond. Using the Roepstroff-Fohlmann-Biemann nomenclature designated to the peptide backbone, the energy on the peptide cleaves the amide, or peptide, bonds forming b and y type ions (Figure 8) [7]. This “fingerprinting” technique allows identification of specific amino acids, molecular groups, and label tags.



**Figure 8:** The Roepstroff-Fohlmann-Biemann nomenclature for peptide backbone fragment ions [7].

### 1.3.1 Collision-induced dissociation

Collision-induced dissociation (CID) is a low energy dissociation technique used in mass spectrometry to fragment selected precursor ion in the gas phase[26]. The molecular ions collide with an inert molecule such as helium or argon, and the collision causes some of the kinetic energy to be converted into internal energy distributed throughout the peptide until a threshold of dissociation is reached, leading to bond breakage. The fragmented product ions, formed by cleavage along the peptide backbone causing formation of b- and y-type ions can be analyzed using liquid chromatography-tandem mass spectrometry (LC/MS/MS) to provide identification and structural information about the ion [7], [26], [27].

### 1.3.2 Higher-energy collisional dissociation

Higher-energy collisional dissociation (HCD), despite its name, is a low energy dissociation technique specific to orbitrap instruments. Unlike CID which occurs in the ion trap, HCD fragmentation uses a higher RF voltage and takes place in a separate cell. In HCD, the ions that have been identified in orbitrap in the MS scan can be passed back through the C-trap, into the HCD cell for dissociation [23], [28]. The fragmented ions are returned to the C-trap and then to the orbitrap for analysis. HCD can be useful for label-based quantification of molecules and have been shown to result higher quality spectra for proteomics studies [23], [29].

### 1.3.3 Electron transfer dissociation

Electron transfer dissociation (ETD) is another soft ionization method first described by Skya et al.[30] ETD induces fragmentation of molecules, including large ones such as peptides and proteins, by transfer of electrons to higher charge state. Radical anions react with peptide/protein cations to cause transfer of electrons and subsequent dissociation. ETD cleaves the amide bond along the peptide backbone to produce c- and z- type ions [26], [31]. As this method does not cleave the most labile bond, it can be useful for post-translational modification analyses.

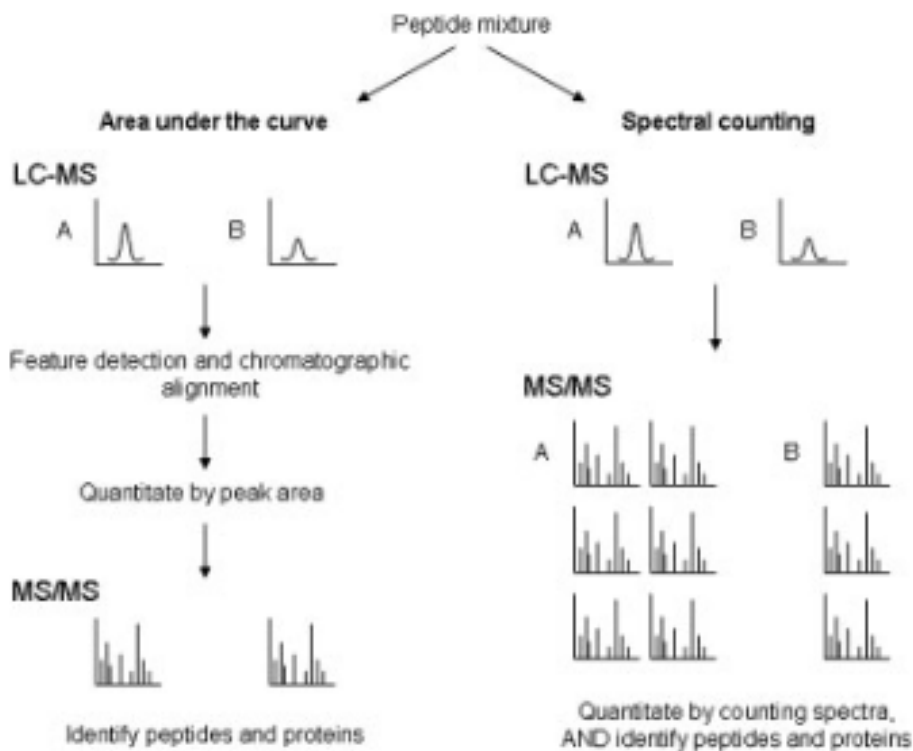
### 1.3.4 Data dependent vs. Data independent acquisition

Data dependent acquisition (DDA) is the most widely used approach for discovery-based analysis, where the data selection for fragmentation and sequencing depends on the precursor abundance [32]. For example, in a Top 5 DDA method, the top 5 most intense peptides from a full MS scan are fragmented. One disadvantage of the method is the loss of identification of lower abundant peptides. Parameters such as using peptide exclusion after a certain event can help increase identification. Data-independent acquisition (DIA) mode is where all precursor

ions in a given window are simultaneously fragmented regardless of the intensity [33]. While DIA can provide more information, it is usually more complicated, potentially more noise, requiring more post acquisition processing to deconvolute the data.

#### **1.4 Label free quantitation**

Mass spectrometry-based quantitation can be used for comparison of proteins under different conditions. Most quantitative proteomic studies involve comparison of one sample to a control, through relative quantification. Label free quantitation obtains quantitative information from mass spectrometry without any form of labeling, instead using the relevant protein abundances. Quantification of peptides for discovery-based quantitative approaches on a global scale is useful for identifying patterns from a large dataset. It is a more common method as it is more accessible, but it is hard to incorporate for certain samples types. It can be divided into two categories: area under the curve (AUC) or spectral counting measurements (Figure 9). With AUC, the peak areas of the precursor  $m/z$  are integrated and compared between samples[34]. Retention time of the peptide is also considered for different samples as peptides under the same conditions should elute around the same time. Additionally, post-data acquisition programs can also be used to correct small chromatographic drifts in retention time [35]–[38].



**Figure 9:** Label free quantification using area under the curve and spectral counting [34].

Spectral counting is where the highly abundant peptides are selected for fragmentation and produce the most abundant product ion spectra [34]. The results rely on a data-dependent acquisition parameter, and more MS/MS scans lead to more peptide quantification. Label free methods are advantageous over labeled strategies as no additional sample preparation is required. The quality of the data will highly determine the quantification and requires mass spectrometers with high mass resolving power and data analysis tools [39]. Target peptide analysis can also be used by monitoring the optimal precursor-product transitions. The transitions can be determined using different programs and can be a great tool to validate results obtained from global discovery experiments. Some problems associated with quantification include, decrease in sensitivity due to the general use of the mass spectrometer over time. There is also the

requirement for very good and consistent chromatography, and the quality of data will determine the quality of results.

## **1.5 Data analysis tools**

### **1.5.1 Proteome discoverer**

Proteome discoverer (ThermoFisher Scientific) is a commercial program used for analysis of data received from mass spectrometry, and it involves analysis of data using a range of proteomic workflows, protein and peptide identification, including labeled or label free quantitation. Proteome Discoverer software supports multiple dissociation techniques, database search algorithms for protein identification and characterization. It can also combine output from multiple algorithms to validate results. The ability to identify PTMs more confidently using multiple dissociation techniques such as HCD, CID, and ETD is extremely useful.

### **1.5.2 Mascot**

Mascot (Matrix Science), is a commercial program for protein identification using mass spectrometry data to identify proteins from databases. It uses a probability-based algorithm to determine significant results, false positives, and comparison using sequence homology. Mascot compares the molecular weights of peptides obtained from a mass spectrometer and compares it against a database of known peptides. The sequence databases that can be searched include Uniprot, Swissport, and NCBI. The program cleaves every protein in the database *in silico*, using the specific enzyme used for digestion, to obtain theoretical mass for each peptide. A score is assigned to each peptide based on the probability that those peptides match proteins in the selected database. More peptides identified from a protein, the higher mascot score, resulting in a more accurate identification. Mascot also estimates a false discovery rate (FDR) based on the comparison of the decoy database matches to target database matches. The decoy database is

generated by obtaining random protein sequences of the same length in the target database, with the same average amino acid composition.

### 1.5.3 Scaffold

Scaffold (Proteome Software) is a commercial bioinformatic tool for comparison and validation of protein identifications across multiple samples and data types [40]. Scaffold viewer allows for easier visual inspection of data along with several statistical methods to classify proteins with high confidence and reduce false identifications. Data from Proteome Discoverer and Mascot can be inputted into Scaffold for further analysis.

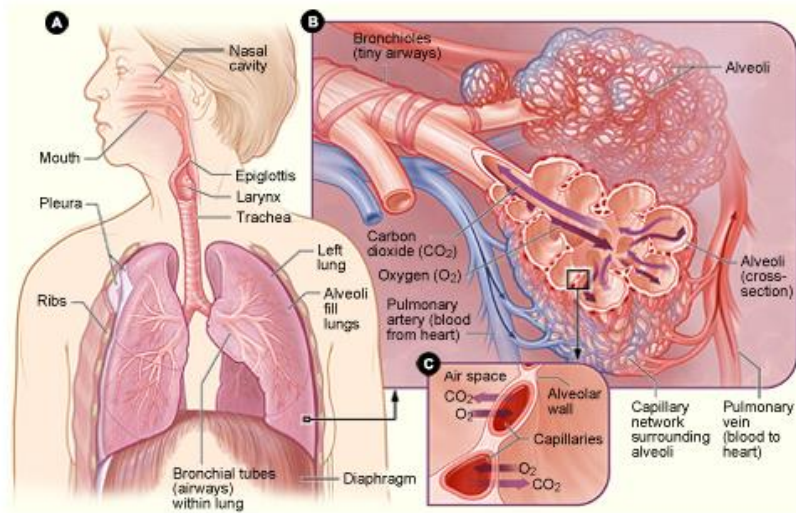
### 1.5.4 Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) is a commercial analysis tool by Qiagen that allows for interpretation of proteomic data, along with other omics data. IPA has databases for human, mice, rat, and bovine proteins. IPA allows for visualization of data, analysis of protein abundance, grouping proteins into categories based on the regulatory networks, protein-protein interactions, and their subcellular location [41].

## **1.6 Introduction to human lung**

Human lungs are a pair of respiratory organs located in the thoracic cavity of the chest in which gas exchange, lungs intake of oxygen and removal of carbon dioxide, occurs. Air enters through nose or mouth passing the pharynx, larynx, and trachea before splitting into left and right bronchi. The bronchi further branch off into thousands of smaller and thinner tubes called bronchioles which are made of elastin fibers and smooth muscle tissue. The bronchioles terminate at the alveoli of the lungs, which are functional units of the lungs that permit gas exchange from lungs and blood of the capillaries of the lungs. Alveoli contain small cluster of

alveolar sacs that are surrounded by tiny capillary that connect to network of arteries and veins to move blood through the body (Figure 10) (National Heart, Lung, and Blood Institute, 2017; American Lung association, 2017).



**Figure 10:** Schematic of human respiratory organs.

a) Location of the respiratory system in human body b) Zoomed in view of bronchioles, alveoli, and capillaries; c) closeup view of gas exchange between capillaries and alveoli. Image from the national heart, lung, and blood institute. <https://www.nhlbi.nih.gov/health/health-topics/topics/hlw/system>

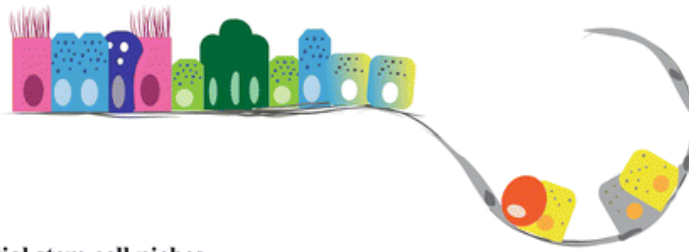
### 1.5.1 Human lung cells

The lung is extremely complex organ with interaction among more than 40 different cell lineages, and scarring or fibrosis can lead to disruption in the lung function [42]. Lung is a highly quiescent tissue but has a remarkable reparative capacity involving facultative progenitor cells that respond to injury by proliferating and differentiating into one or more cell types [42]. While one single stem cell might not be able to generate all the various lineages in the lung, multiple progenitor cell lineages might be used for the response to injury and disease. Lung could respond to injury and stress by activating stem cell population or by reentering the cell cycle to repopulate lost cells.

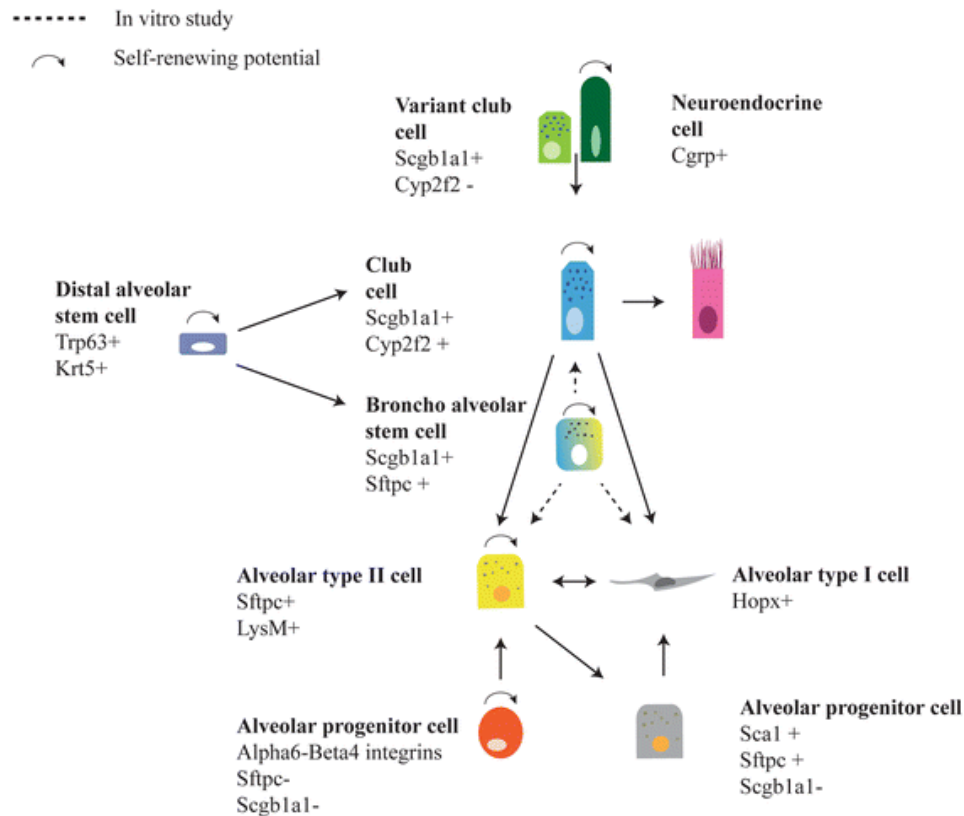
The human airways are lined by basal cells, secretory cells such as club cells, ciliated cells, and neuroendocrine cells [43]. The alveolar epithelium is populated by two cell types: alveolar epithelial type 1 (AT1) and alveolar epithelial type II (ATII) with ATII being the prominent cell type, as combination of ATII and endothelial cells act as gas exchange barrier at the end of the alveolar sacs. ATII cells are capable of differentiating into ATII or AT1 cells; therefore, damaged AT1 are replaced by ATII. [44] Basal cells are multipotent cells and have the capacity to self-renew as well as form secretory and ciliated cells, as determined by notch signaling. They are characterized by the expression of Trp63, Ngfr, and cytokeratin Krt5 [43]. Kr14, a self-renewing basal cell types, are thought to be involved in the maintenance of cell proliferation and differentiation. Two specific types of basal cells found in adult lung are basal stem cells (BSCs) and basal luminal precursor cells (BLPCs) [43]. Goblet cells are abundant in the human epithelium but are rare in mouse.

The distal and alveolar epithelium can regenerate itself after injury (Figure 11 a). There are many potential stem cell niches present including the variant club cells, bronchoalveolar stem cells, and distal alveolar stem cells (Figure 11b). In general, variant club cells give rise to neuroendocrine cells or club cells. Club cells can also be derived from distal alveolar stem cells. These club cells further divide into, bronchoalveolar stem cells, ATII, or AT1 cells. ATII can further divide into alveolar progenitor cells and to AT1 cells. AT1 cells are the most differentiated of the lung epithelial cell types (Figure 11 b).

## A Cuboidal and Alveolar Epithelium



## B Other potential stem cell niches



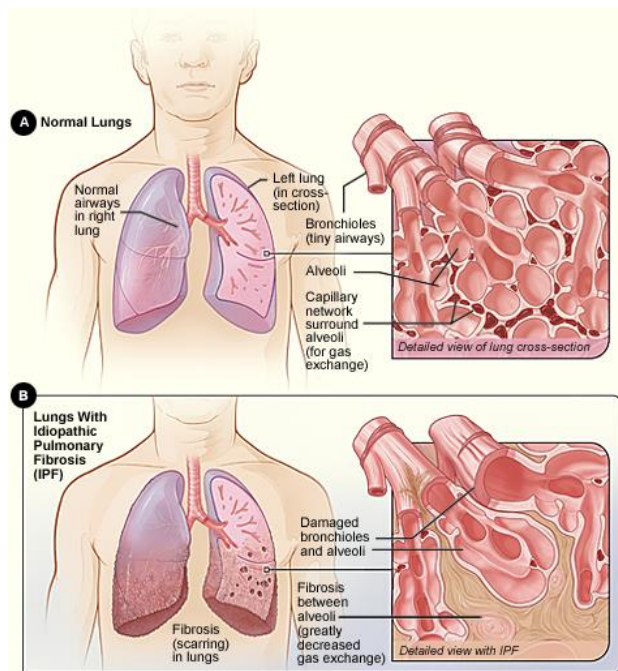
**Figure 11:** Lung stem cell niches after cellular injury.

a) The alveolar epithelium with the various cell types; b) potential stem cell niches in the lung [43].

### 1.5.2 Idiopathic Pulmonary Fibrosis

Idiopathic Pulmonary Fibrosis (IPF) is a chronic, irreversible, progressive disease of unknown cause [45]–[50]. IPF is the most common form of interstitial lung diseases, more prominent in elderly adults, and the incident is approximately 40 per 100,000 persons, with slightly greater prevalence in men [49], [51]. Patients with chronic dry cough and exercise

induced breathlessness seek medical attention, and exclusion of other known causes of interstitial lung diseases, such as pneumonia, is a diagnostic criteria for IPF [51]. Even though the exact cause of the disease is still unknown, the incident of IPF is rising with the median survival of patients after diagnosis between 2-4 years.



**Figure 12:** Comparison of normal lung airways to IPF lung airways.

<https://www.nhlbi.nih.gov/health-topics/idiopathic-pulmonary-fibrosis>

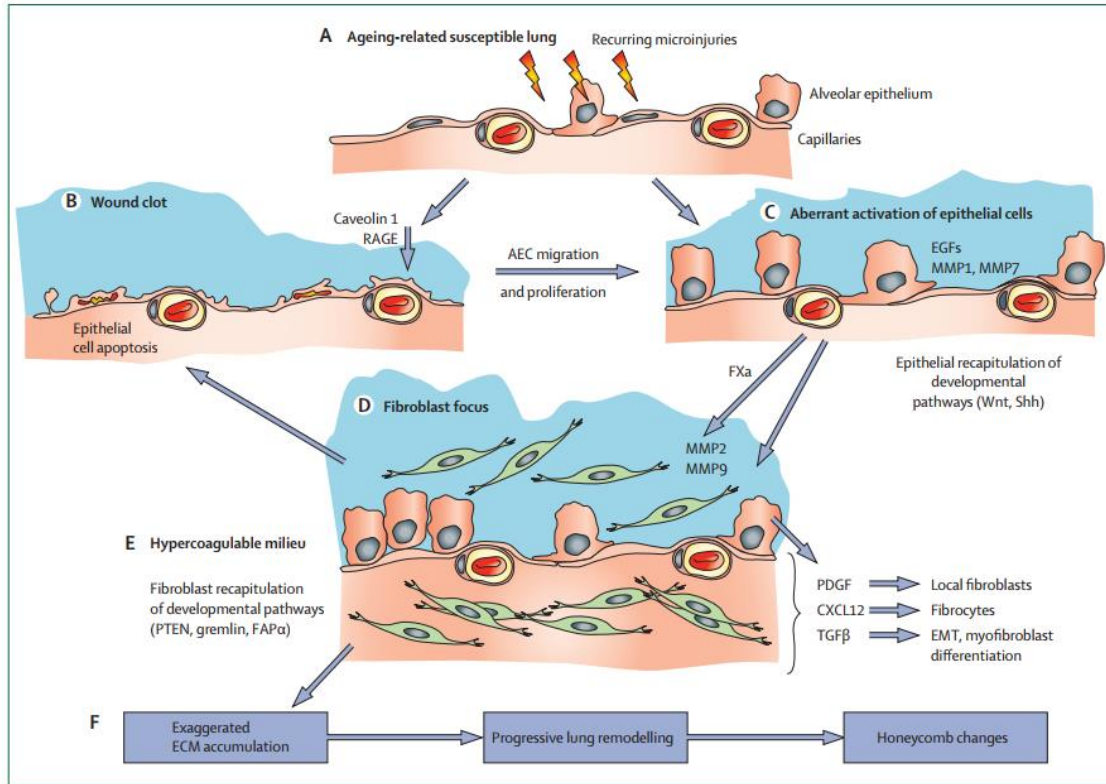
A lung biopsy obtained from either an open thoracotomy or a thoracoscopy is used for histological analysis. A pattern of interstitial pneumonia, with honeycombing, and areas of fibrosis with normal parenchymal, is usually the best way to confirm diagnosis [49], [51]. Other methods such as chest renography can be useful, however, approximately 10% of patients with IPF have normal rentograph [51]. Furthermore, as biomarkers from blood sample or bronchoalveolar lavage are not yet defined, these methods are still considered limiting alternative diagnoses [46], [51]. IPF features tissues deep in lungs becoming thick and stiff, otherwise known as fibrotic, over time (Figure 12). Two theories have been established for the

pathogenesis of IPF: epithelial injury and inflammation [49]. The epithelial theory suggests multiple injuries to the lung results in epigenetic changes leading to abnormal alveolar epithelial cells in the epithelium. Distortion of normal lung architecture is achieved when fibroblast cells secrete excessive amounts of extracellular matrix and collagen, causing excessive thickening and scarring of the epithelium (Figure 13) [45], [50], [51].

The second theory states that inflammation of lungs due to either exposure to damaging agents, such as cigarette smoke, dust particles, viral infections, and silica, or genetic predeterminations plays a vital role in the evolvement of fibrosis [45], [52]. The recruitment of neutrophils, macrophages, among other profibrotic proteins such as TGF, induce release of collagen from tissue fibroblast [49]. Even though there is no complete understanding of pathogenesis in both theories of disease progression, IPF causes acute damage to lung epithelia by chronic degeneration of airway and alveolar tissues [42], [49].

Considering all uncertainties and complex mechanisms in the progression of fibrotic process in IPF, it is increasingly difficult to develop effective therapies. The standard treatment of the disease has been oxygen therapy, pulmonary rehabilitation, pain management, and most effectively, a lung transplant. Recently, two new drugs to treat IPF, Roche's Esbriet (pirfenidone) and Boehringer Ingelheim's Ofev (nintedanib) have been approved. They are both anti-fibrotic drugs, and while they may slow down the decline in lung function, they do not decrease the existing fibrosis [53]. Stem cells to promote correct re-epithelization of the alveoli has also been pursued to stop the disease progression [52]. Signaling pathways in the lung maintain both a proliferative or quiescent lung epithelium, and crosstalk between the stem cells and epithelial cells is important. Hedgehog signaling in the adult human lung balances between these two states of the lung [43]. This hypothesis is supported by significant reduction in

collagen deposition and severity of pulmonary fibrosis in bleomycin induced lung injury in rats and mice treated with mesenchymal stem cells and lung spheroid cells [54], [55]



**Figure 13:** Cellular remodeling in Idiopathic pulmonary fibrosis.

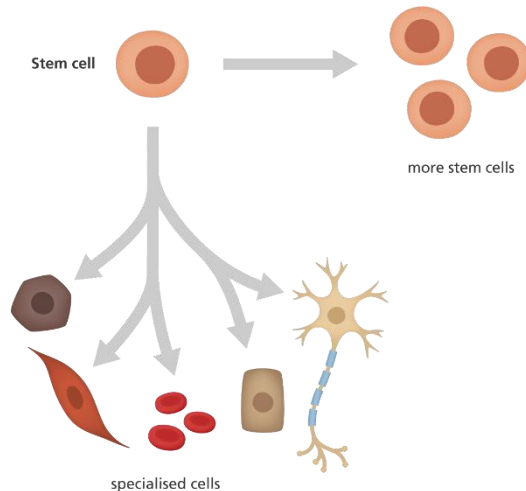
a) Alveolar epithelium damage due to microinjuries. b) formation of a clot on the injury surface followed by c) activation of epithelial cells and recruitment of d) fibroblasts and matrix metalloproteinases for wound healing. E) extracellular matrix related remodeling leading to f) accumulation of extracellular matrix proteins for formation of scars [45].

### 1.7 Stem cells and regenerative medicine

Regenerative medicine is a therapy technique in which specific types of cells or cell products are introduced into diseased tissue or organs for potential tissue repair. Regenerative medicine in the past decade has been a candidate for therapeutics, and advancement in stem cells technologies have made the cell-based regeneration more of a reality [56]. Stem cells are defined as cells that show indefinite self-renewal and multiple differentiation potential (Figure 14).

Previously, human stem cells were not able to be expanded and maintained for a long period of

time, hampering their ability for *in vitro* use [57]. Recent advances in differentiation capability and maintenance have developed technologies for stem cell maintenance.



**Figure 14:** Schematic of stem cell differentiation [43].

### 1.6.1 Embryonic stem cells

Embryonic stem cells are derived from inner cell mass of pre- or peri-implantation mammalian embryos [56], [58]. These are totipotent, largely undifferentiated cells, that have unlimited self-renewal and differentiation capacity. These cells are able to give rise to all three of the embryonic germ layers: ectoderm, endoderm, and mesoderm [56]. However, despite the totipotency of ES cells, these come with considerable problems and controversies. First is the innate risk of immune rejection and toxic effects as they are not autologous. Studies of transplantation of cells in the immunocompromised host indicate there is formation of teratomas [56]. Second is the ethical and political issue of having cells that are derived from fertilized eggs [58]. Third is the need for bulk culture of ES cells required for these cell therapies and the inherent genetic and batch to batch variability that makes these cell more difficult to work with [56].

### 1.6.2 Mesenchymal stem cells

Mesenchymal cells (MSCs) are the major types of stem cells that are used for regenerative therapy. MSCs are adult pluripotent progenitor cells that are derived from sources such as bone marrow, umbilical cord blood, adipose tissue, cartilage, and connective tissue [59]. These cells can differentiate into osteoblasts, adipocytes, myocytes, and neurons. The degree of differentiation of these cells can vary depending on the cell types, age of the donor, and the time (passage) in culture. MSCs have been shown promising in regenerative therapies for heart disease, liver disease and many others; however, the teratogenicity potential of these in a foreign host questions their safe use for these therapies [60]. Currently there are over 700 clinical trials being conducted for the development of mesenchymal cell-based therapies for various diseases (clinicaltrials.gov).

### 1.6.3 Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are tissue specific progenitor cells that represent autologous source of organ regeneration. Pluripotent cells have the ability to differentiate into the various stem cell types, and reprogramming of somatic cells into pluripotent cells, similar to MSCs. [61]. These iPSCs have been used for heart remodeling and vascularization, [61], [62] along with modeling lung stem cells

## **1.8 Introduction to cell culture**

Cells in a host grow naturally in a 3D environment. For *in vitro* studies, cells are typically grown as standard monolayer in culture flasks or Petri-dishes with added media for the source of nutrition. The medium is supplemented with serum, antibiotics, and L-glutamine for cell growth and incubated at body temperature (37°C) [63]. While 2D culture has been widely used as an

initial model for cell-based studies, they lack the overall structural architecture as observed *in vivo*, such as cell-cell and cell-extracellular matrix signaling, that are essential for cell differentiation, proliferation, and other cellular functions [63]. Three-dimensional (3D) cell spheres are being used widely in regenerative method as an alternative to traditional two-dimensional cell culture, as the 3D systems are multicellular models that better represent the organ niche *in vivo* [64].

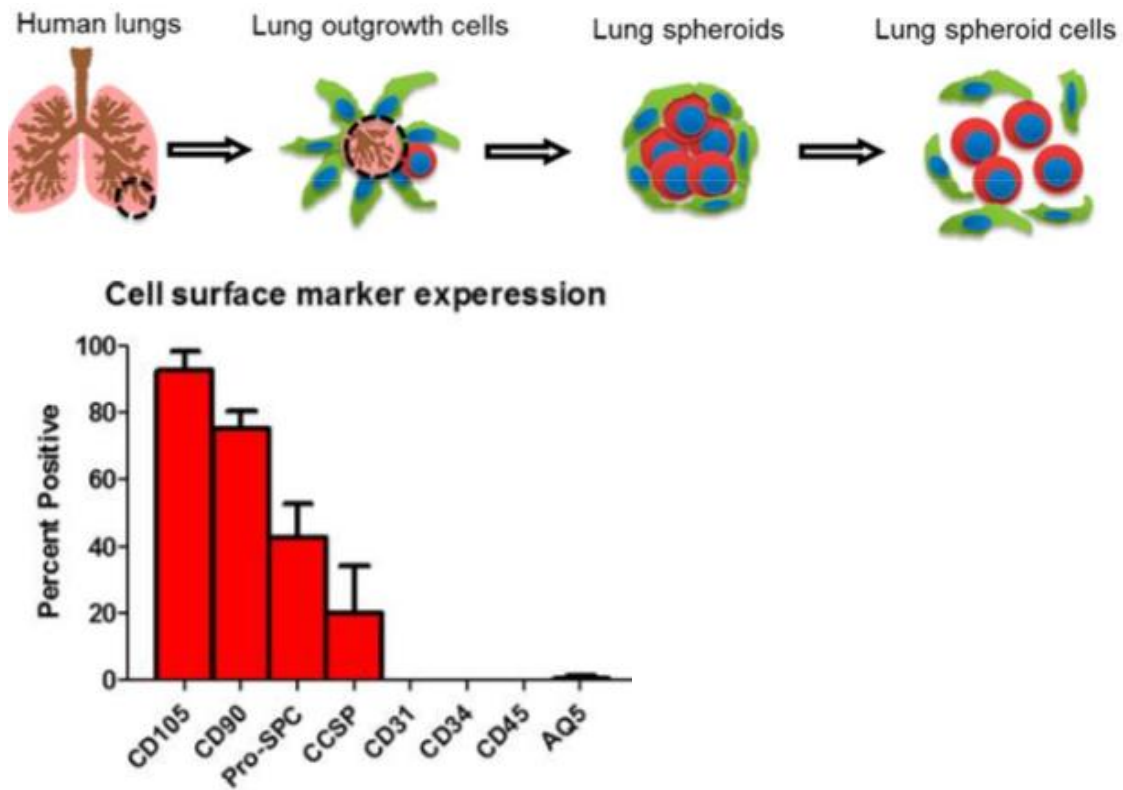
### 1.7.1 Three-dimensional cell culture

The 3D cells are cultured in an environment that prevent the attachment of cells to the surface, encouraging the cells to adhere to each other to form spheroids. Multicellular spheroids provide space for cell adhesion and are an improved model for cell survival and growth. Previously, methods such as forced floating [65], hanging drop [66], matrices [67], [68], scaffolds [69], and organoids[57], [70], [71] have been used widely for the formation of these spheroids [63]. 3D culture provides functional units of the tissue with distinguished architecture, morphology, rather than single cells. Spheroid cell cultures have been used in cancer biology [72], [73] and the formation of neural stem cells[74], and cardiac stem cells[75], [76].

### 1.7.2 Lung spheroid cells

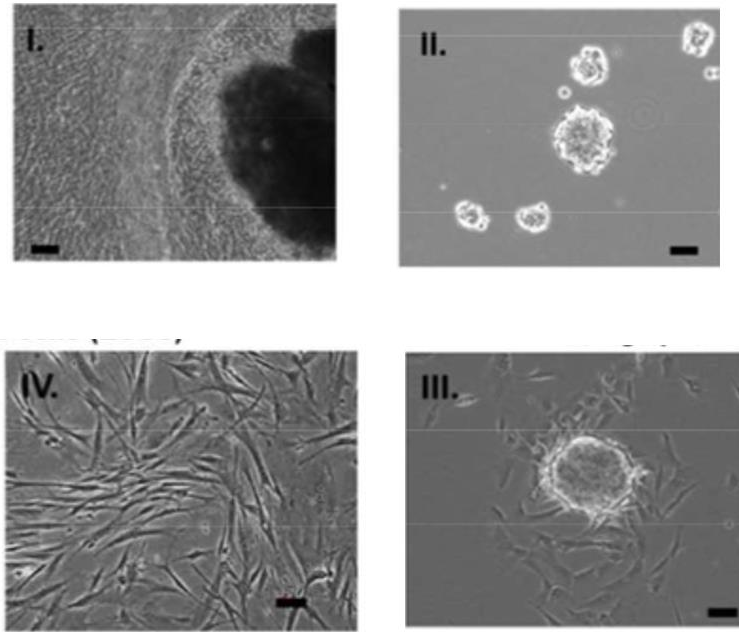
Lung spheroid cells (LSC) are one such type of multicellular cells that are generated from healthy lung tissue using a three-stage adhesion-suspension-adhesion method. Outgrowth cells from human lung tissues can be generated from lung tissue samples in adherent culture when maintained in appropriate cell culture medium. In the first step, approximately 6 mm minced lung tissues are placed in a fibronectin coated flask with cell culture media. After approximately a week, cells start growing from the tissue explants. These cells are then seeded into ultra-low attachment flasks to allow for spheroid formation by aggregation. Phase bright lung spheroid

cells form in the suspension culture after approximately a week after seeding. These cells can be kept in spheroid forms or re-plated into adherent fibronectin coated flasks for growth in a monolayer, in the final adhesion step to form what are called the lung spheroid cells. This allows the cells to be easier to handle and allows them to be more stable. Flow cytometry analysis of the cells showed that these lung spheroids contain clusters of lung progenitor cells (Pro-SPC, CCSP+, p63+, and KRT5+ cells). These progenitors reside in the spheroid core surrounded by stromal like CD105+ and CD90+ cells [55]. Previously, cardiac stem cells also formed spheroids with similar structural properties [75], [76]



**Figure 15:** Formation of LSC.

a) Schematic of LSC expansion from human lungs and b) Cell surface marker expression for LSC [55].

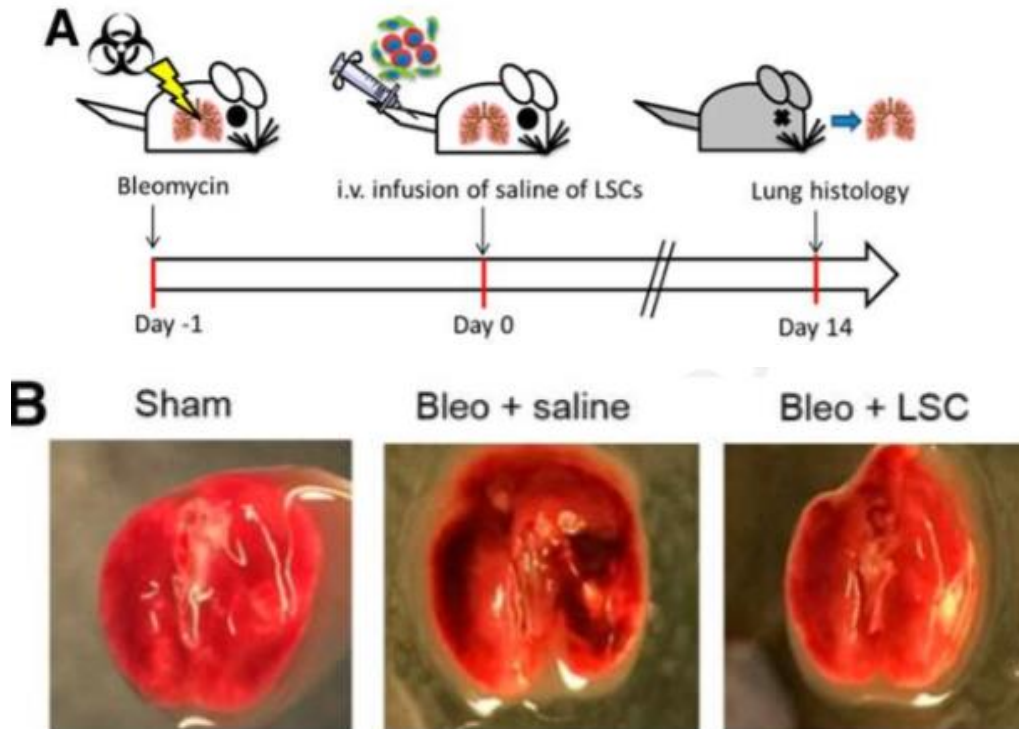


**Figure 16:** Microscopic images showing the generation of lung spheroid cells.

1) lung tissue explants with outgrowth ii) lung spheroid formation in suspension culture iii) plated lung spheroid in fibronectin coated plates for formation of lung spheroid cells iv) expansion of lung spheroid cells [55].

### 1.7.3 Regenerative potential of LSC

LSC generated from healthy lung tissues have been tested for their therapeutic application in mice models. SCID mice were intrathecally given bleomycin to induce pulmonary fibrosis. After 24 hours, LSC or saline control were intravenously infused into the fibrotic mice. The animals were sacrificed at 14 days for endpoint analysis (Figure 17a). The endpoint analysis showed significant damage in the lungs that were fibrotic and treated with saline in contrast with the mice that were injected with LSC. LSC treatment reduced the scarring and thickening of the lungs, seen with visual inspection (Figure 17b) as well as determined by the Ashcroft score [55].



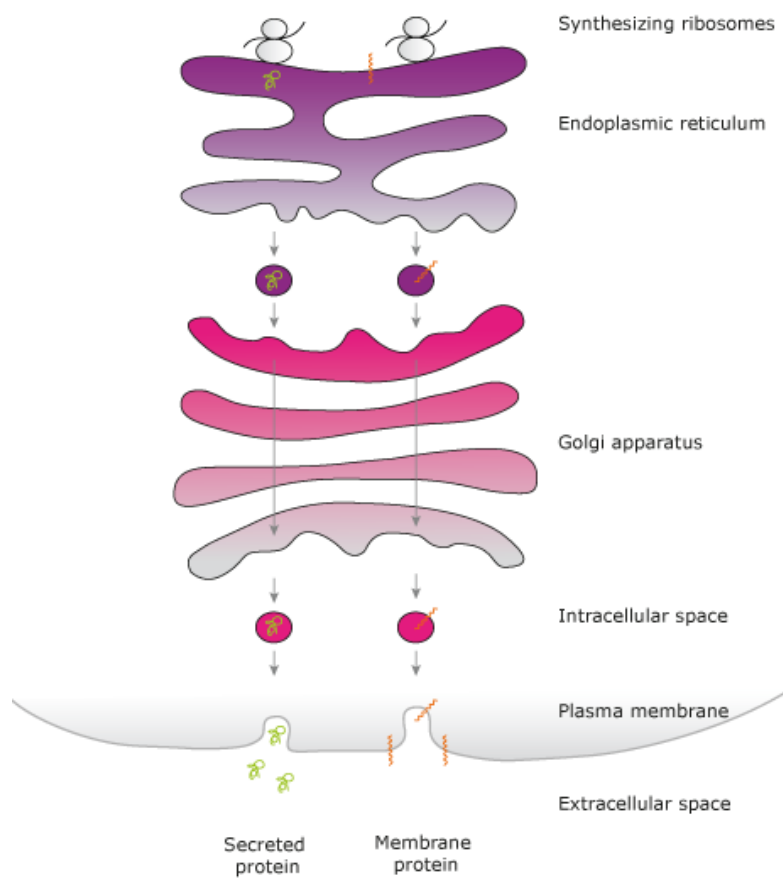
**Figure 17:**Therapeutic benefits of human LSC in bleomycin induced pulmonary fibrotic mice.

A) Lung spheroid cells mice study model. B) Macroscopic view of lungs after 14 days in the different treatment groups [55].

### 1.8 Protein Secretion

Secreted proteins are transported out of the cells and allow for both intercellular and intracellular communication. Secreted proteins play a crucial role in control of physiological and developmental processes in the body. For example, some cells secrete hormones, such as estrogen, and signaling proteins, and neurotransmitters like serotonin. Proteins that enter the secretory pathway contain an ER signal sequenced at the N-terminus, usually consisting of 6 to 12 amino acids with hydrophobic side chains. The signal is recognized by a signal recognition particle, which binds to the signal to stop the translation, and direct the protein synthesizing ribosome to the rough endoplasmic reticulum (ER) to be co-translated in the lumen. The newly synthesized protein is folded and sent to the Golgi apparatus to be transported through the trans-

Golgi network to the plasma membrane using secretory vesicles. This is referred to as classical or Golgi dependent trafficking (Figure 18). Another mechanism is often referred to as non-classical, or Golgi independent trafficking where the proteins do not contain signal peptide and do not travel through the Golgi [77]–[81]. Cells receive signal via signaling molecules and binding to an appropriate receptor triggers a signaling cascades in the intracellular environment to induce physiological changes. Secreted proteins may play a major role as therapeutics in treatment of various diseases.

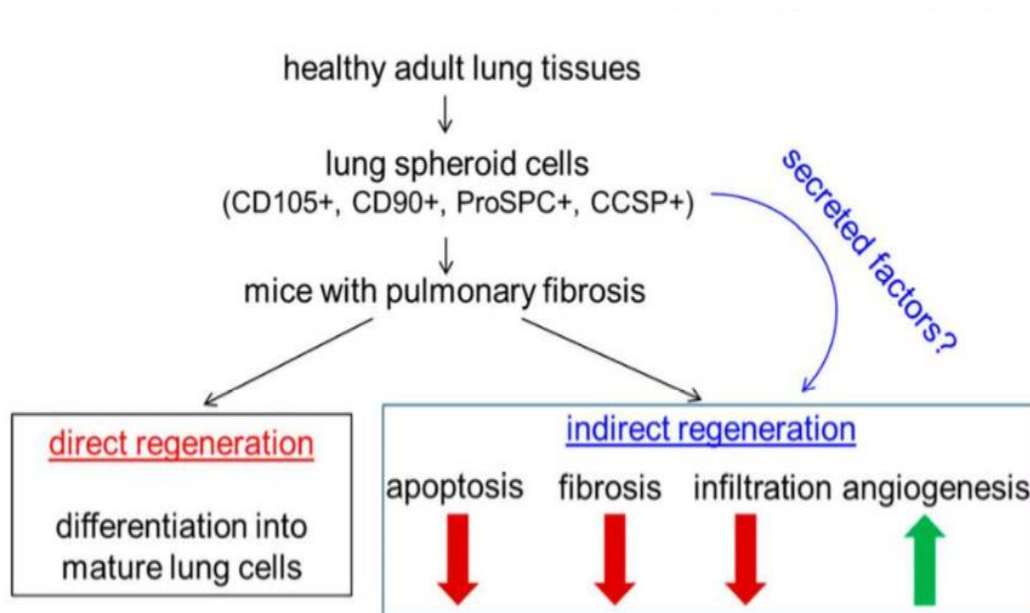


**Figure 18:** Overview of the protein secretion pathway.

(The human protein atlas, [www.proteinatlas.com](http://www.proteinatlas.com))

## 1.9 Scope of my thesis research

While LSC have been successful in treatment of IPF by regeneration of lung tissue, there are inherent limitations to using cell therapy. Cells must be carefully preserved to maintain their viability, there are teratogenic risks associated with the cell transplantation, and there is a high risk of immune incompatibility. To avoid the teratogenic risks, it is more advantageous to isolate cells from the patient, direct the transition into stem cells, and transplant those back into the patient. In all of the preclinical and clinical studies, it was believed that the stem cells engrafted into the damaged tissues via migration to the tissue to repair and regenerate the organ for the specific disease (Figure 20) [82].



**Figure 19:** Pathways for tissue regeneration using LSC.

Furthermore, these therapeutic effects could also be caused by paracrine effects, or by a combination of both. While ESCs and MSCs could be effective due to their paracrine effects, LSC being more specific could be more targeted for repair of the lung, enhancing the therapeutic potential. The goal of my thesis research is to identify the secreted proteins from the conditioned

media of LSC to identify proteins potentially involved in tissue regeneration using quantitative LC/MS/MS analysis. Identification of these protein factors could be explored further for more detailed studies regarding the application of a combination of these proteins for therapeutic intervention.

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## CHAPTER 2

### **Elucidating the Secretome of Human Lung Tissue Derived Lung Spheroid Cells for Identification of Potentially Key Regenerative Therapeutic Proteins.**

#### **2.1 Introduction**

Lung diseases, such as idiopathic pulmonary fibrosis (IPF), are a major cause of mortality worldwide. IPF is a chronic, progressive, and irreversible interstitial lung disease with a mean survival time of three years after diagnosis [47]. It affects approximately 5 million people worldwide, with prevalence slightly greater in men than women [49], [51]. IPF manifests over several years and is characterized by thickening and formation of scar tissue within the lungs [51], [83]. The disease is believed to be influenced by both genetic and environmental factors, such as cigarette smoking and exposure to metal and wood dust [84]. Medical therapies have been ineffective in the treatment of IPF, and Esbriet and Ofev, the two FDA approved pharmaceuticals are not curative and come with number of side effects, making pulmonary transplantation as the only curative option [53].

The lung has extensive reparative capacity, and stem cell therapy has recently been speculated as alternative treatment using pluripotent cells such as ESCs and MSCs [85]–[89]. These cells have been applied to regenerate and repair a wide variety of diseases, including lung diseases, in mice models; however, the clinical translation is hampered by potential teratogenic risk in humans. Furthermore, the clinical use of these cells is limited by ethical and legal considerations. LSC are rapid and reproducible method to generate tissue-specific multicellular three-dimensional cell culture using a three-stage “adhesion-suspension-adhesion” culture process, and have been established as an intrinsic source of therapeutic lung stem cells[55]. The

spheroid method has also been used to generate cancer stem cells models [90], [91], cardiac stem cells models (CSCs)[92], and neural stem cell models [93]. LSC are mixture of progenitor and supporting stromal cells and in a previous study, intravenous infusion of LSC into mice with bleomycin-induced pulmonary fibrosis showed regenerative potential by enriching the stem cell population and formation of mature lung epithelial cell [55]. Furthermore, with their multicellular and 3D component, LSC better represent the tissue composition *in vivo*. In head to head mice treatment with adipose-derived MSCs (ASCs) and LSC to treat bleomycin induced IPF, LSC were superior in reducing fibrotic thickening and tissue infiltration [55]. While cell infusion has shown promising results in rodent models, there are limitations due to risk associated with cell transplantation such as immune compatibility and infections. In previous stem cell studies, MSCs were thought to migrate to the sites of the injury and integrate into the damaged tissue to differentiate into specialized cells; however, only small portion of cells have been observed to survive in the damaged host tissue, and it was later believed that the MSCs might exert their therapeutic effects through secreted trophic factors [14]. A previous cytokine array study on LSC showed the presence of anti-apoptotic, proangiogenic factors, and cytokines in the conditioned media [55]; however, there has not been a comprehensive profiling of the paracrine proteome.

Secretome proteins are released by a cell, tissue or organism through classical (signal peptide-dependent) and non- classical secretion mechanisms [94]. In the classical secretion, proteins are transported through classical endoplasmic reticulum-Golgi secretory pathway and contain the ER signal sequence that directs their release into the extracellular environment through exocytosis [94][95]. The secretome may contain proteins that regulate cell-cell and cell to extracellular matrix interactions to affect the growth and productivity of cells via

paracrine/autocrine mechanism. Secreted proteins are encoded by approximately 10% of the human genome [94] and may include serum proteins, extracellular matrix proteins, growth factors, hormones, and chemokines that have a direct impact on targeted cells by regulating the immune response, cell growth, cell adhesion, differentiation, and angiogenesis. Currently, secretome have had increasing interest as potential biomarker and therapeutic targets in diseases [96]–[98]. Recently, secretome analysis of bone marrow derived- MSCs, adipose tissue derived- MSCs and ESCs have been performed to identify some common as well as unique protein profile; however, the secretome from LSC has not been characterized [97], [99].

In this study, we analyzed conditioned media of LSC from three human donors to determine comprehensive secretory proteome using mass spectrometry. The primary aim of this study was to identify proteins secreted in the conditioned media by LSC that have the potential to act as paracrine tissue repair factors, as determination of these biological factors secreted by LSC can provide a different dimension to study tissue regeneration. In a head to head comparison of MSCs and LSC conditioned media treatment in bleomycin induced fibrotic mice showed that LSC outperformed in reducing fibrotic thickening and tissue infiltration. The proteomic profile of LSC was like the protein profile of other stem cell sources with some potentially key regenerative proteins.

## **2.2 Methods and Materials**

### **2.2.1 Materials**

Human lung tissues were acquired from the Cystic Fibrosis and Pulmonary Disease Research and Treatment Center at University of North Carolina-Chapel Hill. Tissue culture reagents were purchased from Life technologies (Carlsbad, CA [www.lifetechnologies.com](http://www.lifetechnologies.com)),

Sigma- Aldrich (St. Louis, MO, [www.sigmaaldrich.com](http://www.sigmaaldrich.com)) and, Corning Life sciences (Corning, NY [www.corning.com/lifesciences](http://www.corning.com/lifesciences)). Sample preparation and analysis reagents were purchased from ThermoFisher Scientific (Waltham, MA [www.thermofisher.com](http://www.thermofisher.com)), New Objective (Woburn, MA [www.newobjective.com](http://www.newobjective.com)) and Promega (Madison, WI [www.promega.com](http://www.promega.com)).

### 2.2.2 Cell culture

LSC were generated as described previously [55]. Adherent LSC were plated in a T-175 cm<sup>2</sup> fibronectin coated plate in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies) supplemented with 20% fetal bovine serum (FBS; Corning Life Sciences, Acton, MA), 50 ug/mL of gentamicin, 2mmol/L of L-glutamine (Life Technologies) and 0.1 mmol/l 2-mercaptethanol (Life Technologies). Passage 2-8 LSC were used for all the *in vitro* analysis. The cells were grown to eighty-ninety percent confluency, approximately 10 million, before incubation in serum free IMDM for 24 hours, followed by six additional 30-minute incubation washes. LSC were conditioned in 15 mL of phenol red free IMDM for three days at 37°C. Conditioned media was collected and used for further analysis. For the time point studies, 2 mL of the conditioned media were collected after 24, 48, and 72 hours of incubation.

### 2.2.3 Conditioned media preparation

To concentrate the samples, 15 mL of the conditioned media were lyophilized. The samples dried to completion in 24 hours and were re-suspended in 1 mL 100mM ammonium bicarbonate (ABC; Sigma Aldrich) buffer. For protein precipitation, 10 mL of cold acetone (ThermoFisher Scientific) were added to the samples incubated at 20° C overnight and then centrifuged for 30 minutes at 10,000 rpm to separate the precipitated proteins from the supernatant. Total protein concentration was determined using Bradford Assay (Pierce,

ThermoFisher Scientific). All samples were loaded in triplicates onto a microtiter plate and protein concentrations estimated using reference absorbance of BSA standard protein.

Precipitated proteins were digested using Filter Aided Sample Preparation (FASP) method as described [100]. In a 30kDa molecular weight cutoff Vivacon filters (Sartorius, ThermoFisher Scientific), equal amount of proteins from each sample were added, reduced with 5mM dithiothreitol (DTT, Thermo Fisher Scientific) at 37°C for 30 minutes and alkylated with 10mM iodoacetamide (IAA; Sigma-Aldrich) for 20 minutes. Digestion was carried out using sequencing grade trypsin (Promega) at a 1:100 trypsin to protein ratio overnight at 37°C. Peptides were eluted with 100mM ABC, and solvent was evaporated using vacuum centrifugation before storage for further processing.

#### 2.2.4 Cell lysis

For the cellular proteomic analysis, cells were cultured as described previously in section 2.2.2. The adherent cells were detached from T-175 flask using TrypleSelect (Invitrogen, ThermoFisher Scientific) and cell culture media before spinning down for 10 minutes at 5000 rpm. To the pelleted cells, 500 uL of lysis buffer containing 10 mL of M-PER (ThermoFisher Scientific), 100 uL of Halt protease inhibitor cocktail (ThermoFisher Scientific), and 100 uL of 0.5mM ethylene diamine tetra-acetic acid (EDTA; ThermoFisher Scientific) were added. The samples were vortexed and lightly shaken for 10 minutes on ice, before centrifugation for 10 minutes at 14,000 rpm to get rid of the cell debris. The supernatant was collected for further analysis.

### 2.2.5 In-gel protein digestion

Supernatant from cell lysis were precipitated using acetone precipitation as described previously. For the protein concentration determination, Bicinchoninic Acid Assay (BCA assay) was used (ThermoFisher Scientific). To pre-separate proteins, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used. From each of the donors, 30 ug of protein was loaded onto a well of a Nupage 4-12% bis-tris gel (10mm x 10 well; Invitrogen, ThermoFisher Scientific), containing Nupage MES buffer (Invitrogen, ThermoFisher Scientific) and stained with Coomassie brilliant blue kit (Invitrogen, ThermoFisher Scientific). The proteins were digested using an in-gel digestion protocol [101]. Briefly, protein bands were excised from the gel and cut into cubes (1 X 1 mm). To the gel pieces, 200 uL of 50 mM ABC/CAN (Acetonitrile) (1:1, vol/vol) and rotated for 15-20 minutes. This wash step was repeated until the bulk of Coomassie stain was removed. Gel pieces were shrunk using 100 uL of neat acetonitrile before reduction using 100 uL of 10 mM DTT for 30 minutes at 37° C. For alkylation, 100 uL of 55 mM IAA were added to the tube and incubated for 20 minutes at room temperature. The reduction and alkylation mix were removed, and the gel pieces were washed with 500 uL of 50 mM ABC. The gel pieces were shrunk with 200 uL acetonitrile and swollen in 200 uL of 50 mM ABC. This process was repeated twice. The gel pieces were shrunk once more before addition of 50 uL of 10ng/uL of trypsin in 50mM ABC and digesting overnight at 37° C. The supernatant around the gels were removed and 50 uL of extraction buffer (1%FA (Formic Acid), 2% ACN) in H<sub>2</sub>O) was added to the pieces before vortex and sonicated for five minutes. The gel pieces were left in extraction solution for additional 30 minutes, vortexing occasionally, and the extraction solution was removed and combined with the initial supernatant. Samples were dried to completion before filtration. The digested samples were re-suspended in 30 uL of 0.1% FA in

H<sub>2</sub>O and sonicated for 60 seconds. The samples were filtered to remove any remaining gel particle using an in-house filter tip, then placed in an autosampler vial for LC/MS/MS analysis.

#### 2.2.6 LC/MS/MS data acquisition

All LC/MS/MS analysis were performed on an Easy nano ultra-pressure liquid chromatograph coupled to LTQ Orbitrap Elite mass spectrometer (ThermoFisher Scientific). Samples were injected onto a PepMap C18, 5µm 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% ACN/0.1% formic acid in water and mobile phase B was 0.1% formic acid in ACN. The Orbitrap Elite operated in a data-dependent mode, where the five most intense precursors were selected for subsequent fragmentations. 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 the 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 [24]. Monoisotopic precursor selection was enabled, and precursors with unknown charge or a charge state of +1 were excluded.

#### 2.2.7 Data analysis

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](http://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 cysteine was set as a fixed modification; oxidation of methionine was set as a variable modification. Scaffold (4.8.4 Proteome software, [www.proteomesoftware.com](http://www.proteomesoftware.com)) was used to filter the data, quantify peptides/proteins, and perform statistical analysis. A minimum of 2 peptides per proteins at a peptide and protein threshold of 95% was required for high confidence identification. Ingenuity Proteomic Analysis (IPA, QIAGEN Redwood City, [www.qiagen.com/ingenuity](http://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 .

## **2.3 Results**

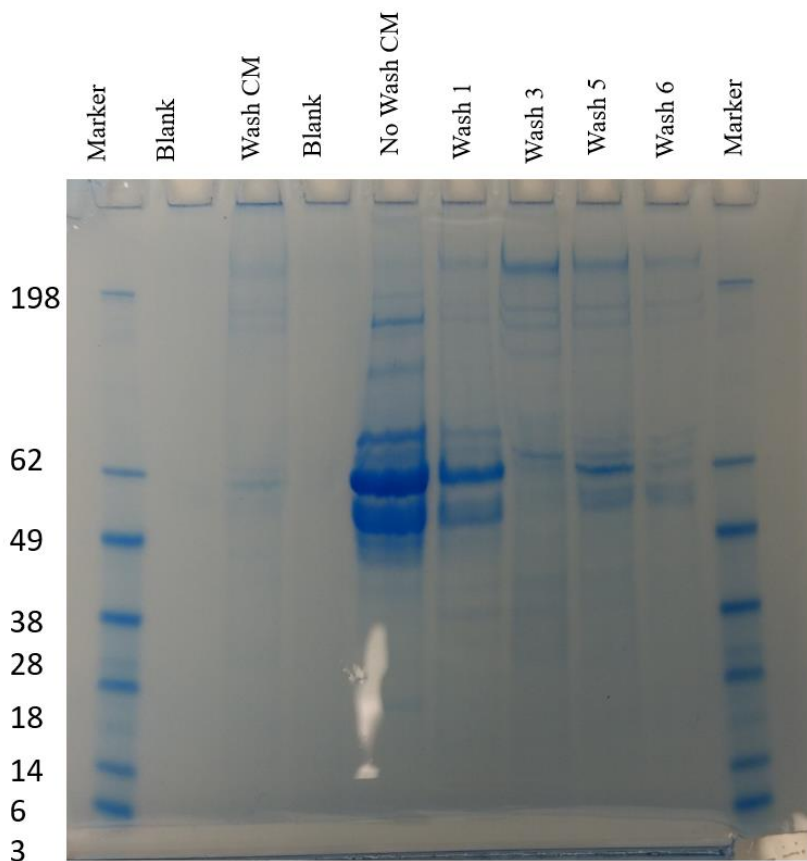
### **2.3.1 Method development for LSC conditioned media preparation for LC/MS/MS acquisition**

To determine a comprehensive secreted protein profile, conditioned media derived from the LSC of three different donors were analyzed. Donor comorbidity information is listed in Figure 20. One of the primary complications with the analysis of the secreted proteins was the contamination of the conditioned media by serum protein BSA, which is essential for cell culture. The secreted proteins tend to be at a lower concentration in the media, and with MS analysis that identifies the most abundant protein, the presence of BSA leads to suppression of other protein signals. To deplete the serum level of BSA, cells were incubated in serum free IMDM for 24 hours before conditioning for an additional 72 hours; however, the incubation itself was not enough to deplete all the BSA. To accommodate for this, six additional 30-minute

incubation washes with 10 mL of IMDM, after the initial incubation with the serum free media, was performed. Washing the cells for these 6 times depleted BSA and increased the sensitivity of the lower level secreted proteins. This was visually confirmed through gel electrophoresis (Figure 21), where the visible BSA band around 66.5 kDa representing BSA was monitored and shown to after each wash.

	sex	age	race	cause of death	smoking
<b>Lung Donor 1</b>	Female	50	Hispanic	Anoxia 2 <sup>nd</sup> Cardiovascular	No
<b>Lung Donor 2</b>	Female	52	Black	Cerebrovascular Accident	No
<b>Lung Donor 3</b>	Male	18	Hispanic	Head Trauma 2nd Self-Inflicted Gunshot Wound	No

**Figure 20:** Lung Donor information.

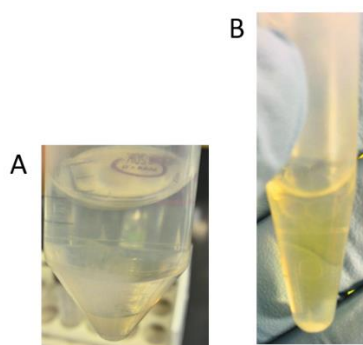


**Figure 21:** Depletion of BSA from CM.

Lane 1 and 10, MW standards; Lane 2 and 4, Blank; Lane 3 final conditioned media; Lane 5, conditioned media before any washes; Lane 6, first wash; Lane 7, third wash; Lane 8, fifth wash; Lane 9 sixth wash. The band around 65kDa is decreases in the washes.

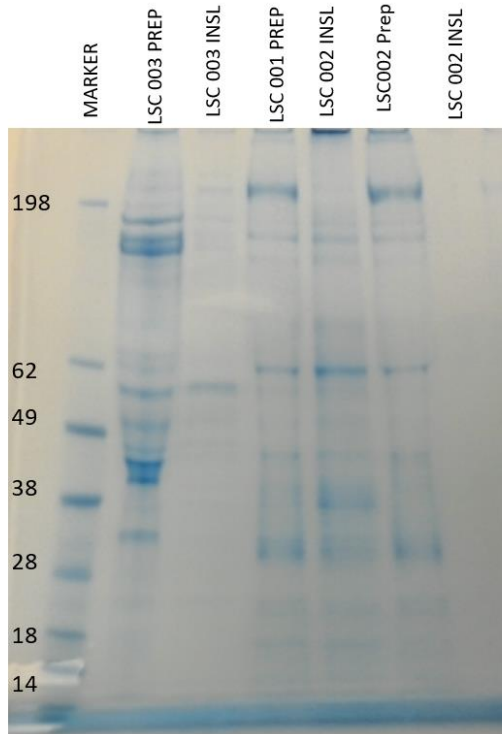
For mass spectrometry analysis, sample preparation steps are critical for detection of low level secreted proteins. A total of 15mL of conditioned media was collected from each 175 cm<sup>2</sup> flask, and due to this high volume, the sample had to be concentrated using a lyophilizer before further cleanup and concentration using acetone precipitation. However, addition of acetone to the lyophilized conditioned media created an acetone insoluble layer, regardless of the phenol red or phenol red free media (Figures 22b). The acetone insoluble layer was not soluble in organic solvents including: methanol, ethanol, and isopropanol, or in aqueous solvents such as 100 mM ABC and 8 M guanidine. Protein quantitation, using Bradford Assay, of the insoluble

layer showed the presence of almost equal amount of protein as the precipitate, which was further confirmed by gel electrophoresis (Figure 23). To bypass this issue conditioned media could be added to the FASP filter to directly concentrate the proteins on the filter. Direct concentration of the proteins on the FASP filter was performed successfully; however, due to the large volume of the conditioned media, it was not a feasible method, as the FASP filter reservoir holds a maximum volume of 500 uL. Additionally, sample cleanup of smaller volume of CM using only a vivacon FASP filter did not get rid of some of the contaminants present in the CM, which lead to the clogging of the LC columns and further contamination of the mass spectrometer itself. Other protein concentration techniques such as trichloroacetic acid (TCA) precipitation, methanol-chloroform extraction, and reversed-phase column concentration were also explored; however, both the precipitation methods did not produce a precipitate, and using a reversed-phase column, with both *in situ* digestion or just on-column concentration, the protein/peptide yield dropped to lower than 20%, making these techniques inefficient. The only method that produced consistent reproducible results, despite some issues with insolubility, was the lyophilization of the conditioned media followed acetone precipitation of the proteins (Figure 24).



**Figure 22:** Protein precipitation using acetone.

A) White precipitate B) yellow acetone insoluble layer.



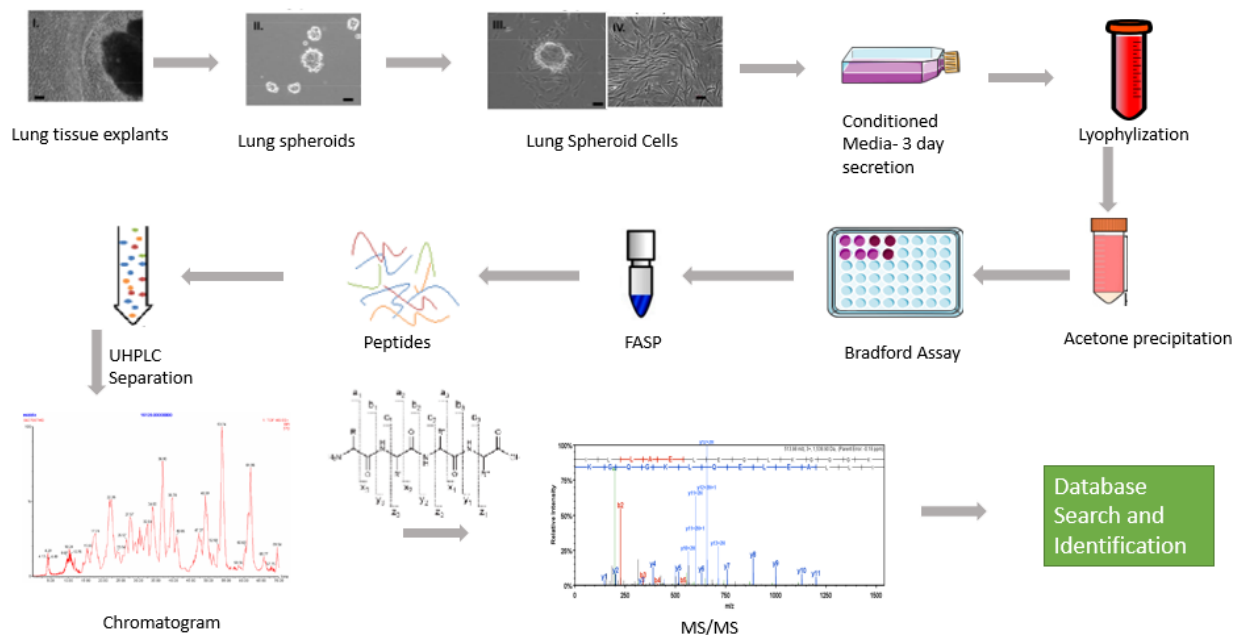
**Figure 23:** Acetone precipitated conditioned media.

Precipitation (prep) and insoluble layer (insl) for LSC 001, 002, and 003. Lane 1, molecular weight marker; Lane 2, LSC 003 prep; Lane 3 LSC 003 insl, Lane 4, LSC 001 prep; Lane 5, LSC 001 insl, Lane 6, LSC 002 prep; Lane 7, LSC 002 insl). The last three lanes of the gel were accidentally torn off during gel handling.

### 2.3.2 Secreted protein analysis with optimized sample preparation method

For LC/MS/MS analysis, 72-hour conditioned media from donors LSC were concentrated by lyophilization, then proteins were precipitated using acetone precipitation. The average secreted protein concentrations from approximately 10 million cells were 150 ug/mL for Donor 1 and Donor 2 and 200 ug/mL for Donor 3 (n=3). Filter aided sample preparation (FASP) was used to filter and concentrate the samples before *in situ* tryptic digestion. Bottom-up proteomic analysis of CM from the different donors were performed in triplicates using an orbitrap elite. Over 1000 proteins total, with 258 proteins commonly expressed in all three CM for each donor and 368 proteins commonly expressed in two out of three donors (Figure 25). This set of 368

proteins were used for further analysis as it represents high confidence LSC secretome profile, since the proteins considered must be observed in at least two of the donors and have two peptides detected with high mass accuracy.

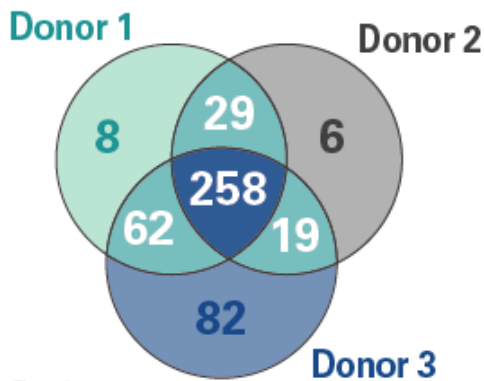


**Figure 24:** Optimized protein workflow for conditioned media analysis.

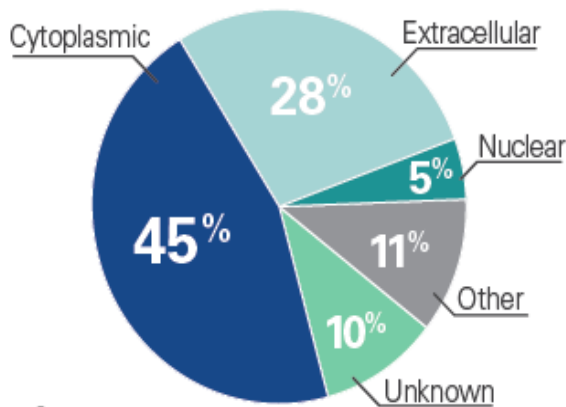
The lung tissue explants were seeded to form lung spheroid cells. The cells were conditioned in IMDM for 3 days to secrete. The CM was lyophilized, acetone precipitated, and quantitated using Bradford. Proteins were tryptically digested, and the resulting peptides were separated using uHPLC, coupled to an orbitrap mass spectrum. The LC/MS/MS data was analyzed with database search algorithms.

To focus on the proteins that were commonly secreted in the donors, Ingenuity Pathway Analysis (IPA) software was used to annotate and classify their subcellular location. IPA provides tools for analysis of signaling and metabolic pathways and was used not only to determine subcellular location of the proteins, but also the canonical pathways of the secreted proteins. Cellular localization component showed 45% of proteins to be cytoplasmic, 5% nuclear, 21% unknown or other and 28% of proteins were identified as extracellular proteins

(Figure 26). The majority of the proteins' subcellular localization was identified to be cytoplasmic, and is consistent with other reported MSC and ASC secretome profile [5]. Many of the secreted factors present in the list include well known factors linked with MSCs [96], [97], [102] and ASCs [103] with 49 of the annotated protein reported as secreted in a previous MSC conditioned media profile [97] and 46 secreted in various adipose derived stem cell secretome [103]. This demonstrates that LSC could contain stem cell like properties as the other derived stem cells, and it is comparable to MSC and ASC in terms of the proteins that are secreted.



**Figure 25:** Analysis of conditioned media proteins derived from Donor 1, 2, and 3.



**Figure 26:** Subcellular localization of CM proteins.

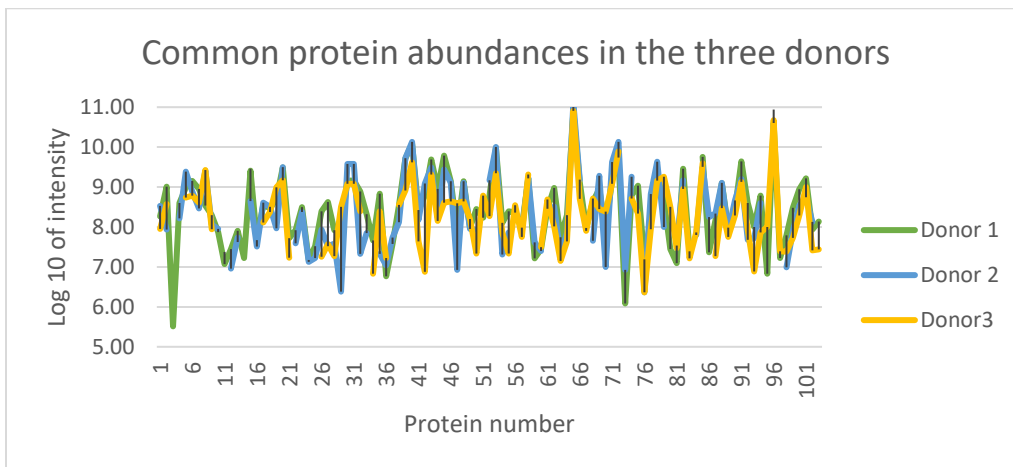
Subcellular localization of the proteins common in 2 out of 3 donors. Annotated using Ingenuity Pathway Analysis software.

### 2.3.3 LSC donor variability

To determine if CM can be used from autologous cell sources, and to determine if there is variation in the protein based on donors, LSC from three different donors were used for the study. Label free proteomics was used for comparison of proteins from the different samples. Overall, the proteins derived from each donor were present in similar abundance, defined by their relative intensity. Figure 27 shows relationship between the 103 extracellular proteins and the relative abundances between the three donors. It was also observed that the age of the donor from which the LSC were derived had an impact on cell proliferation, along with the protein concentration. In general, cells from Donor 3, the younger donor, had more cell proliferation and an increased number of proteins secreted compared to donors 1 and 2. However, even with the increase in total number of proteins, the number of extracellular proteins remained comparable. It is unknown if this donor LSC or their conditioned medium had a superior therapeutic potential. The coefficient of variation (CV), ratio of the standard deviation to the mean, was determined to examine the relationship between the three donors. Overall the data suggested the donors' protein abundances were similar, with some outliers that were present in each donor (Figure 28). For example, CV analysis determined that Annexin A5 protein, which is highest in Donor 2 and the lowest in Donor 1, had a 3 fold difference in abundance among the donors.

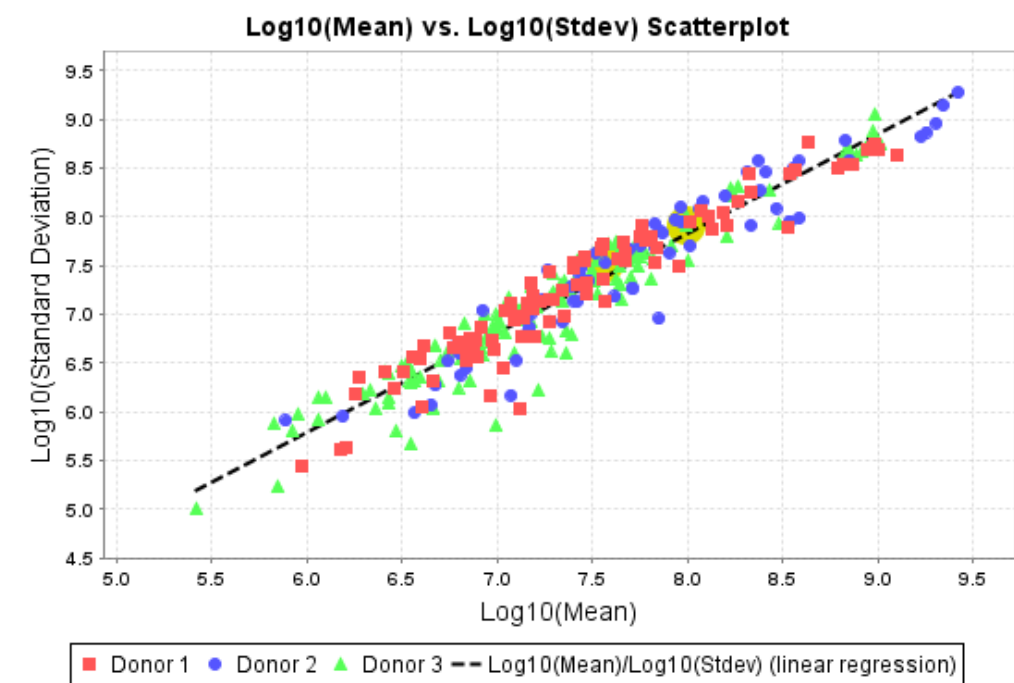
Further analysis of the donors was performed to determine the inherent differences between the individual donors, as well as the biological replicates. Donor 3 vs. Donor 2 samples show optimal protein abundances, with few outliers. Donor 2 vs. Donor 1 also shows most of the proteins falling at within one standard deviation of the mean abundance. These data show the tight correlation between the proteins present in the different donors; however, there is inherent variability in batch to batch processing. This variation could occur due to sample handling, the

amount of different types of cells present in the LSC in the flask at the time of secretion, or even with LC/MS/MS proteomic analysis as data dependent acquisition, where only the top five most intense ions during a scan event get fragmented, is used. This has a possibility of potentially missing some low abundant proteins. To avoid this, longer gradients for separation could be used. Furthermore, an ion can be placed on a dynamic exclusion list where if an ion is seen consecutively in three scans, it can be put on a fragment exclusion list for an indicated amount of time, usually 30 sec. This increases the chance of lower abundant proteins being fragmented. Regardless, presence of these common extracellular and cytoplasmic proteins in the conditioned media further demonstrated that humans secrete similar proteins from their respective LSC, and they could potentially be used as allogenic sources for regenerative therapy.



**Figure 27:** Protein relative abundances in the CM.

X axis: Protein number, Y axis: Log of intensity of donor proteins. Mean of the donor samples (n=3)



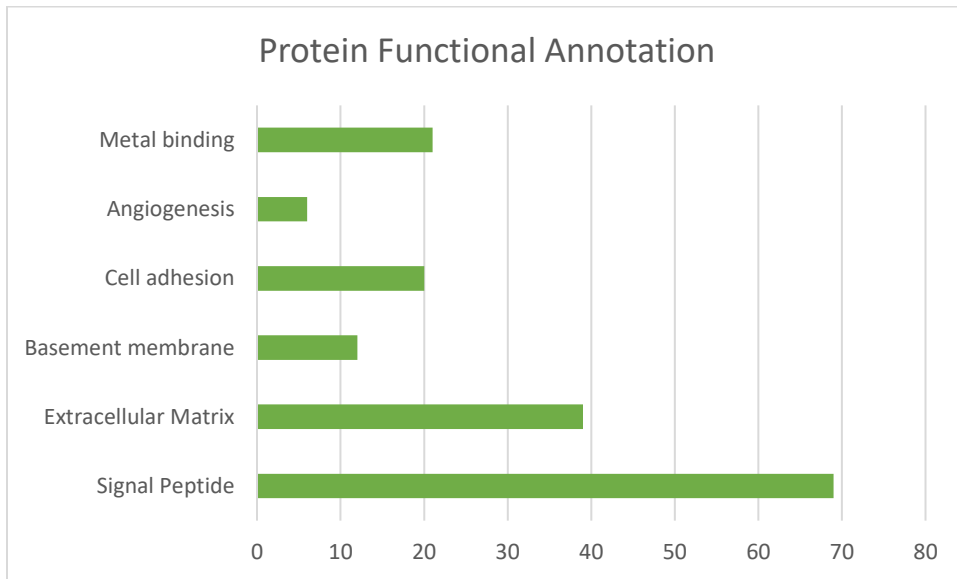
**Figure 28:** Mean vs Standard Deviation Plot for donor relative abundances.

X axis: log 10 of the mean value of the estimated protein abundances across all samples. Y axis: log 10 of standard deviation of the estimated protein abundance computed across all samples.

#### 2.3.4 Protein functional analysis

Paracrine signaling factors, implicated in cellular regeneration, are usually secreted by the cells into the extracellular environment; therefore, the 103 proteins that were annotated as being extracellular were explored further to look for these factors. Those proteins are listed in Table 1, along with their molecular weights and relative intensity for each donor. The list includes some known stem cell cytoskeletal proteins such as fibronectin (FN1), collagen (COL), extracellular matrix proteins (ECM) and laminin (LAMA). These proteins play a role in providing framework within a cell and have an impact on cell division, motility, and health (ncbi.nlm.nih.gov). Growth factors are exogenous signals that are important for cell growth and proliferation [108]. Growth factors present in the LSC conditioned media are insulin like growth factor protein 6 (IGFBP6), latent transforming growth factor beta binding protein 2 (LTEP2),

along with wound healing and repair protein such as thrombospondin 2 (THBS2), decorin (DCN), matrix metalloproteinase 2 (MMP2), granulins (GRN) and Type IV collagenase (COL4A2). Proteins such as alpha-2-macroglobulin (A2M), apolipoprotein E (APOE), hemoglobin subunit alpha 2 (HBA2), and extracellular matrix protein 1 (ECM1) were annotated as being transporters. In general, the most abundant proteins present in each of the donor samples fall into one of these three categories, which suggests the candidate proteins for therapeutic effects of LSC could be the combination of proteins from these pathways.



**Figure 29:** Protein functional analysis of extracellular sub-localized protein. Extracellular proteins common in two out of three donors (DAVID analysis).

Evaluation of protein functional annotation of LSC showed 58 factors, including some trophic factors such as alpha-2-macroglobulin (A2M), Serpin family E member 1 (SERPINE1), Tenascin C (TNC), TIMP metalloproteinase inhibitor 2 (TIMP2) as being involved in cell movement. There were 36 angiogenic factors including Latent transforming growth factor beta binding protein 1 (LTBP1), TIMP metalloproteinase inhibitor 1 (TIMP1), Vimentin (VIM), Cystatin C (CST3), as well as 68 proteins including Biglycan (BGN), Clusterin (CLU),

Peptidylprolyl isomerase A (PPIA), secreted protein acidic and cysteine rich (SPARC) for proliferation of cells.

**Table 1:** Proteins present in at least two out of three triplicate donor conditioned media annotated as being extracellular proteins by IPA.

The conditioned media proteins were analyzed using the optimized sample preparation method. Proteins in red are also shown in the published secretome profile of MSC and ASC conditioned media [80], [103].

Accession No	Symbol	Protein name	Molecular weight	Donor 1	Donor 2	Donor 3
A6NLG9_HUMAN	BGN	<b>biglycan</b>	35 kDa	3.95E+08	1.64E+08	0.00E+00
A8K5J8_HUMAN	C1R	<b>complement C1r subcomponent</b>	80 kDa	1.43E+09	7.77E+08	6.10E+08
COF2_HUMAN	CFL2	cofilin 2	19 kDa	7.28E+07	8.41E+06	3.93E+08
CH3L1_HUMAN	CHI3L1	chitinase 3 like 1	43 kDa	3.73E+07	5.43E+07	0.00E+00
CLC11_HUMAN	CLEC11A	<b>C-type lectin domain family 11 member A</b>	36 kDa	3.44E+08	1.33E+08	3.54E+08
COCA1_HUMAN	COL12A1	<b>Collagen alpha-1(XII) chain</b>	333 kDa	1.39E+09	1.27E+09	4.35E+08
COIA1_HUMAN	COL18A1	<b>collagen type XVIII alpha 1 chain</b>	333 kDa	1.39E+09	1.27E+09	4.35E+08
CO1A1_HUMAN	COL1A1	<b>collagen type I alpha 1</b>	139 kDa	5.17E+09	5.23E+09	8.14E+08
D3DTX7_HUMAN	COL1A1	<b>collagen type I alpha 1</b>	85 kDa	9.63E+09	1.00E+10	2.24E+09
CO1A2_HUMAN	COL1A2	<b>collagen type I alpha 2 chain</b>	129 kDa	1.21E+10	1.34E+10	4.14E+09
CO3A1_HUMAN	COL3A1	<b>collagen type III alpha 1 chain</b>	139 kDa	2.66E+08	1.49E+08	4.38E+07
CO4A2_HUMAN	COL4A2	<b>collagen type IV alpha 2</b>	168 kDa	6.11E+08	1.24E+09	7.53E+06
B2ZZ86_HUMAN	COL5A1	<b>collagen type V alpha 1</b>	184 kDa	8.08E+07	5.42E+07	4.17E+07
CO6A1_HUMAN	COL6A1	<b>collagen type VI alpha 1</b>	109 kDa	4.90E+09	3.10E+09	2.04E+09
CO6A2_HUMAN	COL6A2	<b>collagen type VI alpha 2</b>	109 kDa	9.02E+08	1.71E+08	1.42E+08
Q9BUM6_HUMAN	COL6A2	<b>collagen type VI alpha 2</b>	47 kDa	3.03E+08	7.98E+07	5.31E+07
CO6A3_HUMAN	COL6A3	<b>collagen type VI alpha 3 chain</b>	344 kDa	6.10E+09	2.76E+09	4.09E+08

**Table 1:** (Continued)

<b>CBPA4_HUMAN</b>	<b>CPA4</b>	<b>carboxypeptidase A4</b>	<b>47 kDa</b>	<b>5.87E+06</b>	<b>1.10E+07</b>	<b>1.68E+07</b>
<b>CSF1_HUMAN</b>	<b>CSF1</b>	<b>colony stimulating factor 1</b>	60 kDa	9.55E+07	8.95E+07	1.58E+08
<b>CYTC_HUMAN</b>	<b>CST3</b>	<b>cystatin C</b>	16 kDa	1.69E+08	0.00E+00	6.15E+08
<b>PGS2_HUMAN</b>	<b>DCN</b>	<b>decorin</b>	40 kDa	4.37E+09	1.58E+09	1.34E+09
<b>B4DID6_HUMAN</b>	<b>DKK3</b>	<b>dickkopf WNT signaling pathway inhibitor 3</b>	40 kDa	3.08E+09	3.13E+09	1.49E+09
<b>ECM1_HUMAN</b>	<b>ECM1</b>	<b>extracellular matrix protein 1</b>	61 kDa	1.65E+09	1.71E+09	2.06E+09
<b>EDIL3_HUMAN</b>	<b>EDIL3</b>	EGF like repeats and discoidin domains 3	54 kDa	1.65E+07	4.13E+07	0.00E+00
<b>A8KAJ3_HUMAN</b>	<b>EFEMP1</b>	<b>EGF containing fibulin like extracellular matrix protein 1</b>	55 kDa	8.93E+08	2.90E+08	4.09E+08
<b>B2RCM5_HUMAN</b>	<b>EFEMP2</b>	<b>EGF containing fibulin like extracellular matrix protein 2</b>	49 kDa	2.79E+07	9.00E+06	0.00E+00
<b>EMIL1_HUMAN</b>	<b>EMILIN1</b>	elastin microfibril interfacier 1	107 kDa	2.81E+07	2.52E+07	3.14E+07
<b>B4DUV1_HUMAN</b>	<b>FBLN1</b>	<b>fibulin 1</b>	70 kDa	4.21E+08	3.31E+07	3.68E+07
<b>FBLN1_HUMAN</b>	<b>FBLN1</b>	<b>fibulin 1</b>	77 kDa	9.54E+08	3.25E+08	1.03E+08
<b>FBLN5_HUMAN</b>	<b>FBLN5</b>	<b>fibulin 5</b>	50 kDa	5.52E+07	2.04E+07	1.40E+07
<b>FBN1_HUMAN</b>	<b>FBN1</b>	<b>fibrillin 1</b>	312 kDa	1.99E+08	7.01E+07	4.38E+07
<b>FINC_HUMAN</b>	<b>FN1</b>	<b>fibronectin 1</b>	263 kDa	8.14E+10	1.29E+11	8.12E+10
<b>H0Y4K8_HUMAN</b>	<b>FN1</b>	fibronectin 1	27 kDa	5.45E+09	1.35E+10	7.78E+09
<b>Q68CX6_HUMAN</b>	<b>FN1</b>	fibronectin 1/ thrombospondin 1 variant	235 kDa	4.05E+10	8.64E+10	4.71E+10
<b>Q6MZM7_HUMAN</b>	<b>FN1</b>	fibronectin 1/ uncharacterized protein	35 kDa	6.22E+07	9.82E+06	2.41E+07
<b>FSTL1_HUMAN</b>	<b>FSTL1</b>	<b>follistatin like 1</b>	35 kDa	1.32E+09	1.52E+09	5.06E+08
<b>B4DZY7_HUMAN</b>	<b>GAS6</b>	<b>growth arrest specific 6</b>	60 kDa	8.28E+07	3.80E+07	1.88E+07
<b>GPX3_HUMAN</b>	<b>GPX3</b>	glutathione peroxidase 3	26 kDa	8.81E+07	1.00E+07	2.44E+08
<b>B4DJI2_HUMAN</b>	<b>GRN</b>	<b>granulin</b>	57 kDa	5.20E+07	0.00E+00	1.70E+07
<b>B7Z4U6_HUMAN</b>	<b>GSN</b>	<b>gelsolin</b>	76 kDa	9.10E+07	2.40E+06	3.21E+08
<b>E9M4D4_HUMAN</b>	<b>HBA1/HBA2</b>	hemoglobin subunit alpha 2	11 kDa	2.28E+08	0.00E+00	3.49E+08
<b>G3V1N2_HUMAN</b>	<b>HBA1/HBA2</b>	hemoglobin subunit alpha 2	12 kDa	9.47E+07	0.00E+00	7.96E+07

**Table 1:** (Continued)

<b>HMCN1_HUMAN</b>	HMCN1	<b>hemicentin 1</b>	613 kDa	1.21E+06	8.50E+06	0.00E+00
<b>PGBM_HUMAN</b>	HSPG2	<b>heparan sulfate proteoglycan 2</b>	469 kDa	2.76E+08	4.61E+08	1.94E+08
<b>B3KRN4_HUMAN</b>	HTRA1	<b>HtrA serine peptidase 1</b>	48 kDa	4.67E+07	3.25E+07	0.00E+00
<b>A6XND0_HUMAN</b>	IGFBP3	<b>insulin like growth factor binding protein 3</b>	29 kDa	9.64E+08	2.41E+09	5.47E+08
<b>IBP4_HUMAN</b>	IGFBP4	<b>insulin like growth factor binding protein 4</b>	28 kDa	6.33E+08	1.81E+09	5.15E+08
<b>IBP5_HUMAN</b>	IGFBP5	<b>insulin like growth factor binding protein 5</b>	31 kDa	1.07E+09	0.00E+00	2.15E+08
<b>F8VYK9_HUMAN</b>	IGFBP6	<b>insulin like growth factor binding protein 6</b>	25 kDa	2.87E+08	0.00E+00	4.93E+08
<b>LAMA2_HUMAN</b>	LAMA2	laminin subunit alpha 2	344 kDa	1.56E+07	2.31E+06	2.31E+06
<b>B7Z938_HUMAN</b>	LAMA4	laminin subunit alpha 4	157 kDa	1.45E+09	3.77E+09	1.20E+09
<b>H0UI49_HUMAN</b>	LAMA4	<b>laminin subunit alpha 4</b>	202 kDa	1.54E+09	4.24E+09	1.21E+09
<b>G3XAI2_HUMAN</b>	LAMB1	<b>laminin subunit beta 1</b>	200 kDa	8.90E+08	1.93E+09	2.83E+08
<b>LAMB2_HUMAN</b>	LAMB2	laminin subunit beta 2	196 kDa	1.49E+08	6.70E+08	8.84E+07
<b>LAMC1_HUMAN</b>	LAMC1	<b>laminin subunit gamma 1</b>	178 kDa	2.05E+09	4.28E+09	1.46E+09
<b>LEG1_HUMAN</b>	LGALS1	<b>galectin 1</b>	15 kDa	4.81E+08	9.96E+07	1.82E+09
<b>LEG3_HUMAN</b>	LGALS3	<b>lectin, galactoside binding soluble 3</b>	26 kDa	2.79E+07	0.00E+00	3.20E+08
<b>LYOX_HUMAN</b>	LOX	lysyl oxidase	47 kDa	2.47E+07	0.00E+00	1.63E+07
<b>LOXL2_HUMAN</b>	LOXL2	<b>lysyl oxidase like 2</b>	87 kDa	1.25E+07	3.42E+07	2.95E+07
<b>B7ZLY3_HUMAN</b>	LTBP1	<b>latent transforming growth factor beta binding protein 1</b>	148 kDa	7.74E+08	2.13E+07	2.45E+08
<b>C9JD84_HUMAN</b>	LTBP1	latent transforming growth factor beta binding protein 1	147 kDa	6.78E+08	2.13E+07	2.45E+08
<b>G3V3X5_HUMAN</b>	LTBP2	<b>latent transforming growth factor beta binding protein 2</b>	190 kDa	4.31E+08	4.51E+07	5.20E+08
<b>LUM_HUMAN</b>	LUM	<b>lumican</b>	38 kDa	2.89E+09	1.49E+09	8.95E+08

**Table 1:** (Continued)

<b>B4DUI7_HUMAN</b>	MASP1	<b>mannan binding lectin serine peptidase 1</b>	41 kDa	2.48E+08	8.63E+07	1.80E+07
<b>Q6MZL2_HUMAN</b>	MASP1	<b>mannan binding lectin serine peptidase 1/ uncharacterized protein</b>	11 kDa	1.66E+07	0.00E+00	2.45E+07
<b>B3KTQ2_HUMAN</b>	MFGE8	<b>milk fat globule-EGF factor 8 protein</b>	42 kDa	3.63E+08	4.08E+08	1.29E+08
<b>MIF_HUMAN</b>	MIF	macrophage migration inhibitory factor	12 kDa	0.00E+00	6.30E+07	7.32E+07
<b>B4DN15_HUMAN</b>	MMP1	matrix metallopeptidase 1	46 kDa	3.11E+08	2.39E+08	2.70E+08
<b>MMP2_HUMAN</b>	MMP2	<b>matrix metallopeptidase 2</b>	74 kDa	5.67E+09	3.23E+09	3.91E+09
<b>MMP3_HUMAN</b>	MMP3	matrix metallopeptidase 3	54 kDa	2.33E+07	1.76E+08	0.00E+00
<b>NID1_HUMAN</b>	NID1	<b>nidogen 1</b>	136 kDa	1.47E+08	2.15E+08	1.87E+07
<b>NID2_HUMAN</b>	NID2	<b>nidogen 2</b>	151 kDa	1.25E+09	1.28E+09	2.96E+08
<b>B4DNG0_HUMAN</b>	OLFML3	<b>olfactomedin like 3</b>	39 kDa	1.49E+07	1.33E+07	0.00E+00
<b>B4DTA8_HUMAN</b>	PAPPA	pappalysin 1	74 kDa	3.32E+07	1.66E+07	0.00E+00
<b>A4D2D2_HUMAN</b>	PCOLCE	<b>procollagen C-endopeptidase enhancer</b>	48 kDa	1.02E+09	8.63E+07	3.77E+08
<b>B3KUE5_HUMAN</b>	PLTP	phospholipid transfer protein	57 kDa	2.91E+08	3.21E+08	2.37E+08
<b>C0IMJ3_HUMAN</b>	POSTN	<b>periostin</b>	87 kDa	2.09E+08	7.07E+07	0.00E+00
<b>PRELP_HUMAN</b>	PRELP	proline and arginine rich end leucine rich repeat protein	44 kDa	3.98E+08	4.77E+07	8.44E+07
<b>B4DEK5_HUMAN</b>	PSAP	<b>prosaposin</b>	51 kDa	5.08E+08	9.37E+07	9.93E+08
<b>PTX3_HUMAN</b>	PTX3	<b>pentraxin 3</b>	42 kDa	9.95E+07	5.28E+07	7.73E+06
<b>PXDN_HUMAN</b>	PXDN	<b>peroxidasin</b>	165 kDa	6.15E+08	4.00E+08	7.25E+07
<b>B2R950_HUMAN</b>	PZP	PZP, alpha-2-macroglobulin like	164 kDa	1.17E+07	0.00E+00	2.39E+07
<b>B7ZAB0_HUMAN</b>	SERPINE1	<b>serpin family E member 1</b>	157 kDa	1.45E+09	3.77E+09	1.21E+09
<b>B4DMR3_HUMAN</b>	SERPINE2	<b>serpin family E member 2</b>	37 kDa	8.27E+07	3.90E+07	0.00E+00
<b>PEDF_HUMAN</b>	SERPINF1	<b>serpin family F member 1</b>	17 kDa	9.59E+07	7.78E+07	5.59E+07

**Table 1:** (Continued)

<b>E9PIG2_HUMAN</b>	SERPINH1	<b>serpin family H member 1</b>	17 kDa	9.59E+07	7.78E+07	5.59E+07
<b>D3DQH8_HUMAN</b>	SPARC	<b>secreted protein acidic and cysteine rich</b>	36 kDa	6.14E+08	1.47E+09	1.89E+08
<b>SPON2_HUMAN</b>	SPON2	<b>spondin 2</b>	36 kDa	8.88E+08	3.46E+08	1.93E+08
<b>B3KNF2_HUMAN</b>	STC2	stanniocalcin 2	32 kDa	1.68E+07	0.00E+00	0.00E+00
<b>C9JBB3_HUMAN</b>	TFPI	tissue factor pathway inhibitor	33 kDa	4.60E+07	0.00E+00	6.80E+06
<b>TSP2_HUMAN</b>	THBS2	<b>thrombospondin 2</b>	130 kDa	8.58E+07	1.34E+08	2.59E+07
<b>B3KQF4_HUMAN</b>	TIMP1	<b>TIMP metalloproteinase inhibitor 1</b>	23 kDa	2.59E+09	4.43E+08	2.89E+09
<b>TIMP2_HUMAN</b>	TIMP2	<b>TIMP metalloproteinase inhibitor 2</b>	24 kDa	1.65E+09	5.53E+08	9.96E+08
<b>D3YHM4_HUMAN</b>	TNC	<b>tenascin C</b>	201 kDa	1.26E+08	2.06E+07	7.19E+07
<b>TWSG1_HUMAN</b>	TWSG1	twisted gastrulation BMP signaling modulator 1	25 kDa	1.38E+08	0.00E+00	2.76E+07
<b>CSPG2_HUMAN</b>	VCAN	<b>versican</b>	373 kDa	2.79E+08	8.41E+07	2.17E+07
<b>D6RGZ6_HUMAN</b>	VCAN	<b>versican</b>	136 kDa	2.46E+08	7.26E+07	2.17E+07
<b>Q53GN4_HUMAN</b>	WDR1	<b>WD repeat domain 1</b>	66 kDa	6.77E+06	0.00E+00	1.00E+08

Protein functional analysis, using DAVID, also showed 69 proteins contained a signal peptide, where the synthesized proteins contain a signal peptide at the N terminus and are translocated into the lumen of ER, where using the endoplasmic reticulum and Golgi complex, the proteins are released by exocytosis. However, there are also over 30 proteins that are not annotated as containing a signal peptide. Recently, there have been studies in both plants and animals that show proteins can be secreted to the extracellular space using non-classical pathways and do not contain signal peptides. The exact mechanism of secretion of these proteins is unknown and is speculated to be via exosome process.

While majority of the proteins present in the LSC CM were common with the MSCs and ASCs CM, there were also some proteins, annotated as being extracellular, potentially exclusive

to LSC (Table 2). For example, proline and arginine rich end leucine rich receptor protein is important to anchor basement membrane to underlying connective tissue. Some of the isoforms of these proteins are present in the other CMs, but proteins such as carboxypeptidase A4, laminin subunit alpha 2, and twisted gastrulation BMP signal modulator 1 have some wound healing activity and are present in the LSC CM. These proteins, in combination with the other secreted proteins, might give LSC a therapeutic edge over other stem cells.

**Table 2:** List of potentially LSC-exclusive extracellular proteins. Functions obtained from uniprot.org.

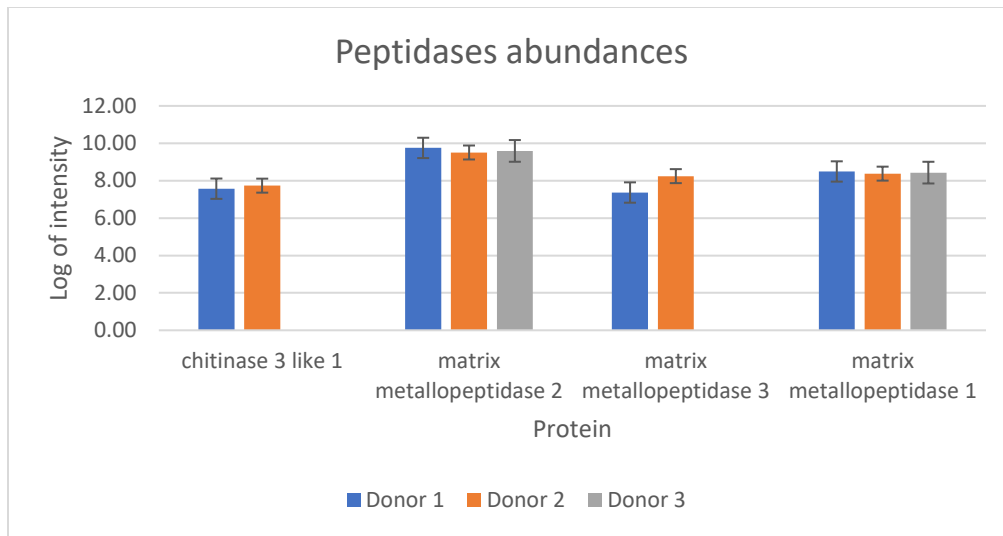
Protein	Function
Cofflin 2	Actin polymerization and depolymerization. Intracellular and actin rods
Carboxypeptidase A4	Metalloprotease for involved in histone deacetylation
elastin microfibril interface 1	Formation of elastic fibers
Laminin subunit alpha 2	Mediation of attachment, migration and organization of cells
Proline and arginine rich end leucine rich repeat protein	Anchoring of basement membrane to connective tissue
Serpin family H member 1	Binds to collagen; involved as a chaperone
Stanniocalcin 2	Action on calcium and phosphate homeostasis
Tissue factor pathway inhibitor	Antithrombotic action
Twisted gastrulation BMP signal modulator 1	Role in thymocyte development
Mannan-binding lectin serine protease 1	Role in recognizing pathogens through sugar moiety

### 2.3.5 Pathways characterization

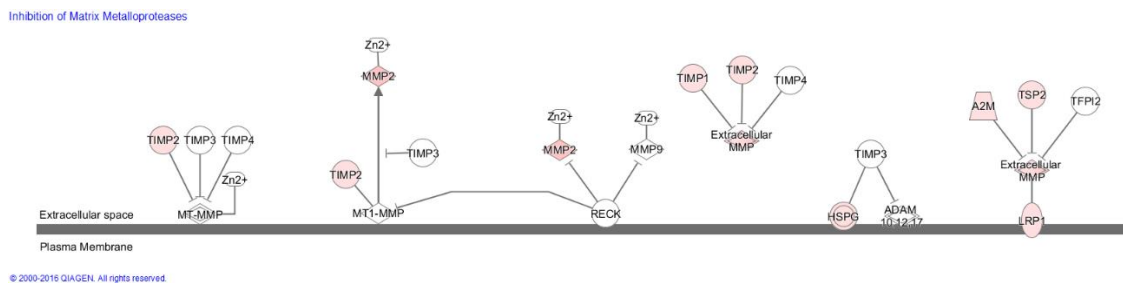
To better understand the functional characteristics of the secreted proteins, the commonly identified extracellular proteins were also classified according to pathways they impact. Some of the common known pathways these proteins are involved in include hepatic fibrosis and matrix metalloprotease activity (Figure 30). Hepatic fibrosis represents wound healing response to liver

injury by response of various proteins [109]. Proteins involved in the injury repair models include extracellular matrix remodeling molecules such as SPARC, thrombospondin 1 and 2, and fibrilin along with proteoglycans such as biglycan, decorin, heparin sulphate proteoglycan, and lumican. For example, proteoglycans are present in extracellular matrices of connective tissues and provide hydration and swelling pressure to tissue allowing it to withstand compression forces [110].

Matrix metalloproteinases, matrixins, are proteins that function in degradation of extracellular matrix molecules to release growth factors for cell receptors [111]. Humans have 24 matrixin genes that are transcriptionally controlled by cytokines, hormones, and cell-cell interactions. These matrix metalloproteinases are known for degrading stiffness and fibrosis inducing extracellular matrix and this pathway has been shown to cause long lasting structural and functional alteration in cardiac muscles [112]. IPF is defined by the presence of fibrosis and scarring in lungs and MMPs might be some critical proteins recruited by the cells to combat this damage. Inhibition of this pathway could prevent alteration and reorganization in lungs due to fibrosis. MMP1, found in LSC, is a collagenase that digest other ECM molecules and soluble proteins. MMPs are complicated as they have various substrates and their activities are also regulated by inhibitors such as alpha-2-macroglobulin and TIMPs, most of which are also present in the conditioned media of LSC. Comparison of some of these peptidases across the different donors shows similar relative abundances (Figure 30). Additionally, Figure 31 shows the pathway and the proteins involved in the matrix metallopeptidase pathway.



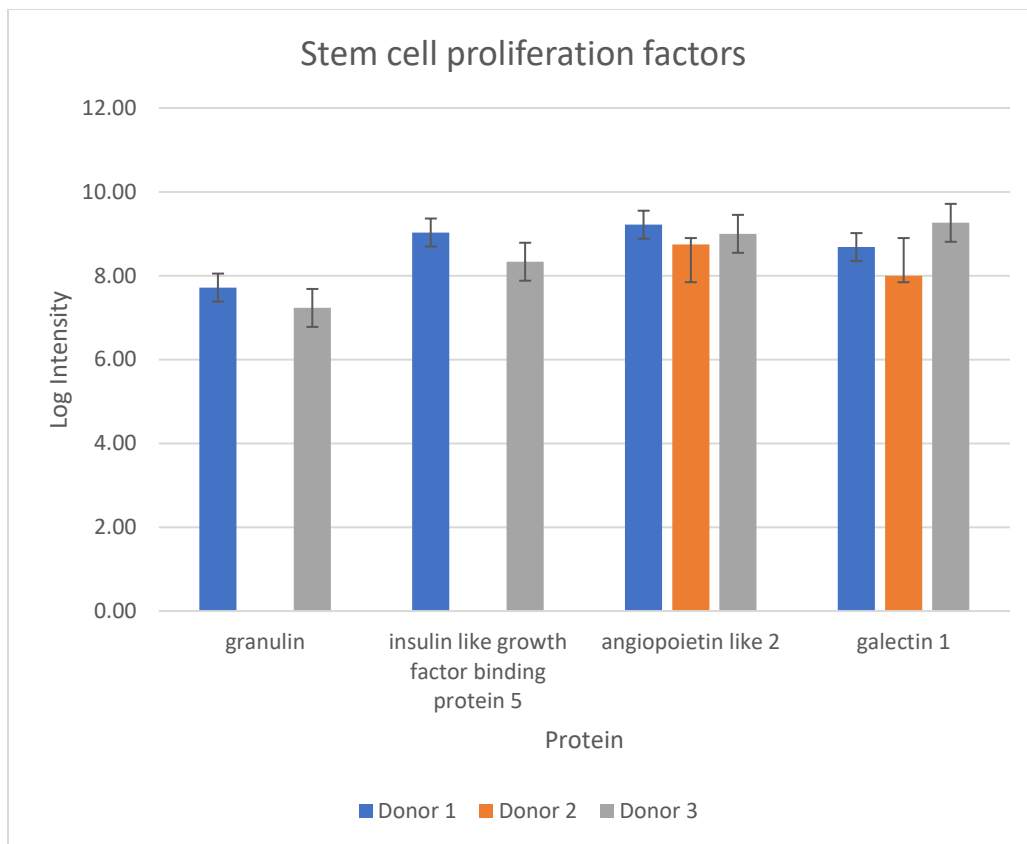
**Figure 30:** Abundances of matrix metallopeptidase proteins.



**Figure 31:** Proteins present in conditioned media (pink) affecting the matrix metallopeptidase pathway.

Other more abundant proteins that were present in the conditioned media were growth factor and stem cell proliferation factors. Platelet derived growth factor and vascular endothelial growth factors are some that are involved in formation of new blood vessels, regulation of cell growth, and cell proliferation (Figure 32). For stem cell proliferation, granulins, insulin-like growth factor 1, galectin and angiopoietin related protein 2, were present. Comparison of these protein abundances between the different donors indicates that the abundances were similar across the donors. Angiopoietin like 2 had a lower abundance in Donor 2, but not significantly so. Furthermore, there were also some proteins, such as granulin, that were just present at least

two out of three donors (either 1 and 2, or 1 and 3 etc.) consistently between all the different sample analysis. This might suggest that these proteins are not present in the third donor; however, low abundance of the proteins may preclude detection in all three samples. The specific variability could also be due to the donors themselves as it shows that the older donor (Donor 2) did not secrete some of the stem cell proliferation factors (Figure 32), and the youngest donor (Donor 3) did not secrete some peptidases (Figure 31). Additionally, for more stringent analysis, proteins containing only two peptides and higher were considered. These proteins might only be identified by one peptide, and further data mining is necessary to determine this possibility.

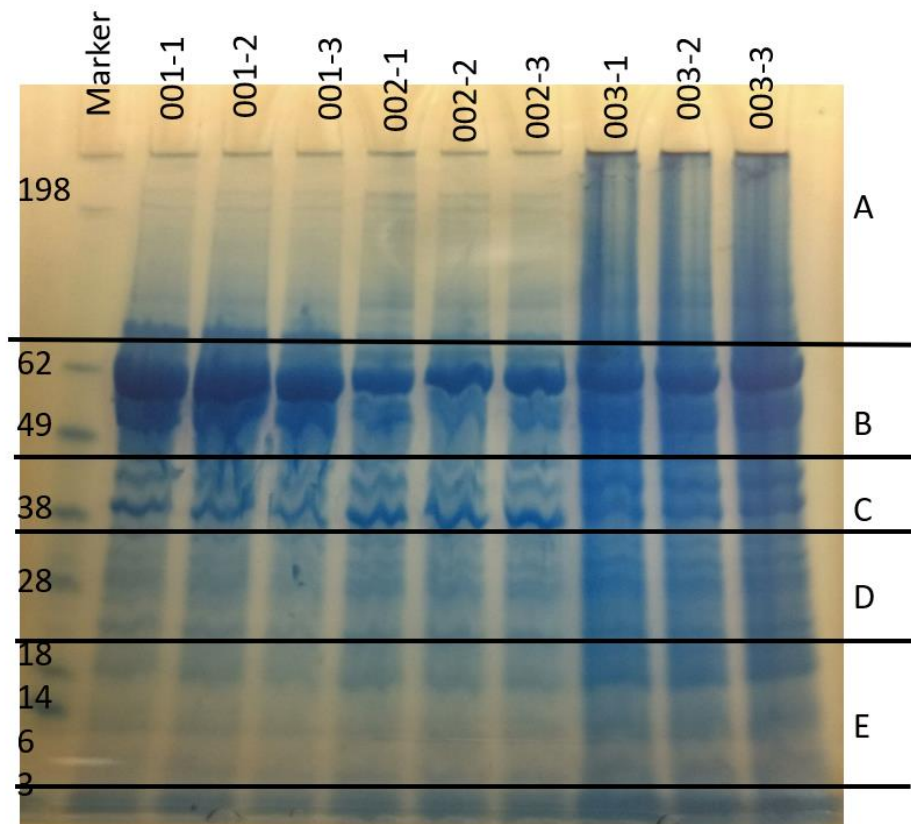


**Figure 32:** Abundances of stem cell growth factors across all donors.

### 2.3.6 Cytoplasmic protein comparison

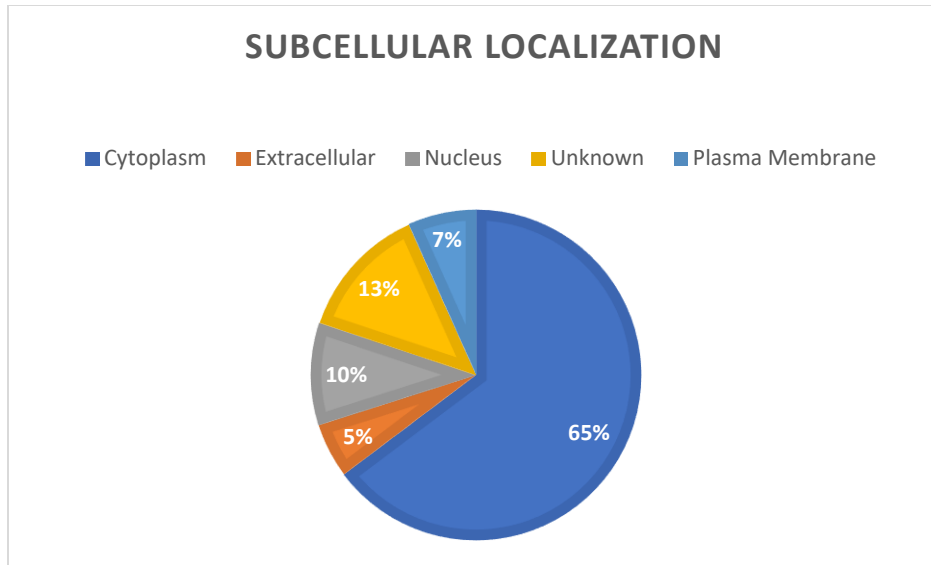
In the secretome, nearly half of the proteins are annotated as cytoplasmic and include cytoskeletal proteins and heat shock proteins. However, it could not be accurately determined if these intracellular proteins were released due to unavoidable cell death or unconventional secretion pathways, like exosome secretion. Exosomes are membrane vesicles that are released extracellularly and are known to participate in pathological processes, though their exact function is not completely understood [113]. Exosomes contain chaperones, cytoskeletal proteins, and enzymes which is consistent with the proteins present in the conditioned media that were classified as cytoplasmic. Folding proteins such as calreticulin, heat shock 70kDa protein, elongation factor 1-alpha 1 are some of the proteins present in the conditioned media that were annotated as being cytoplasmic but are present in secreted media [80] [103]. To determine the secretion factors that could be present in the conditioned media due to cell lysis and elucidate a more comprehensive secretome profile, parallel analysis of cytoplasmic proteins, obtained by lysing the cells, was performed. LSC were lysed using M-PER, and proteins were concentrated using acetone precipitation, fractionation using SDS-PAGE, and the excised section of each lane were subjected in-gel tryptic digestion (Figure 33) before analysis by LC/MS/MS. Three replicate lanes from each donor was analyzed and yielded 448 commonly expressed proteins. Out of these proteins, only 5% were annotated as being extracellular, and contained mostly extracellular matrix proteins such as collagen (Figure 34). Comparison of the cytoplasmic proteins from this analysis to the cytoplasmic proteins identified from conditioned media analysis showed 56 common proteins, most of which were cytoskeletal proteins such as myosin, tropomyosin, vimentin or proteins involved in folding such as those of the heat shock protein family (Figure 35). These proteins are generally found in both the intracellular and extracellular

spaces for cell support and structure; therefore, it is likely that they were not present in the conditioned media due to cell lysis. The cellular protein composition contained mostly ribosomal proteins, proteasomes, and histones, and lack of those being defined in the conditioned media showed there was minimal cell lysis or these “contamination” proteins were present at a low enough level to elude detection. In fact, some of these structural proteins could be some of the key proteins involved in tissue regeneration.

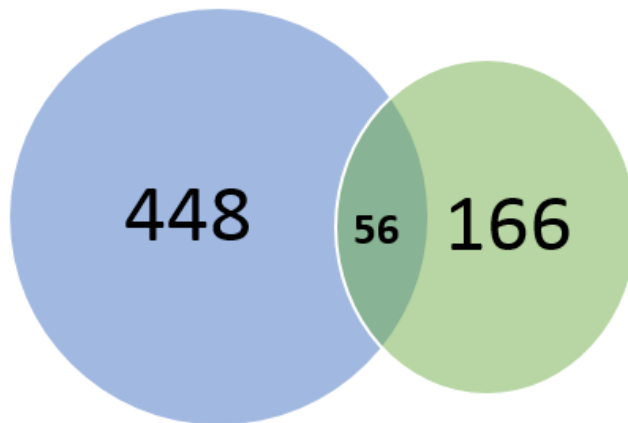


**Figure 33:** SDS PAGE of LSC cytoplasmic fractions.

The line represents the location of excision for the in-gel digestion of the proteins. Lane 1, molecular weight marker; Lane 2,3,4 are cytoplasmic extracts obtained from lysis of Donor 1 LSC; Lane 5,6,7 are extracts obtained from Donor 2 LSC; and Lane 8,9,10 are extracts from Donor 3 LSC.



**Figure 34:** Subcellular localization of the proteins present in the cytoplasmic fraction of LSC.



**Figure 35:** Cytoplasmic proteins from cell lysis and secretome.

cell lysis (cytoplasmic fraction, blue) to proteins annotated as being cytoplasmic in the conditioned media (green).

## 2.4 Discussion

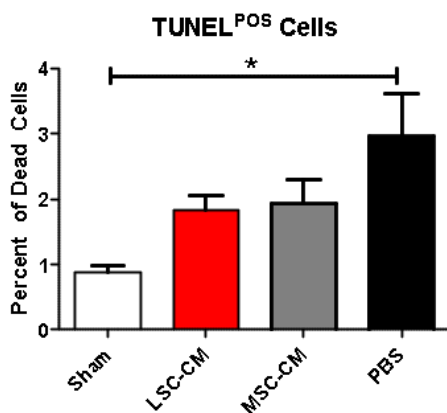
LSC are isolated from healthy lung tissues and are a robust and reproducible method to generate a natural mixture of lung progenitor cells, mesenchymal stem cells, airway progenitor cells, and alveolar progenitor cells. LSC can be used as autologous source of proteins and do not have inherent ethical or political issues that arise from ESCs and MSCs. Previous study using LSC

derived from rats showed that allogenic cell sources could be used for treatment of bleomycin-induced fibrotic rats, further showing the potential of LSC as cell therapy sources for individuals with idiopathic pulmonary fibrosis. Furthermore, lung spheroids have been used to grow lung cancer cells, and spheroids from healthy lungs to generate therapeutic lung progenitor cells that represents the lung niche in vivo [114], [115]. Human LSC injected in immune deficient fibrotic mice have shown tissue repair, but even with the regeneration, the underlying mechanism of action remains unclear. It is speculated that LSC secrete paracrine factors to modulate the environment and recruit an endogenous repair mechanism [55]. A previous cytokine array study showed that human LSC secreted proangiogenic factors such as insulin-like growth factor binding protein 2 and hepatocyte growth factor [55]. Proteomic analysis of both MSCs and ASCs, derived from varied sources, have been performed, but LSC have not been explored previously.

In this study, proteomic analysis of conditioned media from cultured LSC, isolated from three healthy human donor lung tissues, was analyzed using mass spectrometry to identify paracrine factors secreted by the cells. Compared to cell cultures, conditioned media are less likely to have inherent limitations such as the need to preserve and maintain cell viability and bypass the risks associated with cell transplantation. Many experiments on MSCs have reported beneficial outcome from infusion of the MSCs derived from various cultures and therapeutic effects through secreted factors have also been explored [97], [106], [116], [117]. As with MSCs, comprehensive profile of LSC secreted proteins can provide biological insights, including the potential for identification of therapeutic proteins or combination thereof.

One major goal of the study was to use proteomics to compare LSC derived from different donor lungs. The different donors were prepared using the same batches of medium, protocols, buffer and analyzed at the same time to minimize the heterogeneity related to different

procedures, columns, and instrument sensitivity. Comparison of the list of proteins secreted by LSC to that of conditioned media protein from various stem cells in the literature show a high similarity between these different cell lines, demonstrating their stem cell like properties. However, this raises the question for use of LSC and MSC interchangeability in therapy, and the progression to define the LSC signature proteins will require more studies, and comparison of the protein abundances with MSCs and other stem cells. To test the regenerative potential of LSC conditioned media *in vivo*, immunodeficient mice with bleomycin-induced pulmonary fibrosis were treated with either control, MSC or LSC conditioned media by nebulization. Head to head comparison of MSC to LSC conditioned media treatment showed that LSC outperformed in reducing fibrotic thickening and tissue infiltration, and the percent of dead cells were higher in the MSCs (Figure 36, Dinh). Infusion of VEGFA, IL6, or FGF2, candidate therapeutic factors secreted by MSC CM, did not completely substitute the effects of MSCs, suggesting there are combination of factors necessary for full substitution for MSC therapy [97]. Further studies are required to profile the secreted proteins that can potentially replace cell therapy.



**Figure 36:** Conditioned media treatment on fibrotic mice.

Based on the functional annotation of the extracellular proteins, there are some possible candidate protein that are implicated in wound repair and tissue modeling, which might be proteins to be explored further, such as 72 kDa type IV collagenase, a protein that is known for remodeling inflammation and tissue repair. Granulins is another type of protein with cytokine like activity that has role in inflammation, wound repair and remodeling along with proliferation of epithelial cell line. The full list of potentially key proteins, along with their function, are present in Table 3. To determine if these proteins are indeed the key ones for regeneration, further studies performed by isolating these proteins or generation of a cocktail of these proteins with subsequent injection into mice model of fibrosis will be required.

**Table 3:** Potential key proteins secreted by LSC for reduction of lung tissue scarring and promoting repair.

Most of these proteins are involved in the hepatic fibrosis and/or matrix metalloproteinase inhibition pathways. Function determined by the annotation contained in the Uniprot database ([www.uniprot.org](http://www.uniprot.org)).

<b>Protein</b>	<b>Function</b>
<b>Metalloprotease Inhibitor 1</b>	<ul style="list-style-type: none"> <li>○ Forms complexes with collagenase and irreversibly inactivates them by binding to their catalytic zinc cofactor.</li> <li>○ Functions as a growth factor to regulate cell differentiation, migration, and cell death.</li> </ul>
<b>Sulfhydryl oxidase 1</b>	<ul style="list-style-type: none"> <li>○ Secreted and in Golgi</li> <li>○ Oxidation of sulfhydryl groups in peptide and protein thiols to disulfide.</li> <li>○ Contributes to disulfide bond formation in variety of secreted proteins</li> <li>○ Tumor suppressing capabilities being involved in growth regulation</li> </ul>
<b>72 kDa type IV collagenase</b>	<ul style="list-style-type: none"> <li>○ Remodeling of vasculature, tissue repair, tumor invasion, inflammation and plaque rupture.</li> </ul>
<b>Carboxypeptidase A4</b>	<ul style="list-style-type: none"> <li>○ Metalloprotease that could be involved in the histone hyperacetylation pathway</li> </ul>

**Table 3:** (continued)

<b>Angiopoietin related protein 2</b>	<ul style="list-style-type: none"><li>○ May play a role in the wound healing process. May promote epidermal proliferation, remodeling and regeneration. May promote the chemotactic activity of endothelial cells and induce neovascularization.</li></ul>
<b>Galectin 1</b>	<ul style="list-style-type: none"><li>○ May regulate apoptosis, cell proliferation and cell differentiation. Binds beta-galactoside and a wide array of complex carbohydrates</li></ul>
<b>Biglycan</b>	<ul style="list-style-type: none"><li>○ Involved in collagen fiber assembly.</li></ul>
<b>Nidogen</b>	<ul style="list-style-type: none"><li>○ Secreted- basement membrane</li><li>○ Sulfated glycoprotein widely distributed in basement membranes.</li><li>○ Binds to collagen and perlecan</li><li>○ Cell-extracellular matrix interactions</li></ul>
<b>Cathepsin B</b>	<ul style="list-style-type: none"><li>○ Thiol protease participate in intracellular degradation and turnover of proteins.</li><li>○ Implicated in tumor invasion and metastasis.</li><li>○ Hydrolysis of protein with broad specificity</li></ul>
<b>Granulins</b>	<ul style="list-style-type: none"><li>○ Cytokine like activity, role in inflammation, wound repair, tissue remodeling.</li><li>○ Proliferation of epithelial cell line.</li></ul>
<b>Decorin</b>	<ul style="list-style-type: none"><li>○ Affects rate of fibrilis formation</li></ul>
<b>Insulin like growth factor binding protein</b>	<ul style="list-style-type: none"><li>○ Have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture. They alter the interaction of IGFs with their cell surface receptors</li></ul>
<b>Metalloprotease inhibitor 2</b>	<ul style="list-style-type: none"><li>○ Complexes with metalloproteinases and irreversibly inactivates them by binding to catalytic zinc cofactor. There are many different types</li></ul>
<b>Latent transforming growth factor beta binding protein 1 and 2</b>	<ul style="list-style-type: none"><li>○ Assembly secretion and targeting of TGFB 1 to sites where it is stored.</li><li>○ Control and direction of activity of TGFB1</li><li>○ Structural role in extra cellular matrix</li></ul>
<b>Extracellular matrix protein 1</b>	<ul style="list-style-type: none"><li>○ Proliferation of endothelial cells and promotes angiogenesis</li></ul>

**Table 3:** (continued)

<b>Fibrillin 1- Human</b>	<ul style="list-style-type: none"><li>○ Structural components</li><li>○ Extracellular calcium binding microfibrils</li></ul>
<b>Matrix Metalloproteinase 2</b>	<ul style="list-style-type: none"><li>○ Plays a role in wound healing</li><li>○ Degradation of basement membrane components, leading to destruction of cell-basement membrane junction</li></ul>
<b>Transforming growth factor beta 1</b>	<ul style="list-style-type: none"><li>○ Controls cell proliferation and differentiation.</li><li>○ Regulates other growth factors</li><li>○ Can induce epithelial to mesenchymal transition and cell migration in various cell types.</li></ul>

The analysis of secreted proteins can be challenging due to low abundance in the culture media, thus requiring sample concentration. The media could also contain interfering compounds that could co-precipitate with the proteins, providing a poor yield of desired proteins. Furthermore, even some cell lysis could release more abundant cytoplasmic proteins, contaminating the conditioned media. However, even with their low abundance, soluble factors released into the extracellular environment can act as paracrine factors that play a key role in cell renewal and differentiation.

The study presented in this chapter does have some inherent limitations. We are hypothesizing that proteins secreted by the cells are involved in the regeneration; however, conditioned media contain other soluble factors, such as metabolites and small molecules that could act as the paracrine signaling molecules. The smaller proteins and peptides might not be identified using mass spectrometry due to low abundance or sensitivity of the instrument. To ensure the smaller and low-level proteins are identified, pre-fractionation of proteins using 2-DE gel analysis can be used. Additionally, even with the identification of biologically important factors, the mechanism of action and the combination of these factors required to substitute for the cell therapy has yet to be determined. To do so, fractionation of the conditioned media using

size exclusion column and treatment of mice model using these different fractions could help narrow down potential factors. A multi-omics approach to study the condition media, metabolite analysis alongside the protein secretome, can also be utilized to better characterize the components for regeneration. Also, in this study we have only explored the function and characterization of proteins that are annotated as being extracellular; however, the proteins annotated as being cytoplasmic might also be secreted using exosomes or unconventional secretion pathways. Additional studies to determine mechanism of action of LSC and the protein secretion can be performed by blocking the classical signaling pathway using Brefeldin A treatments, a fungal metabolite that binds to Cop1 vesicle to block the Golgi anterograde protein transport pathway [118]–[120]. This will provide a different set of extracellular proteins, without the signal peptide, giving another dimension to study the protein mechanism of action in tissue regeneration.

## **2.5 Conclusion**

In conclusion, we have reported a profile of LSC secretome. Development of new pharmacological treatments for various lung diseases, including IPF, are in great need. Compared to the published human MSC and ESC secretome profile, a specific signature for LSC is difficult to pinpoint. We have identified several proteins that are specific to LSC that can be potentially useful in identifying key proteins that are involved in therapeutic lung regeneration. Using LSC provides cell cultures that can be used for long term study and without the fear of immune compromise. For more feasible regenerative therapies, key proteins can be studied to determine their effect on the cell. Identification of common proteins among different donors shows the possibility of using autogenic proteins for therapy, and options, including stem cell therapies, conditioned media injection are being pursued. Replacement of the cell therapy with

secreted molecules would minimize biological variability, origin, and immune compatibility for safer and more effective therapy. More detailed analysis to identify and characterize the mechanism of action of these proteins can lead to better understanding of drug development and targeting for clinical applications.

## 2.6 References

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