ISOLATION AND CHARACTERIZATION OF MULTIPOTENT LUNG
STEM CELLS FROM p53 MUTANT MICE MODELS

Venkat Sundar Gadepalli
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd

Part of the Bioinformatics Commons, Biological Phenomena, Cell Phenomena, and Immunity
Commons, Cancer Biology Commons, and the Cell Biology Commons

© The Author

Downloaded from
https://scholarscompass.vcu.edu/etd/3644

This Dissertation is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.
ISOLATION AND CHARACTERIZATION OF MULTIPOTENT LUNG STEM CELLS
FROM p53 MUTANT MICE MODELS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By
Venkat Sundar Gadepalli, M.S (Bioinformatics)
Virginia Commonwealth University, 2009

Adviser: Raj R. Rao
Associate Professor
Department of Chemical and Life Science Engineering
Department of Human and Molecular Genetics

Virginia Commonwealth University
Richmond, Virginia
October, 2014.
ACKNOWLEDGEMENT

Life sciences research encompasses exciting questions but at the same time include many challenges to overcome. In such a demanding line, the guidance and support from Prof. Raj R. Rao, my research adviser and many other professors has played a vital role. I express deep gratitude to my adviser for his research guidance, moral strength and financial support. He has been very insightful by allowing me to work on various research questions and was instrumental in training me as a researcher. I am equally thankful to the PhD committee members Prof. Karnam S. Murthy, Prof. Andrew W. Yeudall, Prof. Michael H. Peters, and Prof. Hu Yang. I am grateful for their time and efforts in serving on the committee and providing invaluable inputs. I also thank collaborators at Massey Cancer Center and Prof. Sumitra Deb for his timely advice and helpful critique during the course of the project.

I express my sincere gratitude to Integrative Life Sciences (ILS) Program Director, Prof. William B. Eggleston, Jr., Prof. Karnam S. Murthy, and the administration staff of ILS & Chemical and Life Science Engineering departments for their acceptance, financial support, and constant encouragement. I am fortunate to have a cooperative work environment and extend my appreciation to current and past cell-mates in Dr. Rao’s lab and Ms. Catherine Vaughan of Dr. Deb’s lab. They have been the driving force for my scientific aptitude and technical abilities. I also thank Prof. Xuejun Wen and Prof. Christopher A. Lemmon for allowing me to use research equipment in their respective labs. I am honored to associate with professors of varied scientific expertise at VCU. Their wisdom and inspiration were instrumental in my research progress.
The work of this nature could not have been completed but for the sacrifice and support from the domestic front. I am extremely indebted to my wife who endured inattention from me. I owe her a lot; she put my successful graduation ahead of everything else. She deserves much love! I am grateful to my parents and family who have been very supportive and provided the strength to overcome challenges. I thank them for their love and constant financial support. I am glad to have good friends and well-wishers; without them I would have missed honest opinions, good times, and the charms of life.
# TABLE OF CONTENTS

List of Tables ............................................................................................................................................... viii

List of Figures ........................................................................................................................................... ix

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

Abstract ................................................................................................................................................... xiv

Chapter 1: Literature Review .................................................................................................................. 1

1.1 Adult stem cells ................................................................................................................................. 1

1.2 Stem cells in the lung ......................................................................................................................... 9

1.3 Lung cancer ....................................................................................................................................... 14

1.4 Cancer stem cells in the lung ........................................................................................................... 15

1.5 Lung cancer and role of tumor protein p53 ................................................................................... 19

1.6 Summary .......................................................................................................................................... 25

Chapter 2: Comparison and correlation of Sca-1 expressing lung stem/progenitor cells across p53 normal and cancer-sensitized p53 mutant mouse models ....................................................................... 27

2.1 Abstract ............................................................................................................................................ 27

2.2 Introduction .................................................................................................................................... 28

2.3 Materials and methods ..................................................................................................................... 30
2.3.1 Lung isolation, enzymatic digestion, and cell sorting.................................30
2.3.2 Cell culture components ........................................................................32
2.3.3 FACS sorting and analysis of cell surface marker expression.......................32
2.3.4 Immunocytochemical analysis for Sca-1 expression.....................................33
2.3.5 AlamarBlue® cell proliferation assay.........................................................34
2.3.6 Cell cycle profile analysis.........................................................................35
2.3.7 In-vitro scratch assay for analysis of cell migration.......................................35
2.3.8 Soft agar non-adherent cell culture assay ..................................................36
2.3.9 Mesenchymal lineage differentiation analysis..............................................37
2.3.10 RNA isolation, real time reverse transcription polymerase chain reaction, and gene expression analysis.................................................................38
2.3.11 Western blot analysis.............................................................................39
2.3.12 Genotyping assay by PCR......................................................................39
2.3.13 Colony formation assay..........................................................................40
2.4 Results........................................................................................................40
2.4.1 Sca-1 expressing putative lung stem cells across p53 backgrounds.............40
2.4.2 Sca-1 expressing lung stem cells from p53 mutants possess higher proliferation and self-renewal characteristics.................................................................44
2.4.3 Conditional point mutant p53 are expressed in lung subpopulations that are sorted based on cell surface marker Sca-1 .............................................................................. 53
2.4.4 Cell lines from p53 mutant mice possess higher percentage of Sca-1 expressing cell populations ................................................................................................................... 56
2.4.5 Sca-1 expressing lung cells from wild type p53+/+ cells show higher differentiation ability ........................................................................................................................... 59
2.4.6 Sca-1 expressing lung subpopulations from p53 mutant mice show selective tumorigenic effects ....................................................................................................... 62
2.5 Discussion ................................................................................................................. 66

Chapter 3: Isolation and Characterization of lung stem/progenitor cells based on sca-1 & PDGFR-αlPHA ........................................................................................................... 70
3.1 Abstract ..................................................................................................................... 70
3.2 Introduction ............................................................................................................... 71
3.3 Materials and methods ............................................................................................. 73
3.3.1 Lung isolation, enzymatic digestion, and cell sorting........................................ 73
3.3.2 Cell culture components .................................................................................... 75
3.3.3 FACS sorting and analysis of cell surface marker expression ........................... 75
3.3.4 AlamarBlue® cell proliferation assay ................................................................ 76
3.3.5 Mesenchymal lineage differentiation analysis ................................................... 77
3.3.6 RNA Isolation, real time reverse transcription polymerase chain reaction, and gene expression analysis ............................................................ 78

3.4 Results ....................................................................................................................... 79

3.4.1 Lung comprises of subpopulation of cells that differentially express Sca-1 and PDGFR-α ............................................................................................................. 79

3.4.2 Sca-1Low PDGFR-αHigh expressing lung subpopulation show higher proliferation

3.4.3 Sca-1Low PDGFR-αHigh expressing lung subpopulation exhibit no differentiation ability .................................................................................................................. 89

3.5 Discussion ................................................................................................................. 95

Chapter 4: Conclusions and future directions ..................................................................... 98

4.1 Conclusions ............................................................................................................... 98

4.1.1 Chapter 2 ............................................................................................................ 98

4.1.2 Chapter 3 .......................................................................................................... 103

4.2 Future directions ..................................................................................................... 104

4.2.1 Chapter 2 .......................................................................................................... 104

4.2.2 Chapter 3 .......................................................................................................... 105

list of References ............................................................................................................ 107

Appendix ......................................................................................................................... 114

Vitae ............................................................................................................................... 130
LIST OF TABLES

Table 1.1-1: Commonly studied cell surface markers in identification and isolation of adult stem cells. ................................................................................................................................................ 8

Table 1.2-1: Cell surface markers studied to identify adult lung stem cells........................................... 13
LIST OF FIGURES

Figure 1-1: Schematic representation of adult stem cell self-renewal ................................................. 2
Figure 1-2: Schematic representation of two possible somatic stem cell divisions............................... 5
Figure 1-3: Schematic representation of conditional point mutations in p53 gene exons .................. 23
Figure 1-4: Schematic representation of point mutations in p53 protein domain structure ............. 24
Figure 2-1: Schematic representation of lung isolation, digestion, and sorting............................... 31
Figure 2-2: FACS sorting of Sca-1 expressing lung stem cells across p53 backgrounds..................... 42
Figure 2-3: Differential Sca-1 expression in Sca-1^High and Sca-1^Low sorted cell lines ................. 43
Figure 2-4: Lung stem cells from p53 mutant mice show distinct mesenchymal morphology.
Distinct morphological differences are observed between p53^+/+ wild type and the mutant cell lines. The p53^+/+ wild type cells possess small spindle shape morphology and the cell lines from mutant backgrounds possess longer mesenchymal morphology. Scale bar 20x magnification. .. 46
Figure 2-5: Sca-1 expressing lung cells from mutant p53^R172H/+ mice show higher N-cadherin expression ..................................................................................................................................... 47
Figure 2-6: Sca-1 expressing lung cells from p53 mutants show higher proliferation ......................... 48
Figure 2-7: Sca-1 expressing lung cells from p53 mutant mice show lower percentage of cells in G-1 phase and higher percentage in S and G2/M phase of cell cycle................................................................. 49
Figure 2-8: Sca-1 expressing lung cells from p53 mutant mice show colony formation ability at low seeding density.......................................................................................................................... 50
Figure 2-9: Sca-1 expressing lung cells from mutant p53 show higher levels of BMI-1 mRNA expression ................................................................. 51

Figure 2-10: Higher expression of BMI-1 is observed in human lung cancer lines ..................... 52

Figure 2-11: Genotyping by PCR amplification of conditional mutant alleles results in a 270 bp mutant product and a 166 bp control product. ................................................................. 54

Figure 2-12: Sca-1 expressing lung cells from p53 mutant cell lines show accumulation of p53 protein .......................................................................................................................... 55

Figure 2-13: Cell lines isolated from p53 mutant mice lung possess higher percentage of Sca-1 expressing cells ........................................................................................................ 57

Figure 2-14: Sca-1 expressing lung subpopulations from p53 mutant cell lines show higher transcript levels of Sca-1 ....................................................................................................... 58

Figure 2-15: Sca-1High lung subpopulations from p53+/+ wild type mice show distinct adipogenic differentiation ability ................................................................. 60

Figure 2-16: Sca-1High lung subpopulations from p53+/+ wild type mice show distinct osteogenic differentiation ability ................................................................. 61

Figure 2-17: Sca-1 expressing lung cells from p53 mutant mice show anchorage independent growth ........................................................................................................ 64

Figure 2-18: Sca-1 expressing lung cells from p53R172H/++ mutant mice show higher migration rate ........................................................................................................ 65

Figure 3-1: Schematic representation of lung isolation, digestion, and cell sorting .................. 74

Figure 3-2: FACS sorting of lung cells differentially expressing Sca-1 & PDGFR-α ............... 81
Figure 3-3: Subpopulations established based on both Sca-1 & PDGFR-α expression consists of homogenous cells. .......................................................................................................................... 82

Figure 3-4: Lung subpopulations established based on Sca-1 & PDGFR-α expression show representative Sca-1 and PDGFR-α mRNA transcript levels. ......................................................................................................................... 83

Figure 3-5: Sca-1<sup>High</sup> PDGFR-α<sup>High</sup> expressing lung subpopulations are significantly increased in mice carrying p53 point mutations. ......................................................................................................................... 84

Figure 3-6: Sca-1<sup>Low</sup> PDGFR-α<sup>High</sup> expressing lung subpopulation show higher BMI-1 transcript levels. .................................................................................................................................................. 86

Figure 3-7: Sca-1<sup>Low</sup>PDGFR-α<sup>High</sup> expressing lung subpopulation exhibit higher N-cadherin mRNA expression. .......................................................................................................................... 87

Figure 3-8 PDGFR-α expressing lung subpopulation exhibit higher proliferation potential. .............................................................................................................................................................................. 88

Figure 3-9: Sca-1<sup>High</sup> expressing lung subpopulation show higher adipogenic differentiation. .............................................................................................................................................................................. 90

Figure 3-10: Sca-1<sup>Low</sup> PDGFR-α<sup>High</sup> expressing lung subpopulation lack adipogenic differentiation potential. .............................................................................................................................................................................. 91

Figure 3-11: Sca-1<sup>High</sup> expressing lung subpopulations demonstrate higher osteogenic differentiation potential. .............................................................................................................................................................................. 92

Figure 3-12: Sca-1<sup>Low</sup> PDGFR-α<sup>High</sup> expressing lung subpopulation lack osteogenic differentiation potential. .............................................................................................................................................................................. 93

Figure 4-1: Possible role of p53 in regulating expression of genes investigated in this study. .............................................................................................................................................................................. 102
LIST OF ABBREVIATIONS

APC            Allophycocyanin
BMI-1          B lymphoma Mo-MLV insertion region 1 homolog
CD             Cluster Differentiation
CSC            Cancer Stem cells
DAPI           4',6-diamidino-2-phenylindole
DMEM           Dulbecco's Modified Eagle's medium
DMSO           Dimethyl sulfoxide
DNA            Deoxyribonucleic acid
EMT            Epithelial-mesenchymal transition
FACS           Fluorescence-activated cell sorting
FBS            Fetal bovine serum
FITC           Fluorescein isothiocyanate
GOF            Gain Of Function
HSC            Hematopoietic stem cells
IBMX           3-isobutyl-1-methylxanthine
ICC            Immunocytochemistry
iMEFs          Inactivated Mouse Embryonic Fibroblasts
MDM2           Mouse double minute 2 homolog
MLSC           Mesenchymal Lung stem cells
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEAA</td>
<td>Non-Essential Amino Acids</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer cells</td>
</tr>
<tr>
<td>PBS(^{−})</td>
<td>Phosphate-buffered saline without Mg(^{+2}) and Ca(^{+2})</td>
</tr>
<tr>
<td>PBS(^{+/−})</td>
<td>Phosphate-buffered saline with Mg(^{+2}) and Ca(^{+2})</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGFR-(\alpha)</td>
<td>Alpha-type platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Stem cell antigen-1</td>
</tr>
<tr>
<td>SCLSC</td>
<td>Small cell lung cancer cells</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor Protein 53</td>
</tr>
</tbody>
</table>
ABSTRACT

ISOLATION AND CHARACTERIZATION OF MULTIPOTENT LUNG STEM CELLS FROM p53 MUTANT MICE MODELS

By, Venkat Sundar Gadepalli, M.S (Bioinformatics)

Virginia Commonwealth University, 2009

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2014

Major Director: Raj R. Rao Ph.D, Associate Professor, Department of Chemical and Life Science Engineering, Department of Human and Molecular Genetics
Recent advances in understanding lung biology have shown evidence for the existence of resident lung stem cells. Independent studies in identifying and characterizing these somatic lung stem cells have shown the potential role of these cells in lung repair and regeneration. While identifying a homogenous stem cell pool with unique cell surface markers is still a challenge, the currently available data support the existence of different lung cells with stem cell characteristics of self-renewal and differentiation. Understanding the functional characteristics of these tissue resident stem/progenitor cells has gained much importance with increasing evidence of cancer stem cells, cells in a tumor tissue with stem cell characteristics. Lung cancer is most commonly characterized by loss of p53 function which results in uncontrolled cell divisions. Incidence of p53 point mutations is highest in lung cancer, with a high percentage of missense mutations as a result of tobacco smoking. Certain point mutations in p53 gene results in its oncogenic gain of functions (GOF), with enhanced tumorigenic characteristics beyond the loss of p53 function. However, there are no available data on characterization of lung stem cells carrying GOF mutations and correlating them with those of normal stem cells, in this study, for the first time we show that percentage of Sca-1 expressing subpopulation is significantly higher in the lungs of mice carrying p53 GOF mutations than those in lungs isolated from p53+/+ wild type mice. Additionally, our results show that Sca-1High subpopulations isolated from p53 GOF mutant knock-in mice possess distinct mesenchymal morphology, higher proliferation and limited differentiation potential compared to their wild type counterparts. Further, we noted difference in self-renewal ability, tumorigenic potential, and differentiation ability within the putative lung stem/progenitor cells isolated from two different p53 GOF mutant knock-in mice.
In the second part of our study, we investigated the role of PDGFR-α as a potential stem cell marker. We successfully established lung cells differentially expressing two cell surface markers, Sca-1 and PDGFR-α, with results demonstrating existence of different subpopulations of cells in the lung. We noted that cells expressing Sca-1\textsuperscript{Low} and PDGFR-α\textsuperscript{High} show no differentiation capability, suggesting that these cells lack stem cell characteristics. While all other sorted subpopulations exhibited differentiation potential, Sca-1\textsuperscript{High}/PDGFR-α\textsuperscript{Low} and Sca-1\textsuperscript{High}/PDGFR-α\textsuperscript{High} subpopulations show more robust differentiation ability and higher proliferation respectively. These results suggest that incorporation of PDGFR-α as an additional cell surface marker along with Sca-1 enables us to further identify specific subpopulations with defined stem cell characteristics. Overall, our findings aide to increase the understanding of lung cell biology, highlights the need of additional surface markers to identify and isolate more homogenous stem/progenitor cells, and furthers our understanding of stem cells in the context of lung cancer.
CHAPTER 1: LITERATURE REVIEW

1.1 Adult stem cells

There has been emerging evidence of presence and prospective isolation of somatic stem cells in various tissues and organs. (Kim et al., 2005; Sherwood et al., 2004; Stingl et al., 2006; Uchida et al., 2000). Of all the somatic stem cells studied in mouse and humans, the blood forming hematopoietic stem cells (HSC) were one of the first to be purified and characterized in the mouse (Spangrude et al., 1988). HSCs have been extensively studied in detail with in vitro and in vivo studies resulting in invaluable amount of data about their functions and successful clinical applications (Bryder et al., 2006). The lineage hierarchy of HSCs, from a self-renewing stem cell to lineage restricted cells, has been well-defined and well-characterized than any other tissue specific stem cell (Graf, 2002; Weissman et al., 2001)(Figure 1-1). Each cell type in this hierarchy has been identified with specific or combinations of cell surface markers that define their phenotype. The HSC reside at the top of the hierarchy and are characterized to be a side population of cells with higher self-renewal; and multipotent differentiation capability to generate specialized cells with specific functions. They give rise to multipotent and oligopotent progenitor cells, characterized by limited self-renewal but, higher proliferation ability. Unlike
HSC, the multipotent and oligopotent progenitor cells possess limited differentiation capability, which can form 2 or more mature cells within the tissue they reside in. Oligopotent progenitors constitute common myeloid, lymphoid and other progenitor cells that give rise to mature effector cells such as erythrocytes, platelets-cells, T-cells, B-cells and other mature blood cells which exhibit no self-renewal (except memory T and B cell) and limited proliferation. A comprehensive knowledge on characteristics of each cell type in the HSC lineage hierarchy, research on their gene expression profiles and mouse transplantation studies has enabled progress the use of HSCs in clinical applications to treat leukemia blood cancer and other autoimmune blood cell diseases (Weissman, 2000b; Weissman and Shizuru, 2008; Wilson and Trumpp, 2006). With evidence of somatic stem cells in other tissues, it is important to gain in-depth understanding of these resident stem cells.
Figure 1-1: Schematic representation of adult stem cell self-renewal

Asymmetric self-renewal properties of somatic stem cells results in a stem cell and a progenitor cell. The progenitor cells divides repeatedly and give rise to post-mitotic terminally differentiated cells, thus maintaining homeostasis and stem cell pool of a tissue.
The human body is comprised of about 200 different types of cells with specialized functions that are unique to the specific tissue or organ in the body. Over the lifetime of a human being, these cells are replenished as a result of cell death or injury to maintain cell homeostasis in the body. This is attained by the resident stem cells in response to a stimuli, which by self-renewing maintains respective stem cell pool of the specific tissue or organ (Slack, 2000; Weissman, 2000a). Few in vivo studies in hematopoietic system suggest development plasticity of these adult stem cells, an ability to generate differentiated cells beyond their own tissue boundaries (Korbling and Estrov, 2003). This versatility of somatic stem cells with potential plasticity is considered a rare phenomenon, which need better understanding and yet to be established across different tissues (Wagers and Weissman, 2004).

The cell divisions that are most commonly noted in human body are symmetrical, where two identical daughter cells are formed from a parent cell. This phenomenon is also noted in different types of cells (e.g., skin cells, lung cells, fat cells, neural cells) that are grown in vitro. However, these lineage committed primary cells have a limited division capability, which are eventually replenished by differentiation of respective somatic stem cells. In order to maintain long-term cell homeostasis during the life time of a human being, it is required that the stem cell pool is preserved while meeting the specific requirements in a tissue. With the current understanding of stem cell biology, researchers have noted that this exquisite balance between self-renewal and differentiation could be achieved by only asymmetric division and are believed to be regulated in the stem cell niche of the respective tissue (Watt and Hogan, 2000). Two self-renewal strategies are proposed for these somatic stem cells, population asymmetry and single cell asymmetric division(Figure 1-2) (Morrison and Kimble, 2006; Shen et al., 2004). In population asymmetry,
the balance between self-renewal and differentiation is attained at the population level, where some stem cells differentiate resulting in progenitor cells that eventually proliferate to form specialized cells, while other stem cells go through symmetric self-renewal to maintain stem cell numbers in the pool. In single cell asymmetry, the somatic stem cell divides asymmetrically into a stem cell and a progenitor cell, thus maintaining a constant stem cell pool in the respective tissue (Klein and Simons, 2011; Simons and Clevers, 2011). It is still unclear if this asymmetry is regulated by internal (asymmetric segregation of fate determinants during cell division) or external (stem cell niche) factors. Since, cell division is a complex internal process involving DNA replication and segregation it is important to maintain high fidelity with no chance for errors (Fuchs et al., 2004; Ho, 2005). Hence, somatic stem cells are considered to be slow-cycling cells and asymmetric division at single cell level supports this view. On the other hand, lineage tracking studies and development of inducible genetic systems has allowed investigating population asymmetry in some tissues (e.g., intestinal crypt), with results suggesting that self-renewal of stem cells follows a stochastic pattern where alongside with single cell asymmetric division, population asymmetry also exists to maintain tissue homeostasis and stem cell pool (Shahriyari and Komarova, 2013). However, in-depth understanding of somatic stem cell division is still an area of active research which requires development of different cellular assays to monitor cell division both in vitro and in vivo.
Figure 1-2: Schematic representation of two possible somatic stem cell divisions.

Population asymmetry (A) is characterized by differentiation of a stem cell into progenitor cell, where the loss of stem cell is replenished by a symmetric division of a stem cell in that niche. On the other hand, Single cell asymmetry (B) is characterized by division of stem cell into two different cells, a progenitor cell and a stem cell.
The evidence for existence of somatic stem cells has increased over years. Our body is comprised of various tissues and organs that demonstrate regenerative potential at respective levels. Some tissues (blood, skin, gut, respiratory tract) are extensively exposed to harsh environments and hence perpetually replenish damaged cells. While some tissues are poorly regenerative (e.g., heart, lung) and others are highly regenerative (e.g. liver), various studies have reported resident stem cells with self-renewal and multipotent capability in different tissue types (Wagers and Weissman, 2004). These adult stem cells were identified based on expression of different cell surface markers, some of these markers that have been commonly used are listed (Table 1.1-1) These differences in regenerative potential are yet to be understood at the cellular level, but their classification based on potency and tissues of origin provide some insights. The stem cells are majorly classified based on their differentiation potential, as pluripotent (ability to generate all body cells, including germ cells e.g. ESC), multipotent (ability to generate all tissue cells, e.g. HSC), unipotent (ability to generate only single cell type; e.g.: spermatogonial stem cells). Depending on their tissue of origin/location, differentiation potential, these somatic stem cells are broadly classified as, epithelial stem cells (e.g. intestinal lining, skin), mesenchymal stem cells (e.g., bone marrow), hepatic stem cells (liver). However, certain organs like the lung contain both epithelial and mesenchymal stem cells. In order to understand self-renewal and differentiation potential of different stem cells, identification and isolation of these cells from their respective tissue is an important necessity. Even though different research studies have identified different cell surface markers that correlate with stem cells of respective tissues, these are not very specific or exclusive for stem cells alone. Some of the cell surface markers that are employed to identify these specific stem cells are listed in Table 1.1-1 below. There is need for improved isolation techniques, cell culture protocols and detailed molecular analysis to identify
stem cells with distinct characteristic of self-renewal and differentiation (Barker et al., 2010; JM., 2000; Ute Bissels, 2013).
<table>
<thead>
<tr>
<th>Cell surface markers studied</th>
<th>Tissue/ Organ resident somatic stem cells reported</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone morphogenetic protein receptor (BMPR) CD34+Sca1+Lin- profile c-Kit Hoechst dye</td>
<td>Bone marrow</td>
<td>(Morikawa et al., 2009; Wilson and Trumpp, 2006; Zhau et al., 2011)</td>
</tr>
<tr>
<td>Sca-1, c-Kit, CD34</td>
<td>Lung</td>
<td>(Raiser and Kim, 2009)</td>
</tr>
<tr>
<td>Leucine-Rich G Protein-Coupled Receptor 5 (LGR5), BMI-1, Galectin-1, CD133</td>
<td>Intestine</td>
<td>(Barker et al., 2010; Korbling and Estrov, 2003; Morrison and Kimble, 2006; Wagers and Weissman, 2004)</td>
</tr>
<tr>
<td>Integrin α6, CD 34</td>
<td>Neural stem cells</td>
<td>(Shen et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Epidermal stem cells</td>
<td>(Barker et al., 2010)</td>
</tr>
</tbody>
</table>

Table 1.1-1: Commonly studied cell surface markers in identification and isolation of adult stem cells.

Somatic/adult stem have been studied in a variety of organs and tissues such as bone marrow, brain, lung, and other epithelial tissues. The table summarized the commonly used cell surface markers to identify, isolate and establish self-renewing adult stem cells across these different tissues and organs.
1.2 Stem cells in the lung

The lung is considered to be a highly heterogeneous organ with a variety of cells located in distinct regions of the organ. Functionally distinct putative stem cells were shown to reside in different anatomical regions of the respiratory system, which play a key role in repopulating the cells in their local area (Alamgeer et al., 2013; Hegab et al., 2010; Kajstura et al., 2011; Kim et al., 2005; Singh et al., 2012). Studies have reviewed the role played by the local stem cells found in trachea (basal, mucous secretory), bronchus (basal, mucous secretory), bronchiole (clara), and alveolus (type II pneumocyte) and have shown that they primarily contribute to regeneration of lost cells/tissues in response to injury (Otto, 2002).

Identification of resident multipotent lung stem cells that can differentiate into any lung cell is still an area of active research. Two major types of stem cells, namely epithelial and mesenchymal stem cells have been reported in the lung so far (Hegab et al., 2010; Kim et al., 2005; Summer et al., 2007). These cells were isolated and characterized based on specific cell surface markers that are unique to certain cell lineages. In line with the heterogeneous nature of the lung, the cells that reside in different regions of the lung exhibit differential expression of various cell surface markers. A classic stem cell marker used in the identification of hematopoietic stem cells, Stem cell antigen (Sca-1), has also been found to be expressed in some cells of mesenchymal origin (Holmes and Stanford, 2007). Studies have demonstrated that lung cells expressing Sca-1 were predominantly found in distal regions of lungs and were shown to possess a temporal emergence, indicated by enrichment of Sca-1 expressing cells in adult mouse lungs when compared to neonatal lungs. Sca-1^pos cells have been shown to emerge in postnatal lung during the branching of the airways/lung vasculature and increase exponentially in adult
lungs (McQualter et al., 2009). Based on the expression of Sca-1 and other markers, various studies have identified unique subpopulations in lung that possess stem cell characteristics (Raiser and Kim, 2009). Thus Sca-1 emerged as a representative cell surface marker to identify the lung stem cells.

Bronchioalveolar stem cells (BASCs) isolated, from bronchioalveolar duct junction in adult mouse lungs, based on expression of Sca-1 and CD34 (Hematopoietic and epithelial markers) were shown to exhibit self-renewal and multipotent capabilities. In in vivo studies, the BASCs were shown to participate in lung epithelial cell renewal and maintain bronchiolar, clara and alveolar cell populations in the distal lung (Kim et al., 2005). Gene expression analysis on Sca-1neg, CD45neg, CD31neg lung populations and corresponding Sca-1pos cell lines were shown to possess epithelial and mesenchymal gene expression profiles respectively, signifying the presence of Sca-1pos cells with mesenchymal characteristics (McQualter et al., 2009) (Hegab et al., 2010). Moreover, the Sca-1pos, CD45neg, CD31neg were enriched with mesenchymal progenitor cells in culture as shown by their spindle shaped morphology and expression of mesenchymal markers (CD104a, Vimentin). In contrast, Sca-1neg, CD45neg, CD31neg cells were shown to possess cobblestone epithelial cell morphology and epithelial marker expression (E-cadherin, cytokeratins 5 and 14, and proSP-C) (McQualter et al., 2009). From a functional standpoint, the isolated and characterized stem cells in the lung are believed to play an important role in maintaining lung homeostasis. Bronchiolar stem cells have been functionally defined by their expression of clara cell secretory protein (CCSP), pro-surfactant protein C and were shown to reside in airway epithelium (Giangreco et al., 2002; Hong et al., 2001). A systematic study conducted by Teisanu et al has identified bronchiolar stem cells phenotype as CD45neg, CD31neg,
CD34\textsuperscript{neg}, Sca-1\textsuperscript{Low} AF\textsuperscript{Low} (Teisanu et al., 2009) as opposed to Sca-1\textsuperscript{pos}, CD34\textsuperscript{pos}, CD45\textsuperscript{neg}, CD31\textsuperscript{neg} reported earlier by (Kim et al., 2005). Based on studies with transgenic mice models associated with stem cell expansion, ablation, and lineage tracing, Teisanu and colleagues demonstrated that CD34\textsuperscript{pos} populations do not belong to the airway epithelium, while CCSP expressing cells are found in CD34\textsuperscript{neg}, Sca-1\textsuperscript{Low} and AF\textsuperscript{Low} populations (Teisanu et al., 2009). It is useful to note that evidence of different subpopulations in lung cells with potential stem cell properties has been attributed to the method of isolation, culturing conditions, and choice of markers (Raiser and Kim, 2009). These studies indicate the complex nature of lung and presence of one or more putative stem cells in the Sca-1\textsuperscript{pos} CD45\textsuperscript{neg} CD31\textsuperscript{neg} pool. Details of isolation and characterization of these cells have been detailed in recent articles published by our group and others (Gadepalli et al., 2013; Hegab et al., 2010).

In humans, lung stem cells with self-renewing, clonogenic, and multipotent (in vitro and in vivo) properties were shown to exist. These were identified and isolated based on expression of c-kit in combination with other cell surface markers (Kajstura et al., 2011). The c-kit\textsuperscript{pos} cells were negative for hematopoietic and mesenchymal markers and interestingly demonstrated positive expression of key markers associated with pluripotency: OCT4, NANOG, KLF4, and SOX2. However, a key defining feature of somatic stem cells that differentiates them from pluripotent stem cells is that they undergo asymmetric division that results in the generation of a heterogeneous population of stem cells and progenitor cells. This study has been received with some skepticism putting forward several questions and need for independent studies to ascertain existence of somatic lung stem cells in humans (Stripp BR, 2011).
The fact that lung tissue is composed of a variety of cells with distinct phenotypes and functions complicates our understanding of lung regeneration. This is evident from multiple research studies where lung cells characterized by different markers were shown to possess core stem cell properties of self-renewal, clonality, and multipotent characteristics (Hegab et al., 2010; Kim et al., 2005; Raiser and Kim, 2009). While these research studies promise a step ahead in identifying putative lung stem cells, there are challenges that need to be addressed. One such challenge is to define a unique set of cells that possess multipotent capability as well as play a crucial role in lung regeneration. This is not only expected to strengthen our attempts to develop focused therapeutic strategies in the context of wound healing but also in identifying and targeting putative cancer stem cells in the lung. Some of the studies carried out in identifying lung stem cells in mouse and their characteristic features described by experimental evidence are summarized in Table 1.2-1.
<table>
<thead>
<tr>
<th>Markers for sorting</th>
<th>Anatomical location of cells</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sca1&lt;sup&gt;pos&lt;/sup&gt;, CD34&lt;sup&gt;pos&lt;/sup&gt;, CD45&lt;sup&gt;neg&lt;/sup&gt;, CD31&lt;sup&gt;neg&lt;/sup&gt;</td>
<td>Bronchi alveolar duct junction</td>
<td>These cells were positive for clara cell marker and Surfactant protein marker.</td>
<td>(Kim et al., 2005)</td>
</tr>
<tr>
<td>Sca1&lt;sup&gt;pos&lt;/sup&gt;, CD44&lt;sup&gt;pos&lt;/sup&gt;, CD106&lt;sup&gt;pos&lt;/sup&gt;, CD45&lt;sup&gt;neg&lt;/sup&gt;, CD31&lt;sup&gt;neg&lt;/sup&gt;</td>
<td>Sca1 expression is predominantly found in Distal Lung, restricted to Endothelial and Perivascular Cells</td>
<td>Similar to other mesenchymal progenitors, these cells express Sca-1, CD106, CD140a (PDFGR-a) and CD44.</td>
<td>(Holmes and Stanford, 2007)</td>
</tr>
<tr>
<td>Sca1&lt;sup&gt;pos&lt;/sup&gt;, CD45&lt;sup&gt;neg&lt;/sup&gt;, CD31&lt;sup&gt;neg&lt;/sup&gt;</td>
<td>-</td>
<td>Differentiated into endothelial and lung epithelial (alveolar type I, II, and clara)</td>
<td>(Hegab et al., 2010)</td>
</tr>
</tbody>
</table>

**Table 1.2-1: Cell surface markers studied to identify adult lung stem cells.**

The above table summarizes cell surface markers employed across different research studies to identify and isolate somatic/ adult stem cells in the lung. It also highlights the anatomical location and specific characteristics of lung subpopulations that these studies have established by using a combination of different cell surface markers.
1.3 Lung cancer

One of the leading causes of cancer-related deaths is cancer of the lung, of which 80-90% is attributed to tobacco smoking (Ferlay J, 2013; Jemal et al., 2011). More than 50 different histological variants of lung cancer have been recognized by the World Health Organization (WHO) and classified based on their phenotype, their zone of origin in the lung, or by tumors arising from functionally diverse lung cells (Travis, 2004). This diversity characterizes the neoplasms in lung as heterogeneous with different histological subtypes. Almost 98% of lung cancers are carcinomas (tumors arising from epithelial cells) and based on the size of a cancer cell, categorized as small cell lung cancer (SCLC) or non-small cell lung cancer (NSCLC) carcinomas. SCLCs and NSCLCs constitute approximately 10-15 % and 85-90% of lung cancers respectively (Travis, 2002, 2011). The SCLCs are malignant small epithelial cells with scanty cytoplasm. The NSCLCs are further classified based on their size and shape as large cell carcinoma, squamous carcinoma, and adenocarcinoma. NSCLCs are relatively larger in size and contain a high nucleus to cytoplasmic ratio (Travis, 2002; Travis, 2004; Travis et al., 1995). Additionally, other rare subtypes of lung cancer include bronchioalveolar carcinoma, carcinoid, glandular, and neuroendocrine tumors.

According to NCI PDQ®, of all the subtypes in lung cancer, the incidence of squamous and adenocarcinoma are considered to be the highest (NCI, 2014). Studies comparing the major four subtypes of lung cancer recognized that the rate of development of adenocarcinoma is more common and constitute approximately 40% of these lung cancer subtypes in humans, with the cause strongly associated with tobacco smoking (Kenfield et al., 2008; NCI, 2014; Travis, 2004). Various other factors such as asbestos, arsenic, radon (radioactive gas formed as a result of
breakdown of uranium in soil), and environmental air pollution also pose a risk of lung cancer (Subramanian and Govindan, 2007). Epidemiological studies and molecular biology studies have indicated a high risk of at least 20 carcinogens in tobacco smoke that can cause lung cancer (Hecht, 1999). Research has shown that tobacco carcinogens such as polycyclic aromatic hydrocarbons (benzo[a]pyren) target hot spots in codon regions of TP53 by forming DNA adduct, thus forming sites for mutations in cancer (reviewed and summarized in (Toyooka et al., 2003)). The systematic analysis based on different lung cancer research data uploaded at International Agency for Research on Cancer (IARC) indicates a moderate relationship between smoking exposure and mutation pattern in codon regions (157, 158, 175, 245, 248, 249 and 273) of TP53, suggesting that mutational pattern in cancers arising in smokers is not specific to a single codon. Studies also confirmed a high frequency in G: C to T: A transversions in TP53 coding region of smokers (16%) than in non-smokers (5.8%). One key observation in their analysis is change of mutational spectrum based on gender. For instance, G: C to T: A transversions found to be higher in female smokers (36%) than male smokers (27%) (Ezzati et al., 2005; Toyooka et al., 2003). The advances at molecular level in understanding the cause of cancer and research studies targeting identification of cancer stem cells hold promise for development of novel approaches in diagnosis and treatment of lung cancer.

1.4 Cancer stem cells in the lung

Over the past few decades, advances in cancer research have enabled us to understand the different mechanisms that contribute to the aberrant proliferation of normal cells into abnormal cells that result in tumors. In the pursuit to find cures, researchers have primarily focused on various molecular level changes that are unique to cancerous cells. In humans, about 50% or
More cancers have a mutated tumor suppressor p53 gene thereby resulting in accumulation of p53 protein and losing its function to activate the target genes that regulate cell cycle and apoptosis. Extensive research conducted in murine cancer models with activated p53, loss of p53, or p53 missense mutations has facilitated researchers to understand the role of this key protein. In spite of identification of numerous triggers that cause cancer, specific cures still remain elusive. One of the primary reasons attributed to this is due to the fact that the tumor tissue is heterogeneous and contains numerous subpopulations of cells. Studies have shown that a specific subpopulation of cells termed as cancer stem cells (CSCs) drive the recurrence of cancer in response to standard chemotherapy. These CSCs are mutated cells with core properties similar to those of adult stem cells. They reside in a microenvironment within the tumor tissue that supports their growth and make them less susceptible to drug treatment. These cells possess properties of self-renewal and migration, thereby driving tumor formation and metastasis. Over the last decade, research specifically targeting these cells has gained prominence towards developing new therapeutic agents against cancer.

Numerous studies have demonstrated the presence of subpopulations of cells in tumors that play a critical role in initiating a tumor during post chemotherapy or radiation treatments (Alamgeer et al., 2013; Perona et al., 2011). These cells are present as a small population within the tumor and appear to be more potent in initiating the tumor than other subpopulations and have been classified as cancer stem cells (CSCs). These CSCs were characterized by independent research studies and were shown to sustain their malignant phenotype against drugs targeting cancer (Berns, 2005; Kratz et al., 2010; Perona et al., 2011). Interestingly, these CSC subpopulations were found to possess stem like properties of self-renewal and differentiation similar to those
exhibited by somatic stem cells. Certain signaling pathways such as Hedgehog, Notch, and WNT that are important for maintenance of embryonic stem cells were also shown to have a role in putative CSCs found in the lung (Hassan et al., 2013; He et al., 2005; Singh et al., 2012; Zhang et al., 2013; Zhang et al., 2012). Thus the discovery of CSCs has opened a new area of research in cancer that focuses on understanding and targeting the cells that drive the recurrence of tumor and metastasis.

CSCs share similarities with the resident somatic stem cells in their respective tissues of origin. Somatic stem cells are characterized by their oligopotent property, where they continuously renew themselves as well as differentiate into distinct descendants that are specific to a tissue. Somatic stem cells are found along with specialized cells of an adult tissue or organ as rare side populations. For prolonged periods of time they reside in quiescence (G0/resting) phase of the cell cycle, a stage that is an actively controlled phase involving various epigenetic, transcriptional, and signaling pathways (Cheung and Rando, 2013). In response to injury or stimuli, these somatic stem cells enter mitosis and give rise to a stem cell and a progenitor cell by the process of asymmetric cell division. The stem cell resides back in quiescence stage until the next signal to re-enter the cell cycle, while the progenitor cells undergo a series of amplifications that give rise to post mitotic differentiated cells in respective tissues or organs of an animal. This characteristic asymmetric division not only plays a role in maintaining homeostasis in adult tissues by replacing the dead or aging cells, but also avoids repetitive entry of stem cells into the cell cycle, which may increase the chance of DNA damage. This similar kind of hierarchy is observed in CSCs, where a side population of cells forms the backbone to drive relapse of tumor and metastasis. However, unlike normal somatic stem cells these CSCs possess abnormal characteristics which are currently being explored in the context of understanding their role in
specific cancers. A broad perspective and future directions in identifying cancer stem cells, *in vitro* and *in vivo* assays to characterize them, and developing drug screening strategies have been critically discussed (Beck and Blanpain, 2013; Clarke et al., 2006; Liao et al., 2014).

Identification and isolation of these CSCs from the bulk of tumors have been reported based on presence of specific markers that differ from those used to identify adult lung stem cells have been recently reviewed (Alamgeer et al., 2013). The phenotypic characterization of CSCs include the activity of cytoplasmic enzyme aldehyde dehydrogenase (ALDH) (Jiang et al., 2009), expression of cell surface markers CD133 and CD44, or capacity of cells to efflux membrane permeable dyes such as Hoechst 33342 dye and existing as a side population (SP) in bulk of tumor cells (Ho et al., 2007). CD133 has been identified to be a putative marker for NSCLC and SCLC, while CD44 is found to be enriched only in NSCLC and not in SCLC. Similarly, NSCLC demonstrate positive activity for ALDH. (Chen et al., 2008). Identifying a panel of universal markers to classify CSCs is an active area of research. Signaling pathways such as Hedgehog (Hh), Notch, and WNT are important in the maintenance of stem cells and tissue homeostasis are also found active in CSCs (Alamgeer et al., 2013). It is believed that dysregulation of these pathways in CSCs could drive their tumorigenic activities with several reports focused on developing therapeutic strategies to target these pathways. For example, inhibiting Hh signaling pathway in lung cancer cell lines resulted in loss of side population cells, while targeting Notch and Wnt signaling resulted in reduction of ALDH positive tumor cells or induction of apoptosis or growth inhibition in NSCLC (He et al., 2004; Sullivan et al., 2010; Tian et al., 2012). These clinical trials provide some novel developments in treating lung cancer but further trials are needed to demonstrate efficacy. Despite these encouraging clinical results in treating cancer,
other mechanisms that are being discovered in CSCs still need to be further researched to develop feasible therapies. One such key mechanism is the Epithelial-mesenchymal transition (EMT) in CSCs, first reported in breast cancer stem cells (Mani et al., 2008), where the CSCs were shown to exploit this EMT mechanism that is normally observed during developmental process of the mesoderm. It involves a process by which the epithelial cells lose their morphology and gain migratory and invasive properties to become mesenchymal cells. This EMT mechanism was found to be activated during cancer invasion and metastasis and results in the generation of mesenchymal cells that express the stem cell marker CD44 and form tumors effectively in mammary epithelial cancer cells (Mani et al., 2008) and believed to be associated with drug resistance and cancer progression. Our current understanding is limited on signaling pathways and transcriptional factors that takes place in these CSCs. Expression of EMT associated genes is also being assessed in the context of lung cancer, but the role of EMT in progression of lung cancer is yet to be established (Xiao and He, 2010).

1.5 Lung cancer and role of tumor protein p53

In normal cells, p53 is expressed at low levels but as a result of stress or cellular damage, it activates a host of different proteins that are involved in cell cycle, apoptosis, and senescence, thereby preventing proliferation of cells that carry mutations or DNA damage. In unstressed cells, p53 function is regulated by its specific target murine double minute 2 (MDM2) by a process of ubiquitination. MDM2, an E3 ubiquitin-protein ligase, binds N-terminal transactivation domain of p53, thus mediating p53 degradation by nuclear and cytoplasmic proteasomes. This constant mono-ubiquitination by MDM2 regulates physiological levels and functions of p53 in normal cells (Moll and Petrenko, 2003). Apart from known functions of p53,
recent evidence suggests that p53 plays a crucial role in regulating stem cell homeostasis (Bonizzi et al., 2012). Studies involving re-programming of differentiated cells into induced pluripotent stem cells have noted that inhibition or loss of p53 increases the reprogramming efficiency by 3 to 10 fold (Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009). These studies indicate that p53 has a pivotal role in restricting the reprogramming process. Other studies involving adult mammary stem cells derived from p53−/− mice were shown to possess immortal behavior by increased self-renewal and symmetric division as opposed to limited self-renewal and asymmetric division observed in their wild type counterparts (Cicalese et al., 2009). Similarly in hematopoietic stem cells (HSC), expression of p53 has been found to be critical for regulation of several aspects of HSC behavior. Deletion of p53 in mice was also shown to contribute to increased HSC self-renewal as well as an increase in the HSC pool (Liu et al., 2009). In HSCs, p53 was also found to regulate cellular response to oncogene expression in progenitor cells, where absence of p53 and expression of proto-oncogene KRAs was found to promote acute myeloid leukemia (Zhao et al., 2010). A recent study in hematopoietic stem cells and mammary stem cells has noted that DNA damage by irradiation induces up regulation of p21, a known target of p53, thereby impeding apoptosis, cell cycle progression with symmetric cell divisions (Insinga et al., 2013). These studies indicated that elevated levels of p21 prevent p53 activation and its basal activity, thus preventing stem cells from apoptosis, and allowing them to enter the cell cycle. This study identified a unique mode of p21-dependent response to DNA damage in stem cells wherein p21 activates DNA repair, minimizing DNA damage accumulation, and exhausting the stem cells to divide symmetrically as opposed to less stressful asymmetric division (Insinga et al., 2013). In summary, these studies suggest that, apart from its
normal functional role in tumor suppression and cell cycle regulation, p53 is able to restrain adult stem cell self-renewal, and impose asymmetric mode of cell division.

The loss of tumor suppressor function of p53 is either impaired by deletion of Tp53 gene or expression of mutated p53 protein. Alternatively, in some human cancers even though wild type p53 is active, its function is diminished by its primary cellular inhibitor, MDM2. These functional disparities are the most commonly observed causes of cancers in humans. MDM2 has a dual function towards p53, by acting as a positive regulator of p53 by interacting with p53 mRNA when the ATM (Ataxia telangiectasia mutated) pathway is active. As the ATM activity ceases, MDM2 acts as a negative regulator to suppress the p53 protein activity by mediating its degradation under normal conditions (Gajjar et al., 2012).

The incidence of p53 missense mutations (70%) is highest in lung cancer compared to all cancer types. Approximately 90% of these p53 mutations are missense mutations that result in accumulation of mutant p53 protein (Govindan et al., 2012; Govindan R, 2014). These impact molecular activity of cells and cause novel tumors not commonly observed in p53-/- (p53 null) cancerous mice. These missense mutation effects have been explained based on two primary models: dominant negative (DN) activity or oncogenic gain-of-function (GOF) (Olive et al., 2004). In the first model, it is proposed that the mutant protein forms a hetero tetramer with wild type p53 and exerts a dominant negative effect on wild type p53 function. In the second model, it is projected that the mutant allele confers oncogenic progression irrespective of the wild type p53 allele counterpart. Studies carried out to understand the GOF mutations by transforming p53 null mice with mutant p53 constructs have supported the effects based on the GOF model. Phenotypic characteristics ascribed to GOF activity of mutant p53 include increased
tumorigenicity, growth rate, motility, metastasis, invasiveness and decreased sensitivity to chemotherapeutic drugs (Olive et al., 2004). Understanding the changes in these cancer lines with GOF activities is basically proposed as an important area of research for drug targeting. Towards use in these studies, many researchers have generated p53 mutant mouse models (Olive et al., 2004) and human cell line models based on mutations reported by research studies complied in IARC TP53 database (http://www-p53.iarc.fr/). Recent studies have also highlighted the use of lentiviral approaches (endogenous expression) or transfections (transient expression) to express tumor-derived mutant p53 in cells (Vaughan et al., 2013). It is observed that these p53 mutants show enhanced expression of NF-kappaB2 and receptor tyrosine kinase AXL (Scian et al., 2005; Vaughan et al., 2012b) that could be potential targets for therapies. However, mechanisms underlying these differential expression are unclear and yet to be defined.
In this study, 2 different types of p53 point mutations expressing mice and a p53+/+ wild type mice were employed. The targeting vector were constructed by specific mutations in the codon region of endogenous p53 at exon 5 for p53^{R172H/+} and at exon 2 and 5 for p53^{QSH/+} (red color). To induce stage specific expression *lox-Stop-lox* (LSL) element (yellow color) is introduced in the intron location.

**Figure 1-3: Schematic representation of conditional point mutations in p53 gene exons**

In this study, 2 different types of p53 point mutations expressing mice and a p53^{+/+} wild type mice were employed. The targeting vector were constructed by specific mutations in the codon region of endogenous p53 at exon 5 for p53^{R172H/+} and at exon 2 and 5 for p53^{QSH/+} (red color). To induce stage specific expression *lox-Stop-lox* (LSL) element (yellow color) is introduced in the intron location.
Figure 1-4: Schematic representation of point mutations in p53 protein domain structure

Point mutations in the expressed p53 protein of the respective mice carrying the engineered p53 constructs are represented above. The targeting vector carrying p53R172H/+ mutation express a point mutation in the DNA binding domain of the p53 protein, where arginine is replaced by histidine at position 172 (represented by red line). Similarly, the targeting vector carrying p53Q25S/W26L/R172H mutation express point mutations in the transactivation domain and DNA binding region of the p53 protein, where glutamine is replaced by leucine and tryptophan is replaced by serine at positions 25,26 and arginine replaced by histidine at position 172.
1.6 Summary

Cancer stem cells are found to conserve many properties of normal somatic stem cells that relate to self-renewal and differentiation. Some of these mechanisms, specifically related to self-renewal and differentiation are defining features of somatic stem cells. Along with stem-cell like characteristics, CSCs also carry dysregulated activities in of p53, MDM2 and pathways dependent on these proteins. They develop resistance to the action of drugs by activating new molecular mechanisms and thereby prevent apoptosis. It is thus important to understand pro-tumorigenic effects of missense mutations and their impact on cancer progression. Understanding the molecular mechanisms that lead to tumorigenic properties of CSCs will enable us to develop effective therapeutic strategies.

With the available data on lung stem cells, it is reasonable to expect that Sca-1 positive and lineage negative lung cells possess stem cell characteristics of differentiation and self-renewal. As discussed in this chapter, identification of homogenous lung somatic stem cells is still an active area of research. It is thus important to establish homogenous population of lung stem cells to understand their unique characteristics. Based on the background evidence, we propose to investigate the following specific aims.

Specific Aim (I) Comparison and correlation of Sca-1 expressing lung stem/progenitor cells across p53 normal and cancer-sensitized p53 mutant mice

Experiments under this specific aim are focused to investigate the phenotype, functional and tumorigenic properties of Sca-1 expressing and lineage negative lung cells across p53 normal and cancer-sensitized p53 mutant mice backgrounds.
Specific Aim (II) Isolation and characterization of lung stem/progenitor cells based on Sca-1 and PDGFR-α

Experiments under this specific aim are focused to investigate the phenotype and functional differences of lung cells that are lineage negative but, differentially express Sca-1 and PDGFR-α cell surface marker. We will characterize these sub-sorted populations for their multipotent ability. These results will aide us in determining the role of PDGFR-α as a potential lung stem cell marker.
CHAPTER 2: COMPARISON AND CORRELATION OF SCA-1 EXPRESSING LUNG STEM/PROGENITOR CELLS ACROSS P53 NORMAL AND CANCER-SENSITIZED P53 MUTANT MOUSE MODELS

2.1 Abstract

Incidence of p53 point mutations is most common in lung cancers that result in oncogenic gain of function (GOF). Tumor recurrence after initial treatments in cancer patients is attributed to presence of cancer stem cells. The role of cancer stem cells in lungs is still an area of active research. In this study, we characterized lung stem/ progenitor cells from cancer sensitized mice carrying p53 GOF mutations. Our results show that there is distinct morphological and cell proliferation difference between the Sca-1 expressing cells established from mice lungs carrying different p53 genotypes. Additionally, our results suggest that Sca-1 expressing subpopulation of cells are significantly higher in p53 mutant carrying mice lungs than those in p53+/+ wild type. We also noted that Sca-1 expressing p53 mutants show tumorigenic properties of p53 accumulation, migration, and colony formation in non-adherent culture conditions. When the cells across the different genetic backgrounds were subjected to mesenchymal lineage
differentiation, we noted that Sca-1<sup>High</sup> p53<sup>+/+</sup> wild type readily differentiated into adipogenic and osteogenic lineages. However, the mesenchymal differentiation ability of the cells isolated from p53 mutants is not evident compared to their wild type counterparts. Results from our studies demonstrate the stem cell and tumorigenic nature of Sca-1 cells carrying p53 GOF mutations. This study lays the foundation and rationale for elaborate cellular and molecular level investigation of resident stem/progenitor cells expressing p53 GOF mutations. The insights on these resident stem/progenitor cells will ultimately shed light on cancer progression and development of new therapeutics in treating lung cancer.

### 2.2 Introduction

Cancer is a complex disease condition which results in uncontrolled division of affected cells. It is caused by combinatorial or discrete events of genetic changes (Govindan et al., 2012), environmental exposure to chemicals and radiations (Gorlova et al., 2006; Subramanian and Govindan, 2007), or life style choices, such as smoking (Hecht, 1999; Travis, 2004). According to recent estimates of incidence and mortality rates obtained by the International Agency for Research on Cancer, lung cancer was the most frequently diagnosed cancer of all cancers (Ferlay J, 2013). The incidence and mortality of lung cancer in males is highest at 16.7% and 23.6% respectively (Ferlay J, 2013), which is comparable to the 2008 global statistics suggesting that lung cancer has been the major cause of cancer related deaths in males (Jemal et al., 2011). In females over the last past 5 years, the incidence of lung cancer (8.8%) ranks 3<sup>rd</sup> behind breast (25.2%) and colorectal (9.2%) cancer, but mortality rate ranks second (13.8%) behind breast cancer (14.7%) (Ferlay J, 2013; Jemal et al., 2011). Overall, these data suggest the high occurrence of lung cancer and the need for focused research to understand its complexity and to
reduce the incidence and mortality of lung cancer. Lung cancer is predominantly caused as a result of exposure to tobacco, with global estimates indicating that about 80% of men and 50% of women affected by lung cancer as a result of smoking (Ezzati et al., 2005; Ezzati and Lopez, 2003). In non-smokers, cause of lung cancer is attributed to their genetic susceptibility (Govindan et al., 2012) and environmental or occupational exposure to chemicals (asbestos, burning coal (Subramanian and Govindan, 2007), arsenic, polycyclic aromatic hydrocarbons) or radiations (ultra-violet light, radon) (Gorlova et al., 2006; Jemal et al., 2011; Parkin et al., 2005). As a result of the exposure to carcinogenic agents in smokers and non-smokers, malignant transformation of normal cells takes place. This involves genetic damage and oncogenic signaling that eventually leads to dysregulation of normal cellular functions. Lung cancer is broadly classified based on their cell size as small-cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). More than 85% of lung carcinoma constitutes NSCLC that are further classified based on specific histologic typing (Travis et al., 2013). 70% of SCLC and 50% of NSCLC show mutations in p53 (Lane, 1992; Takahashi et al., 1989). Tumor suppressor gene p53 located on chromosome 17p13 is considered as guardian of a cell genome that functions in repairing DNA damage in G1 phase of cell cycle, before allowing cells to progress to S-phase of cell division. In an event of failure to repair the DNA damage, p53 activates apoptosis pathway that eventually results in cell death. Research studies have indicated that p53 mutations are most frequent in tobacco smokers thereby suggesting p53 as a mutational hot-spot.

The lung is a complex organ with a wide variety of cells with identification of stem/ progenitor cells with specific cell surface markers enabling researchers to characterize lung cells with multipotent stem cell characteristics. However, specific roles and characteristics of resident
stem/progenitor cells expressing Gain of Function (GOF) mutations are still unknown. In the present study, we present evidence for the first time the ability to isolate and establish stem/progenitor cells from mice lung cancer models carrying p53-GOF mutations. The single knock-in GOF p53\textsuperscript{R172H/+} mutation in this study is arginine substituted histidine in the DNA binding region of the p53 protein. The triple knock-in p53\textsuperscript{QSH/+} has substitutions in transactivation domain (L25Q, W26S) (Johnson et al., 2005) and DNA binding domain (R172H) (Olive et al., 2004; Vaughan et al., 2013). Both of these GOF mutations lead to expression of mutant p53 protein which results in selective disrupted biological functions. We hypothesize that multipotent lung stem cells (MLSCs) isolated from mice carrying p53-GOF mutations exhibit limited differentiation potential and higher self-renewal capabilities. Here, we report for the first time, morphological, phenotypical, and functional differences of MLSCs established from mice lungs across the different p53 backgrounds.

2.3 Materials and methods

2.3.1 Lung isolation, enzymatic digestion, and cell sorting

The lungs were dissected from p53\textsuperscript{+/+} Wild type C57BL/6 and conditional mutants p53\textsuperscript{R172H/+} (Olive et al., 2004) and p53\textsuperscript{QSH/+} following the steps described (Gadepalli et al., 2013; Hegab et al., 2010). For the purpose of this study, lungs from two mice (n=2) were harvested from respective p53 backgrounds. Briefly, the mice were anesthetized by intra-peritoneal (IP) route and sacrificed by cervical dislocation. Careful dissection is performed from posterior to anterior end of the abdomen to locate trachea near throat region. 1 ml of dispase (2U/ml) is injected after a slight incision made on trachea. Following the injection of dispase, the inflated lungs were carefully harvested along with trachea and other visceral organs. The lung lobes were separated...
from the other body parts and incubated in sterile solution containing dispase and collagenase to obtain slurry of the lung tissue (Gadepalli et al., 2013). Finally, the red blood cells (RBC) and undigested lung tissue were separated by addition of RBC lysis buffer, rinsing in sterile PBS$^{++}$ (with Mg$^{2+}$ and Ca$^{2+}$) and filtering through 0.45 µm sterile filter. The cells are counted and plated at 1000 cells per cm$^2$ and expanded for 21 days.

Figure 2-1: Schematic representation of lung isolation, digestion, and sorting.

(i) Separation of lung lobes, mincing lung tissue, and enzymatic digestion to prepare single cell suspension. (ii) RBC lysis and filtration of undigested lung tissue. (iii) Rinsing in growth medium, cell counting and plating in appropriate tissue culture-treated flask. (iv) Collection of single cells in trypsin, neutralize with growth medium and PBS$^{++}$ wash. (v) Fluorescent conjugated Antibody staining with appropriate controls, depletion of PE positive cells and FACS sorting of lung subpopulations differentially expressing Sca-1 (vi) Propagating cells in growth medium for downstream cell analysis.
2.3.2 Cell culture components

As the current study aims to establish and characterize primary cells with stem cell characteristics following similar marker combination reported by (Hegab et al., 2010), we opted to use the same medium composition that was shown to be conducive for propagating these cells. The components for growth medium are 10% defined FBS (HyClone Laboratories, Logan, Utah USA), DMEM-High glucose with Glutamine & sodium pyruvate 1x Penicillin/Streptomycin, 1x NEAA. Splitting of cells was carried at near confluence with 0.25% trypsin. Cryopreserved cell stocks were prepared at regular passages using freezing medium containing 10% DMSO and 30% defined FBS. All reagents were obtained from Life Technologies (Grand Island, NY, USA) unless otherwise noted.

2.3.3 FACS sorting and analysis of cell surface marker expression

The goal of the study is to establish lung stem/progenitor cell lines from mice with different p53 genetic background, based on expression of cell surface marker Sca-1. For this purpose, the cells were passaged using 0.25% trypsin, washed with PBS++ and suspended in 100 µl volume of PBS++ with respective concentration of conjugated antibody for staining, as described in (Appendix i). The cells were incubated on ice for 30 minutes and washed twice with PBS++. Sorting of the stained cells is performed on BSC Aria- BD FACSAria™ II High-Speed Cell Sorter (BD Biosciences, Franklin Lakes, NJ, USA) at flow cytometry shared resource, VCU. For the current study, the cells were sorted based on the following expression of cell surface marker, Sca-1^{High}, CD31^{neg}, CD45^{neg} and Sca-1^{Low}, CD31^{neg}, CD45^{neg}. All the sorting procedures were conducted under sterile conditions and respective fluorophore isotype IgG control were employed as negative control.
For analysis of differential expression of Sca-1 marker across the different p53 background mice, unsorted cells were plated in 100 cm dish (in replicates). At near confluence, the uniform staining protocol was employed as described in (Appendix i). The expression differences were evaluated on BD Accuri™ C6 flow cytometry. For this purpose, equal numbers of flow events were collected across all the samples in this study in replicates. Analysis was carried out using respective fluorophore isotype controls. Statistical differences were assessed using Students t-test and p-value <0.05 was considered significant.

2.3.4 Immunocytochemical analysis for Sca-1 expression

Sorted Sca-1$^{\text{High}}$ and Sca-1$^{\text{Low}}$ cells across all the p53 backgrounds were seeded on 0.1% gelatin coated glass bottom 35 mm dishes (MatTek Corporation, Ashland, MA, USA) at 2000 cells per well. After 24 hours or at 80% confluence, the cells were fixed with 4% PFA and stained with mouse Sca-1/Ly-6 primary antibody and Goat Anti-Rat IgG NL557, secondary antibody (R&D Systems, Minneapolis, MN, USA). The nuclei were stained with DAPI (EMD Millipore, Billerica, Massachusetts USA). Briefly, 10 µg/ml of primary antibody in a 300 µl of blocking buffer (3% goat serum in PBS$^{++}$) was added to the cells and the plates were incubated overnight at 4°C. The sample were prepared for imaging by 1 hour incubation with secondary antibody, followed by PBS$^{++}$ washes and nuclear stain with DAPI. Fluorescent images were acquired using a CoolSnap EZ camera 29 (Photometrics, Tucson, AZ, USA) mounted on a Nikon Eclipse TE 2000-S inverted microscope (Nikon, Melville, NY, USA) with attached image analysis software. All images settings were controlled for uniform acquisition between the samples. Specifically, uniform exposure time of 500ms was maintained across the experimental samples and as well as the negative controls for background subtraction.
2.3.5 AlamarBlue® cell proliferation assay

The differences in propagation for Sca-1 sorted cells were determined by AlamarBlue® Cell viability (Life Technologies, Grand Island, NY, USA). In this study, to determine cell growth over a period of 7 days, the Sca-1\(^{\text{High}}\) and Sca-1\(^{\text{Low}}\) subpopulations across the wild type and mutants were trypsin passaged at confluence and cell counts were determined on BD Accuri™ C6 (BD Biosciences, Franklin Lakes, NJ, USA), as described in Appendix ii). 500 cells were plated per well in a 96-well plate in quadruplicates for respective lung cell lines. After allowing the cells to attach to the tissue culture plate for 4 hours the medium is aspirated and a fresh growth medium mixed with 10% AlamarBlue® reagent was added. Absorbance was measured at a wavelength of 570 nm and 600 nm using Synergy H1 plate reader. Continuous readings were obtained every day for a period of 7 days without changing the growth medium. Wells with growth medium alone and growth medium plus 10% AlamarBlue® reagent were run as blanks. The experiment was repeated at two different passages and OD readings were averaged across the experiments. The percent reduction of AlamarBlue® reagent is calculated by the following equation,

\[
\text{AR}_{570} = \left( \frac{\text{A}_{570} - (\text{A}_{600} \times \text{Ro})}{\text{Ro}} \right) \times 100
\]

Where,

\(\text{AR}_{570}\) = Amount of reduced AlamarBlue® reagent

\(\text{A}_{570}\) & \(\text{A}_{600}\) are sample absorbance at respective wavelength minus media blank

\(\text{Ro}\) = Correction factor. This is calculated by \(\text{AO}_{570} \div \text{AO}_{600}\), where \(\text{AO}_{570}\) and \(\text{AO}_{600}\) are the Absorbance of Oxidized (AO) form of AlamarBlue® reagent, at respective wavelengths. \(\text{AO} = \text{OD} 10\%\) AlamarBlue® in medium - OD growth medium, at respective wavelengths.
2.3.6  Cell cycle profile analysis

To evaluate the percentage of actively dividing cells in Sca-1\(^{\text{High}}\) and Sca-1\(^{\text{Low}}\) lines established across the different p53 backgrounds, the cells were stained with propidium iodide. Briefly, at near confluence, cells were subjected to Trypsin treatment and cell counts were determined as described in Appendix ii). For use in cell cycle analysis, 5×10\(^4\) cells were plated per 10 cm\(^2\) (35 mm) tissue culture plates. The cells were allowed to propagate for 24 -48 hours, allowed to reach 80% confluency, collected together after Trypsin treatment, washed with PBS\(^{++}\), fixed in cold 70% ethanol and stored at -20°C until analysis. Further, DNA is stained with 20 µg/ml of propidium iodide (St. Louis, MO, USA). Equal number of events are collected across all the samples using BD Accuri™ C6 flow cytometry and analysis is carried using FCS Express 4. The detailed procedure for staining is described in Appendix. Samples were analyzed in triplicates and statistical significance was determined by student t-test, with p-value <0.05 considered to be significant.

2.3.7  In-vitro scratch assay for analysis of cell migration

The tumorigenic potential of p53 mutant backgrounds with respect to the normal wild type cell lines was determined by quantifying migration ability of cells. For this purpose sterile silicone culture inserts (ibidi LLC, Verona, WI, USA) were used, designed to create cell free gap of 500 µm. The respective wild type and mutant cell lines were passaged with trypsin, quenched in growth medium and cell counts determined as described in Appendix ii). The inserts were placed in a 12-well dish with sterile forceps. Lines were drawn on the external bottom surface of each well of the 12-well dish with a marker pen for proper...
orientation during imaging. Approximately, 500 cells were plated in the culture inserts and placed in the incubator for 24 hours. The inserts were carefully removed with sterile forceps, plates rinsed in with PBS++ and fresh medium was added. At 1st time point (0 hours), images of the cell free gap created after removing the culture inserts was captured in proper orientation based on the markings created underneath the well surface. The plates were returned back to the incubator and images captured after 6 hours in the same orientation and at the same position corresponding to the earlier time point. The experiment was conducted in duplicates with 3 points of measurement for each well. The images were captured with CoolSnap EZ camera (Photometric, Tucson, AZ, USA) mounted on Nikon Eclipse TE2000-S inverted microscope (Nikon, Melville, NY, USA). The distance of the cell free gap was measured with Nikon NIS Elements-Br imaging software between two time points. A total of nine measurements were acquired for each cell line and statistical significance was determined by student t-test. The migration rate (µm/hr.) was calculated using the following formula.

\[
\text{Migration rate (µm/hr)} = \frac{\text{Starting distance (µm)} - \text{Final distance(µm)}}{\text{Time (hours)}}
\]

2.3.8 Soft agar non-adherent cell culture assay

The tumorigenic potential of the p53 mutant cell lines was determined by their ability to form spheres in anchorage independent culture conditions. For this purpose, 1% pure agarose (BioExpress, Kaysville, UT, USA) was autoclaved and appropriate amount (2ml) was poured into 6-well tissue culture plate to prevent cell attachment. The cells were passaged as described in
Appendix ii and 10,000 cells were plated per well of 6-well plate. After addition of the growth medium, the cells were returned to the CO$_2$ incubator and cell growth monitored for a period of 10-14 days. 1 ml of fresh growth medium was added without replacing the old medium every 2 days. The experiment was conducted in duplicates at two different passages. All cell colonies in suspension that grew in size were counted across triplicates. Statistical significance was determined by student t-test, with p-value<0.05 considered to be significant.

### 2.3.9 Mesenchymal lineage differentiation analysis

The multipotent differentiation capability of the Sca-1$^{\text{High}}$ and Sca-1$^{\text{Low}}$ lines established across the different p53 backgrounds was evaluated by their ability to differentiate into osteogenic and adipogenic lineage. For osteogenic differentiation, 10,000 cells were plated per well of a 12-well tissue culture plate in growth medium. At 70 % confluence, the growth medium is replaced with osteogenic differentiation medium containing 10nM Dexamethasone, 50 µM L-Ascorbic acid 2-phosphate and 20mM β-Glycerophosphate disodium salt hydrate. The cells were propagated for a period of 14-21 days. At the end of the differentiation period, the cells were fixed with 4% Paraformaldehyde. The deposition of calcium was detected with 40 mM Alizarin red stain. For adipogenic differentiation, 20,000 cells were plated per well of a 12-well tissue culture plate in adipogenic differentiation medium containing 1:50,000 dilution of 1mM insulin and 1:10,000 dilution of 10 µM T3. At 100 % confluence, the differentiation medium is replaced with adipogenic induction medium containing 0.125M Indomethacin, 2mg/ml dexamethasone, 0.25M IBMX along with 1mM insulin and 10 µM T3. The cells were induced in the induction medium for 2 days and later maintained in adipogenic differentiation medium for 5 more days. At the end of differentiation, the cells were fixed in 10% formaldehyde. The formation of lipid droplets was...
detected with Oil Red O staining solution. Further details of the differentiation protocol and staining procedure are described in Appendix iii. All reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise noted.

2.3.10 RNA isolation, real time reverse transcription polymerase chain reaction, and gene expression analysis.

To quantitate expression of genes of interest listed in Appendix iv, cell pellets were collected and RNA purification was performed using AllPrep DNA/RNA Mini Kit following manufacturer instructions (Qiagen, Valencia, CA, USA). The RNA concentration of the samples was determined by Nanodrop 2000c spectrophotometer (Thermo scientific, Wilmington, DE, USA) and 1µg of RNA across all the sorted cell lines was converted into cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY, USA). Q-PCR reaction was setup using iTaq Universal SYBR Green supermix (BioRad, Hercules, CA, USA) with appropriate concentration of primer and cDNA. The primer efficiencies of the internal control (GAPDH) and genes of interest were determined by a standard curve. Gene expression data (in triplicates) were acquired using CFX 96 real time system (C1000 touch). Details of the q-PCR setup and protocol are described in Appendix v). GAPDH was run as a housekeeping reference gene and analysis was performed by normalizing the expression quantity of gene of interest to the expression quantity of internal control gene using the relative standard curve method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Statistical significance was determined by student t-test, with p-value <0.05 being considered as significantly different.
2.3.11 Western blot analysis

To determine the expression of p53 protein in the sorted Sca-1\textsuperscript{High} and Sca-1\textsuperscript{Low} lines established across the different p53 backgrounds, cells were plated at 5000 cells per cm\textsuperscript{2} in respective 60 mm (area 20 cm\textsuperscript{2}) tissue culture plate and propagated to 90\% confluence. Cells were collected in trypsin, quenched, washed with PBS\textsuperscript{++}, and cell pellets placed on ice. Protein extraction was performed using RIPA lysis buffer following the supplier protocol. Protein concentrations were determined using Bradford assay. The crude protein lysate obtained across the different cell lines were probed for expression of p53 and ERK-2 by Western blot using specific antibodies (Santa Cruz Biotech, Dallas, Texas, USA). Anti-rabbit HRP-conjugated antibody was used as secondary antibody (CST, Danvers, MA, USA). Detection was carried out by developing the membrane in with ECL substrate solution (Thermo scientific, Wilmington, DE, USA) and images were captured using Gel Doc\textsuperscript{TM} XR\textsuperscript{+} system (BioRad, Hercules, CA, USA).

2.3.12 Genotyping assay by PCR

DNA isolation and purification was performed using AllPrep DNA/RNA Mini Kit following manufacturer instructions (Qiagen, Valencia, CA, USA). Standard PCR reaction was setup with appropriate concentration of 3 primers, DNA and reagents. The genotyping strategy is to span the LoxP site in the mutant and to distinguish them from wild type. The PCR products were run on a 2\% agarose gel to determine genotype of the wild type or mutant cell lines based on presence or absence of LoxP. Detailed description of the protocol can be found in Appendix v).
2.3.13 Colony formation assay

To determine the colony forming ability of sorted cells, cells were plated at low density 5 cells per cm² on inactivated mouse embryonic fibroblasts (iMEFs) and allowed to propagate for 7 days. The cells were collected in trypsin, quenched in growth medium and cell counts determined as described in Appendix ii). Later, they were fixed in 10% ice cold methanol and colonies were stained with 1% crystal violet solution (Sigma, St. Louis, MO, USA).

2.4 Results

2.4.1 Sca-1 expressing putative lung stem cells across p53 backgrounds

Taking into consideration the complex nature of the lung and based on previous studies (Hegab et al., 2010; Kim et al., 2005; McQualter et al., 2009), we expect that Sca-1<sup>pos</sup>, [hematopoietic (CD45<sup>neg</sup>) and endothelial (CD31<sup>neg</sup>)] lin<sup>neg</sup> cell population comprises more than one type of putative lung stem/progenitor cells. Additionally, with increasing evidence of two different subpopulations within the Sca-1<sup>pos</sup> cells, it is important to compare and characterize them for a better understanding of lung biology. Towards use in this comparative study, single cell suspensions were obtained by enzymatic digestion of lungs (n=2) dissected from mice from different p53 genetic backgrounds. Since the population of lin<sup>neg</sup> and Sca-1<sup>pos</sup> cells in lungs were determined to be a rare subpopulation, we plated the unsorted single cells into an appropriate tissue culture (TC) flask (T75) and propagated them with regular growth medium changes. In order to minimize cell culture induced changes in in vitro conditions, we avoided splitting and expanding the population before sorting. We noted that the unsorted lung cells obtained from the mutant p53 background mice reached confluence in about 15-20 days while the p53<sup>++</sup> wild type
cells took more than 20 days to reach confluence. After enriching the unsorted lung cells, single cell suspensions were stained with fluorescent conjugated somatic stem cell marker Sca-1-FITC, and lineage markers for hematopoietic (CD45-PE) and endothelial (CD31-PE) cell surface markers. Lin<sup>pos</sup> cells were depleted and Sca-1<sup>Low</sup> and Sca-1<sup>High</sup> subpopulations were collected using BD FACS Aria™ II high-speed cell sorter. Our results indicate that even though Sca-1<sup>High</sup> and Sca-1<sup>Low</sup> subpopulations are not distinctly concentrated in a particular region of the Forward-scatter (FS)-area vs Side-scatter (SS)-area or on the fluorescence (FITC) dot plot, they could be gated as high and low expressing populations based on their spread. We noticed that the percentage of cells staining positive for CD45 and CD31 were lower in cell suspension obtained from in vitro culture than those obtained directly from lung tissue. We speculate that these lin<sup>pos</sup> cells were loosely adhered or have limited propagation ability and were lost during growth medium changes. Upon sorting, the Sca-1<sup>Low</sup> and Sca-1<sup>High</sup> cell subpopulations from different p53 backgrounds were propagated in TC plates (35mm) with growth medium. We further performed immunofluorescence staining to validate our sorting strategy, as shown in (Figure 2-3). Under uniform exposure conditions, we noted the Sca-1<sup>High</sup> populations stained positive, while the Sca-1<sup>Low</sup> populations were predominantly negative with few positive cells.
Figure 2-2: FACS sorting of Sca-1 expressing lung stem cells across p53 backgrounds

FACS sorting is performed with appropriate gating to deplete lin$^{pos}$ cells (CD45 and CD31) from single cell suspension prepared from the whole lung harvested across the respective p53 backgrounds. The remaining lung cell subpopulations were sorted for Sca-1$^{High}$ and Sca-1$^{Low}$ and plated in tissue culture dish for propagation.
Figure 2-3: Differential Sca-1 expression in Sca-1\textsuperscript{High} and Sca-1\textsuperscript{Low} sorted cell lines

Immunocytochemical analysis for Sca-1 shows differential staining patterns across the mutant and the wild type lines. The Sca-1/Ly-6 expression is show in red and nuclei were stained with DAPI (blue). Scale bar represents 50µm.
2.4.2 Sca-1 expressing lung stem cells from p53 mutants possess higher proliferation and self-renewal characteristics

Our phenotypic assays on proliferation and self-renewal ability on the sorted Sca-1 subpopulations have demonstrated differences between the wild type p53 and mutant p53 cell lines. Comparing morphology under inverted microscope (Nikon TS100), we did not observe differences in cell morphology within the Sca-1\textsuperscript{Low} and Sca-1\textsuperscript{High} subpopulations established across the respective p53 backgrounds. However, we noted distinct morphological differences between the p53\textsuperscript{+/+} wild and p53 mutant cell lines. The p53 wild type cell lines were more heterogeneous, with small spindle shaped cells and cobblestone appearances, while the mutant cell lines were predominantly homogenous with elongated mesenchymal cell morphology (Figure 2-4). This observation is supported by our quantitative-PCR studies, where significantly higher mRNA transcript levels of N-cadherin was observed in p53 mutant cells relative to the wild type. Additionally, between the Sca-1\textsuperscript{Low} and Sca-1\textsuperscript{High} of the mutant cell lines, Sca-1\textsuperscript{High} subpopulations possess higher levels of N-cadherin, supporting the mesenchymal nature of the putative cancer stem cells (Figure 2-5). We also observed that lung subpopulations derived from p53 mutant background were more easily detached from the cell culture plate compared to the wild type subpopulation, an indicative of their mesenchymal nature. Our proliferation curves were determined by ability of viable cells to reduce resazurin, an active ingredient in AlamarBlue® reagent, into fluorescent resorufin. As indicated by proliferation curves (Figure 2-6), the subpopulations derived from p53 mutant backgrounds possess higher proliferation compared to those derived from p53 wild type. This is also supported by our cell cycle analysis, where higher percentage cells carrying p53 mutants were analyzed to be in the S and G2/M phase of the cell cycle (Figure 2-7).
Furthermore, we investigated the self-renewal ability of the Sca-1 subpopulations to form colonies at low seeding density over a period of 7 days. Our results show that Sca-1 subpopulations expressing wild type p53+/+ cells fail to form large colonies at low seeding density during this time, while the same subpopulations from p53 mutant mice formed distinct large colonies (Figure 2-8). In order to further ascertain the self-renewal characteristic of the lung subpopulations from different backgrounds, we quantified the expression levels of BMI-1. BMI1 expression plays a crucial role in self-renewal of stem cells and as well as in cancer progression (Siddique and Saleem, 2012). Specifically, in lungs, BMI-1 deficient bronchioalveolar stem cells lack self-renewal property (Dovey et al., 2008; Zacharek et al., 2011). Moreover, BMI-1 is elevated across diverse tumor types, thus suggesting that BMI-1 plays a key role in regulating proliferation of both stem cells and tumor cells (Dovey et al., 2008; Siddique and Saleem, 2012). Our results show that Sca-1 lung subpopulations carrying p53 mutations possess significantly higher BMI-1 levels relative to those derived from p53+/+ wild type mice (Figure 2-9). We also observed significant increase in expression levels of BMI-1 across different human lung cancer lines relative to normal human bronchiolar endothelial cells (Figure 2-9). Overall, our results demonstrate that Sca-1 expressing subpopulations from the p53 mutant mice are an actively dividing and self-renewing population compared to those derived from p53+/+ wild type mice.
Figure 2-4: Lung stem cells from p53 mutant mice show distinct mesenchymal morphology.

Distinct morphological differences are observed between p53\(^{+/+}\) wild type and the mutant cell lines. The p53\(^{+/+}\) wild type cells possess small spindle shape morphology and the cell lines from mutant backgrounds possess longer mesenchymal morphology. Scale bar 20x magnification.
Figure 2-5: Sca-1 expressing lung cells from mutant p53<sup>R172H</sup>/+ mice show higher N-cadherin expression

Relative transcript levels of N-cadherin across the p53 backgrounds and between the sorted cell lines (A) Sca-1 High (B) Sca-1 Low, were determined by Q-PCR and fold change was calculated by relative standard curve method with p53<sup>+/+</sup> wild type as reference cell line. Results are presented together with standard deviation from experiment conducted in triplicates. (* = p < 0.05).
Figure 2-6: Sca-1 expressing lung cells from p53 mutants show higher proliferation

Proliferation curve of the established cell lines was determined over a period of 7 days in medium containing Alamar blue reagent. The percent reduction in Alamar blue reagent was measured by absorbance at 570 nm with 600 nm as reference. The active growth of the cells is proportional to the amount of Alamar blue reagent reduced. The lung sub-population established from p53 mutant background attained confluence at day 4 while, the p53 wild type cells reach confluence at day 6 Results are presented together with standard deviation from two independent experiments conducted in quadruplicates. (* = p < 0.05).
Figure 2-7: Sca-1 expressing lung cells from p53 mutant mice show lower percentage of cells in G-1 phase and higher percentage in S and G2/M phase of cell cycle.

The higher proliferation potential of the p53 mutant cell lines were analyzed by determining the cell cycle phases using propidium iodide (PI) staining and analyzed with Accuri C6 flow cytometry. Error bar represents standard deviation across experiments conducted in triplicates (* = p <0.05).
Figure 2-8: Sca-1 expressing lung cells from p53 mutant mice show colony formation ability at low seeding density

The ability of Sca-1 subpopulations sorted across the p53 mutants to form colonies was evaluated by plating cells at low seeding density on inactivated mouse embryonic fibroblasts (iMEF). The colonies were stained with crystal violet stain after a period of 6 days. Data from experiment performed in triplicates was used for analysis.
Figure 2-9: Sca-1 expressing lung cells from mutant p53 show higher levels of BMI-1 mRNA expression

Relative transcript levels of BMI-1 across the p53 backgrounds were determined by Q-PCR and fold change was calculated by relative standard curve method with p53+/+ wild type as reference cell line. Results are presented together with standard deviation from experiment conducted in triplicates. (* = p < 0.05).
Figure 2-10: Higher expression of BMI-1 is observed in human lung cancer lines

Gene expression levels of BMI-1 across the human cancer cell lines were determined by Q-PCR and fold change was determined by comparative $C_q$ method with normal human bronchiolar endothelial (NHBE) cells as reference cell line. Results are presented together with standard deviation from experiment conducted in triplicates. (* = $p < 0.05$).
2.4.3 Conditional point mutant p53 are expressed in lung subpopulations that are sorted based on cell surface marker Sca-1

The GOF mutations p53\textsuperscript{R172H/+} and p53\textsuperscript{QSH/+} investigated in this study are conditional heterozygous mutant alleles, expressing point mutations in p53 protein, and activated by Cre-mediated recombination as described in an earlier study (Olive et al., 2004). In our study, we determined the genotype of these mutants and wild type by standard PCR with a 3 primer combination based on published protocols (Olive et al., 2004). Our results indicate the expression of these conditional mutants in Sca-1\textsuperscript{Low} and Sca-1\textsuperscript{High} sorted lung cells are as anticipated. Results show the PCR amplification with a 3 primer combination resulted in only a control product of 166 bp in p53 wild type cells, while the p53 mutant background cells express both the control product of 166 bp and 270 bp LOXP LSL element (Figure 2-11). We further determined the ability of the conditional mutants to express aberrant p53 protein that results in accumulation of p53 protein. As expected, Western blot analysis show that p53\textsuperscript{+/+} wild type cell lines did not have any p53 protein accumulation for both Sca-1\textsuperscript{High} and Sca-1\textsuperscript{Low} subpopulations (Figure 2-12). The subpopulations from the p53 mutant cell lines, p53\textsuperscript{R172H/+} and p53\textsuperscript{QSH/+}, shows p53 protein accumulation, suggesting that alleles with point mutations in p53 are expressed in the engineered knock-in mice models.
Figure 2-11: Genotyping by PCR amplification of conditional mutant alleles results in a
270 bp mutant product and a 166 bp control product.

(A) PCR products run on 1% agarose gel (1) Wild type, (2) p53^{R172H/+}, and (3) p53^{Q57/+} cell lines
expressing (a) Sca-1^{High} and (b) Sca-1^{Low}. (L-100 bp ladder). (B) Primer combinations that yields
the respective PCR product wild type allele (166 bp) detected by T037, T035, and (270bp)
detected by T036, T035 flanking the LSL element.
Figure 2-12: Sca-1 expressing lung cells from p53 mutant cell lines show accumulation of p53 protein.

Whole cell lysates were extracted from Sca-1 expressing lung subpopulations across the p53 backgrounds and were analyzed for expression of p53 and ERK-2 (housekeeping control). The conditional point mutants $p53^{R172H/+}$ and $p53^{QSH/+}$ show accumulation of p53 protein while the $p53^{+/+}$ wild type show no p53 accumulation.
2.4.4 Cell lines from p53 mutant mice possess higher percentage of Sca-1 expressing cell populations

We compared the percentage of cell populations expressing Sca-1\(^{High}\) and Sca-1\(^{Low}\) across different p53 backgrounds using flow cytometry analysis. For these comparisons, we performed independent flow cytometry analysis on unsorted lung cells propagated in TC plates (100 mm). We noted that there is a significant difference in the number of Sca-1\(^{High}\) cells across the mice with different p53 genotype backgrounds. Our results indicate that approximately 75% of unsorted lung cells from p53\(^{R172H/+}\) and 74% of p53\(^{Q58/+}\) can be classified as Sca-1\(^{High}\) population, while only 61% of p53\(^{+/+}\) wild types are Sca-1\(^{High}\) (Figure 2-13). Similarly, we noted that percentage of Sca-1\(^{Low}\) is significantly lower in p53 mutant cell lines, but higher (33%) in p53\(^{+/+}\) wild types. This observation was also supported by our q-PCR analysis, where Sca-1 mRNA transcript levels are significantly higher in the Sca-1\(^{High}\) population in p53 mutant cell lines relative to the p53\(^{+/+}\) wild types (Figure 2-14).
Figure 2-13: Cell lines isolated from p53 mutant mice lung possess higher percentage of Sca-1 expressing cells

Unsorted lung cells established across different p53 background were propagated in culture and cells differentially expressing Sca-1 were analyzed by flow cytometry. (A) Scatter plots of unsorted cells stained with Sca-1-FITC (y-axis) and CD45-PE, CD 31-PE (x-axis) (B) Bar plots show significant differences in percentage of cells expressing Sca-1, PDGFR-α between the p53 backgrounds. (* = p < 0.05). Results were presented together with standard deviation from 3 independent experiments.
Figure 2-14: Sca-1 expressing lung subpopulations from p53 mutant cell lines show higher transcript levels of Sca-1

Q-PCR analysis of respective transcript levels of Sca-1 mRNA across the p53 backgrounds. Sca-1 expressing lung subpopulations from p53 mutants show significantly higher mRNA levels of Sca-1 compared to the p53+/+ wild type. Results are presented together with standard deviation from experiment conducted in triplicates. (* = p < 0.05).
2.4.5 Sca-1 expressing lung cells from wild type p53\(^{+/+}\) cells show higher differentiation ability

We determined the multipotent differentiation capability of the sorted cells across different p53 backgrounds by subjecting them to conditions that lead to formation of adipocytes and osteocytes, considered to be traditional mesenchymal lineages. Our results show that the p53\(^{+/+}\) Sca-1\(^{\text{High}}\) subpopulations readily differentiated compared to the Sca-1\(^{\text{Low}}\) subpopulations. Our results indicate that the p53\(^{+/+}\) Sca-1\(^{\text{High}}\) subpopulations show distinct Oil red O staining patterns, indicative of adipogenic differentiation, after a period of 10 days (Figure 2-15), while the Sca-1\(^{\text{Low}}\) subpopulation exhibited fewer oil droplet formation indicative of limited differentiation potential. Similar differentiation potential was noted when p53\(^{+/+}\) wild type cells were subjected to osteocyte differentiation conditions for a period of 14-21 days, with robust calcium deposition in p53\(^{+/+}\) Sca-1\(^{\text{High}}\) cells than in Sca-1\(^{\text{Low}}\) cells (Figure 2-16). However, we observed that p53 mutant cell lines p53\(^{\text{R172H/+}}\) and p53\(^{\text{Q57/+}}\) did not exhibit adipogenic differentiation potential, with differentiation conditions resulting in loss of cell viability during multiple attempts (Figure 2-15). Under osteogenic differentiation conditions, the Sca-1\(^{\text{Low}}\) subpopulations from the p53 mutant cell lines exhibited distinct calcium deposition, while the Sca-1\(^{\text{High}}\) subpopulations from these p53 mutant backgrounds showed some heterogeneous calcium deposition (Figure 2-16), indicating that the p53 mutant cell lines have propensity for osteogenic differentiation. Interestingly, the subpopulations from the p53 mutant mice expressed tissue specific alkaline phosphatase, indicative of propensity for osteogenic differentiation (Appendix vi).
Figure 2-15: Sca-1\textsuperscript{High} lung subpopulations from p53\textsuperscript{+/+} wild type mice show distinct adipogenic differentiation ability

The sorted cell lines across the different p53 backgrounds are propagated in adipogenic differentiation medium for 10 days. Formation of lipid vacuoles that stain with oil red o stain at the end of differentiation period is indicative of adipogenesis. The Sca-1\textsuperscript{High} wild type subpopulations show distinct Oil red O droplet formation compared to the Sca-1\textsuperscript{Low} lung subpopulations. Sca-1 expressing lung cells from p53 mutant mice exhibited loss of viability indicating detrimental effects of the adipogenic differentiation medium.
Figure 2-16: Sca-1^{High} lung subpopulations from p53^{+/+} wild type mice show distinct osteogenic differentiation ability

The sorted cell lines across the different p53 backgrounds are propagated in osteogenic differentiation medium for 21 days. Deposition of calcium that stain with Alizarin red S at the end of the differentiation period is indicative of osteogenesis. The Sca-1^{High} and Sca-1^{Low} subpopulation from p53^{+/+} wild type and Sca-1^{Low} subpopulation from p53 mutant cell lines show distinct Alizarin red S staining.
2.4.6 Sca-1 expressing lung subpopulations from p53 mutant mice show selective tumorigenic effects

The tumorigenic property of the cell lines derived from cancer-sensitized p53 mutant (p53^{R172H/+} and p53^{QSH/+}) mice, were determined by anchorage independent growth and migration assay. Our results clearly indicate that subpopulations derived from p53^{+/+} wild type mice exhibit no colony forming ability in anchorage independent growth on soft agar over a period of 10 days, while the subpopulations derived from the p53 mutant mice demonstrated ability to form colonies and grow in size in anchorage independent growth conditions during the same time period.

Specifically, Sca-1^{High} subpopulations derived from p53 mutant mice demonstrate higher colony formation compared to the Sca-1^{Low} subpopulations (Figure 2-17). To determine the migration rate, we captured images of cells in defined regions at 0 and 6 hrs after initiation of the assay as described in the methods section (Appendix vii). Our observations indicate that the subpopulations from the p53^{+/+} wild type and p53^{QSH/+} mice possess a similar migration rate, while the subpopulation from the p53^{R172H/+} mice exhibited a
significantly higher migration rate (Figure 2-18); Overall, our results at the in vitro level indicate that subpopulations derived from the cancer sensitized p53 mutant mice exhibited tumorigenic properties. However, our observations indicating differences existing between subpopulations derived from different cancer-sensitized p53 mutant mice is noteworthy and alludes to potential differences in tumorigenic properties based on specific p53 mutant genetic backgrounds.
Figure 2-17: Sca-1 expressing lung cells from p53 mutant mice show anchorage independent growth

Colonies formed by lung sub-populations across different p53 backgrounds and histogram representing the total number of colonies counted after 14 days in culture. (A) Sca-1\textsuperscript{High} (B) Sca-1\textsuperscript{Low}. Data represented across two independent experiments conducted in replicates. (* = p < 0.05, ** = p < 0.01).
Figure 2-18: Sca-1 expressing lung cells from p53R172H/+ mutant mice show higher migration rate

Sca-1 expressing subpopulations across different p53 backgrounds were cultured to confluence in wells of silicone culture inserts with defined cell free gap of 500 µm. The monolayer is denuded by carefully lifting the inserts and distance between the denuded areas was measured at 0 and 6 hrs. Migration is shown pictorially at 20x magnification (A) and migration rate graphically (B). Data represented across experiments conducted in triplicates and error bars represent standard deviation. (* = p < 0.05).
2.5 Discussion

Treatment of cancer requires elimination of all tumor cells that are capable of regenerating tumors. A better understanding of tumor-recapitulating cells that are considered to be cancer stem cells is essential for development of successful cancer therapies. Studies have shown that characteristics of cancer stem cells vary with the genotype of the cancer (Curtis et al., 2010). While some studies have shown the role of CD133 expressing lung cancer stem cells in tumor regeneration (Chen et al., 2008; Eramo et al., 2008); there is also evidence for tumor-recapitulating ability of Sca-1 expressing lung cells (Curtis et al., 2010). Here we analyzed lung cells differentially expressing Sca-1 across different p53 genotype backgrounds and characterized the nature of these Sca-1 expressing cells in \textit{in vitro} conditions. The heterozygous mutant genotypes in this study, p53^{R172H/+} and p53^{Q57H/+}, were previously shown to possess tumor spectrums different from p53^{+/-} and p53\textsuperscript{Null} mutants (Olive et al., 2004; Vaughan et al., 2012a). The heterozygous and homozygous targeted point mutations in the p53 allele were reported to possess high frequency of metastasis and distinct carcinoma development. In spite of an active wild type p53 allele, these genotypes show tumor characteristics indicating a dominant negative effect of the point mutation (de Vries et al., 2002). Since p53 point mutations result in expression of mutant p53 protein, which prevents binding of p53 to DNA or MDM2 (de Vries et al., 2002; Olive et al., 2004; Vaughan et al., 2012b), analysis of subpopulations derived from these backgrounds in the context of lung cancer merits further attention.

Our study investigated the selective effects of these point mutations in Sca-1 expressing lung cells which were shown to possess demonstrable self-renewal and differentiation characteristics in normal lung stem cells (Hegab et al., 2010; Kim et al., 2005; McQualter et al., 2009). Our
work indicates that the Sca-1\textsuperscript{High} expressing lung cell subpopulation is significantly higher in p53 mutant mice when compared to the p53\textsuperscript{+/+} wild type mice, suggesting increased numbers of Sca-1 expressing cells in cancer sensitized p53 mutant backgrounds. A similar observation of increased number of Bronchioalveolar stem cells (BASC) in Lox-K-ras mice with atypical adenomatous hyperplasias (AAH) and adenomas was noted (Kim et al., 2005) and were found to play a role in tumor initiation \textit{in vivo} (Curtis et al., 2010; Kim et al., 2005). While we did not test the tumor-initiating capabilities of the Sca-1\textsuperscript{High} and Sca-1\textsuperscript{Low} subpopulations derived from cancer-sensitized p53 backgrounds \textit{in vivo}, we have successfully demonstrated the tumorigenic properties of these cells. Sca-1\textsuperscript{High} cells across both the p53 mutant genotypes have demonstrated higher proliferation, anchorage independent growth, and migration, indicative of their tumorigenic properties. Our results also confirm p53 accumulation in the protein lysates obtained from subpopulations derived from both mutant backgrounds, validating the expression of mutant p53 protein. Even though our tumorigenic assays demonstrate the higher tumorigenic potential of the subpopulations derived from the p53\textsuperscript{R172H/+} mice, these cell lines expressed low amount of p53 protein compared to those derived from p53\textsuperscript{QSH/+} mutant mice. Our observations indicating low levels of p53 accumulation in Sca-1 sorted cells from p53\textsuperscript{R172H/+} cell lines could be attributed to the presence of an altered splice acceptor site in p53\textsuperscript{R172H/+}, that results in reduced expression of the mutant p53 protein (de Vries et al., 2002; Olive et al., 2004). B cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) protein has been shown to play a critical role in self-renewal and differentiation of somatic stem cells in lung and other tissues (Siddique and Saleem, 2012; Zacharek et al., 2011). Conclusions based on numerous studies indicate the role of BMI-1 in progression, recurrence, and chemo-resistance of cancer cells. Our analysis of BMI-1 expression across cancer sensitized p53 mutants and p53\textsuperscript{+/+} wild type genotypes indicate that p53
mutant cell lines express significantly higher BMI-1 mRNA levels. This higher mRNA levels correlates to the observed tumorigenic properties, supporting the significance of BMI-1 expression in cancer sensitized p53 mutant cell lines.

Tissue specific adult stem cells exhibit self-renewal, a process by which stem cells divides to make more stem cells, in order to maintain the stem cell pool throughout the lifespan. The role of proto-oncogenes and tumor suppressor genes in controlling cell cycle, balancing self-renewal and differentiation have been studied with results indicating that proto-oncogenes promote self-renewal, while gate-keeper tumor suppressors limit self-renewal and care-taker tumor suppressors maintain genomic integrity (He et al., 2009). The p53 gene exhibits both gate-keeper and care-taker functions (Jerry et al., 2008) and it is reasonable to expect that point mutations result in disruption of both these functions. The Sca-1 expressing lung stem cells in this study were sorted from mice carrying p53$^{R172H/+}$, a model for Li-Fraumeni syndrome (Olive et al., 2004), and p53$^{QSH/+}$ with compromised transactivation ability to induce apoptosis (Johnson et al., 2005; Vaughan et al., 2013). We tested the stem cell properties of Sca-1 expressing lung stem cells by assessing their multipotent differentiation ability into mesenchymal lineage. Sca-1$^{High}$ and Sca-1$^{Low}$ subpopulations from p53$^{+/+}$ wild type mice exhibited differentiation potential into adipocytes and osteocytes, while those derived from p53 mutant mice exhibited limited differentiation abilities. Our results also indicated that the adipocyte differentiation medium has detrimental effects on the subpopulations derived from the cancer sensitized p53 mutant mice, resulting in restricted growth and loss of cell viability. Our observations of loss of cells under adipogenic differentiation conditions are similar to what has been observed by experiments conducted on putative stem cells isolated from prostate cancer cells (Zhau et al., 2011). Based on
our findings, we conclude that further studies need to be conducted to investigate the uni- and multi-
potent differentiation ability of these cells into specific lung lineages and other lineages.

In summary, our study highlights the existence of higher number of Sca-1 expressing putative lung stem cells in cancer sensitized p53 mutant mice. Additionally, phenotypic and functional studies support their tumorigenic properties and limited differentiation ability, suggesting that the increase in Sca-1 population can be correlated to p53 mutations. Future in vivo studies are needed to further confirm the stem cell nature, self-renewal and differentiation, as *in vivo* studies captures the natural stem cell niche. The tumor initiating role of the Sca-1 expressing lung stem cells needs further validation *in vivo* to investigate the importance of Sca-1 expressing cells from p53 mutant backgrounds as cancer stem cells. Detailed characterization of these putative cancer stem cells is expected to lead to the development of chemoprevention strategies for treating lung cancer.
3.1 Abstract

The lung consists of different anatomically defined regions composed of large airways (bronchi), bronchioles, and alveoli that harbor morphologically and functionally distinct mesodermal and endodermal cell types. Within these complex structures, studies have shown the existence of candidate cells that exhibit stem cell characteristics of self-renewal and differentiation. Identification of Sca-1 expressing lung cells has been the most common approach in isolation of these resident lung/stem progenitor cells. To reduce cellular heterogeneity, studies have focused on combination of different cell surface markers along with Sca-1 to identify and characterize stem/progenitor cells with defined stem cell characteristics. Here we incorporated a two marker strategy to identify and characterize lung cells differentially expressing Stem cell antigen-1 (Sca-1) and Platelet derived growth factor-alpha (PDGFR-α). By employing fluorescence-activated cell sorting (FACS), we have established multiple subpopulations of cells from the murine lung and evaluated their phenotypic and functional properties. Our results suggest that except for Sca-1\textsuperscript{Low}PDGFR-α\textsuperscript{High} subpopulations, all the subpopulations possess mesenchymal differentiation.
potential into adipocytes and osteocytes, suggesting that Sca-1^{Low}PDGFR-α^{High} subpopulation lacks stem cell characteristics. We also noted that these subpopulation possess higher proliferation, and high N-cadherin expression indicating their mesenchymal nature. On the contrary, we observed robust differentiation ability in both Sca-1^{High}PDGFR-α^{High} and Sca-1^{High}PDGFR-α^{Low} subpopulations, but lower proliferation ability in the latter. Initial FACS analysis in p53 mutant lines indicate the presence of a high percentage of Sca-1^{High}PDGFR-α^{High} subpopulation, which alludes to the importance of PDGFR-α as a potential cancer stem cell marker. Overall, our results demonstrate the potential use of PDGFR-α in sorting for subpopulations with defined stem cell characteristics and to further determining its importance in the context of lung cancer.

3.2 Introduction

Understanding the role of somatic stem cells in different tissues to maintain homeostasis and wound healing is still an area of active research. Numerous studies have uncovered existence of different somatic cells, non-hematopoietic stem cells, with multipotent characteristics in various tissues and organs, including lungs (Lundin and Driscoll, 2012; Stappenbeck and Miyoshi, 2009). Identification of homogenous stem cell populations in the lung is a challenge since lung encompasses approximately 40 different types of cells with distinct functions (Filby, 2012; Kajstura et al., 2011). There has been increasing evidence showing lung stem cells with self-renewal and differentiation capability in mouse models, based on unique combinations of specific cell surface marker profiles (Raiser and Kim, 2009; Stripp BR, 2011). Studies have demonstrated that lung cells expressing Sca-1 were predominantly found in distal regions of lungs and were shown to possess a temporal emergence, indicated by enrichment of Sca-1

Back To Table of Contents
expressing cells in adult mouse lungs when compared to neonatal lungs (McQualter et al., 2009). These Sca-1\textsuperscript{pos} lung subpopulations established from adult mice lungs were shown to possess stem cell characteristics of self-renewal and differentiation. Based on the expression of Sca-1 and other markers, various studies have identified unique subpopulations in adult lung that possess stem cell characteristics (Hegab et al., 2010). Thus Sca-1 emerged as a representative cell surface marker to identify the lung stem cells. An important area of research is the identification of novel cell surface markers that would aid in distinguishing lung stem cells and progenitor cells as well as to isolate a homogenous population of these cells. Identification of endogenous lung stem cells and progenitors not only aids in a fundamental understanding of lung biology, but also can contribute to applications in regenerative medicine for treating lung diseases such as pulmonary hypertension and chronic obstructive pulmonary diseases (Lau et al., 2012; Weiss, 2014).

Isolation of a subpopulation from the bone marrow based on expression of both Sca-1 and an early mesodermal marker platelet-derived growth factor receptor α (PDGFRα) has enabled to establish homogenous population of mesenchymal stem cells with minimum contamination of hematopoietic stem cells and other cells (Morikawa et al., 2009). Lung is comprised of different stem cell subpopulations with characteristics that differ with respect to the method of isolation and choice of cell surface (Raiser and Kim, 2009). Recent evidences points to existence of epithelial lung stem cells in Sca-1\textsuperscript{neg} subpopulations and mesenchymal lung stem cells in Sca-1\textsuperscript{pos} subpopulation (Hegab et al., 2010; McQualter et al., 2009). Our rationale to characterize Platelet-derived growth factor receptor-alpha (PDGFR-α) expression in lung cells was based on studies in bone marrow (Houlihan et al., 2012) and heart (Chong et al., 2013) which supports its role as a potential mesenchymal stem cell marker. In this study, we have characterized lung stem cells...
based on expression of Sca-1 and PDGFR-α cell surface markers. We hypothesize that lung cells consist of subpopulations that differentially express PDGFR-α and Sca-1 with different stem cell characteristics of self-renewal and differentiation. Here, we report for the first time, morphological, phenotypical, and functional differences of subpopulations that point to a role for PDGFR-α in sorting for subpopulations with defined stem cell characteristics and to further determining its importance in the context of lung cancer.

3.3 Materials and methods

3.3.1 Lung isolation, enzymatic digestion, and cell sorting

For the purpose of this study, lungs (n=2) were dissected from p53+/+ Wild type C57BL/6 following the steps described (Gadepalli et al., 2013; Hegab et al., 2010). Briefly, the mice were anesthetized by intra-peritoneal (IP) route and sacrificed by cervical dislocation. Careful dissection is performed from posterior to anterior end of the abdomen to locate trachea near throat region. 1 ml of dispase (2U/ml) is injected after a slight incision made on trachea. Following the injection of dispase, the inflated lungs were carefully harvested along with trachea and other visceral organs. The lung lobes were separated from the other body parts and incubated in sterile solution containing dispase and collagenase to obtain slurry of the lung tissue (Gadepalli et al., 2013). Finally, the red blood cells (RBC) and undigested lung tissue were separated by addition of RBC lysis buffer, rinsing in sterile PBS++ (with Mg²⁺ and Ca²⁺) and filtering through 0.45 μm sterile filter. The cells are counted and plated at 1000 cells per cm² and expanded for 21 days.
Figure 3-1: Schematic representation of lung isolation, digestion, and cell sorting.

(i) Separation of lung lobes, mincing lung tissue, and enzymatic digestion to prepare single cell suspension. (ii) RBC lysis and filtration of undigested lung tissue. (iii) Rinsing in growth medium, cell counting and plating in appropriate tissue culture-treated flask. (iv) Collection of single cells in trypsin, neutralize with growth medium and PBS++ wash. (v) Fluorescent conjugated Antibody staining with appropriate controls, depletion of PE positive cells and FACS sorting of lung subpopulations differentially expressing Sca-1 PDGFR-α. (vi) Propagating cells in growth medium for downstream cell analysis.
3.3.2 Cell culture components

As the current study aims to establish and characterize primary cells with stem cell characteristics following similar marker combination reported by (Hegab et al., 2010), we opted to use the same medium composition that was shown to be conducive for propagating these cells. The components for growth medium are 10% defined FBS (HyClone Laboratories, Logan, Utah USA), DMEM-High glucose with Glutamine & sodium pyruvate 1x Penicillin/Streptomycin, 1x NEAA. Splitting of cells was carried at near confluence with 0.25% trypsin. Cryopreserved cell stocks were prepared at regular passages using freezing medium containing 10% DMSO and 30% defined FBS. All reagents were obtained from Life Technologies (Grand Island, NY, USA) unless otherwise noted.

3.3.3 FACS sorting and analysis of cell surface marker expression

The goal of the study is to establish lung stem/progenitor cell lines from mice with different p53 genetic background, based on expression of cell surface marker Sca-1 and PDGFR-α. For this purpose, the cells were passaged using 0.25% trypsin, washed with PBS++ and suspended in 100 µl volume of PBS++ with respective concentration of conjugated antibody for staining, as described in Appendix i). The cells were incubated on ice for 30 minutes and washed twice with PBS++. Sorting of the stained cells is performed on BSC Aria- BD FACSAria™ II High-Speed Cell Sorter at flow cytometry shared resource, VCU. For the current study, first lineage CD45 and CD31 positive lung cells were depleted and a 4-way gating was applied to sort Sca-1^High PDGFR-α^High, Sca-1^High PDGFR-α^Low, Sca-1^Low PDGFR-α^High, and Sca-1^Low PDGFR-α^Low.
subpopulations of lung cells. All the sorting procedures were conducted under sterile conditions and respective fluorophore isotype IgG control were employed as negative control.

For analysis of differential expression of Sca-1 and PDGFR-α expression across the different p53 background mice, single cell suspension were obtained as described earlier and unsorted cells were plated in 100 cm dish (in replicates). At near confluence, the uniform staining protocol was employed as described in Appendix i). The expression differences were evaluated on BD Accuri™ C6 flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). For this purpose, equal numbers of flow events were collected across all the samples in this study in replicates. Analysis was carried out using respective fluorophore isotype controls. Statistical differences were assessed using Students t-test and p-value <0.05 was considered significant.

3.3.4 **AlamarBlue® cell proliferation assay**

The differences in propagation for Sca-1 and PDGFR-α expressing lung subpopulations were determined by AlamarBlue® Cell viability (Life Technologies, Grand Island, NY, USA). In this study, to determine cell growth over a period of 7 days, the respective subpopulations were trypsin passaged at confluence and cell counts were determined on BD Accuri™ C6 (BD Biosciences, Franklin Lakes, NJ, USA), as describe in Appendix ii). 500 cells were plated per well in a 96-well plate in quadruplicates for respective lung cell lines. After allowing the cells to attach to the tissue culture plate for 4 hours the medium is aspirated and a fresh growth medium mixed with 10% AlamarBlue® reagent was added. Absorbance was measured at a wavelength of 570 nm and 600 nm using Synergy H1 plate reader. Continuous readings were obtained every day for a period of 7 days without
changing the growth medium. Wells with growth medium alone and growth medium plus 10% AlamarBlue® reagent were run as blanks. The experiment was repeated at two different passages and OD readings were averaged across the experiments. The percent reduction of AlamarBlue® reagent is calculated by the following equation,

\[
AR_{570} = \left( \frac{A_{570} - (A_{600} \times Ro)}{Ro} \right) \times 100
\]

Where,

\[AR_{570} = \text{Amount of reduced AlamarBlue® reagent}\]
\[A_{570} \text{ & } A_{600} = \text{sample absorbance at respective wavelength minus media blank}\]
\[Ro = \text{Correction factor. This is calculated by } AO_{570} ÷ AO_{600}, \text{ where } AO_{570} \text{ and } AO_{600} \text{ are the Absorbance of Oxidized (AO) form of AlamarBlue® reagent, at respective wavelengths. } AO = \text{OD 10\% AlamarBlue® in medium - OD growth medium, at respective wavelengths.}\]

3.3.5 **Mesenchymal lineage differentiation analysis**

The multipotent differentiation capability of the lung subpopulations established based on differential expression of Sca-1 and PDGFR-α was evaluated by their ability to differentiate into osteogenic and adipogenic lineage. For osteogenic differentiation, 10,000 cells were plated per well of a 12-well tissue culture plate in growth medium. At 70 % confluence, the growth medium is replaced with osteogenic differentiation medium containing 10nM Dexamethasone, 50 µM L-Ascorbic acid 2-phosphate and 20mM β-Glycerophosphate disodium salt hydrate. The cells were propagated for a period of 14-21 days. At the end of the differentiation period, the cells were fixed with 4% Paraformaldehyde. The deposition of calcium was detected with 40 mM Alizarin red stain. For adipogenic differentiation, 20,000 cells were plated per well of a 12-well tissue culture plate in adipogenic differentiation medium containing 1:50,000 dilution of 1mM insulin
and 1:10,000 dilution of 10 µM T3. At 100 % confluence, the differentiation medium is replaced with adipogenic induction medium containing 0.125M Indomethacin, 2mg/ml dexamethasone, 0.25M IBMX along with 1mM insulin and 10 µM T3. The cells were induced in the induction medium for 2 days and later maintained in adipogenic differentiation medium for 5 more days. At the end of differentiation, the cells were fixed in 10% formaldehyde. The formation of lipid droplets was detected with Oil Red O staining solution. Further details of the differentiation protocol and staining procedure are described in Appendix iii. All reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise noted.

3.3.6 RNA Isolation, real time reverse transcription polymerase chain reaction, and gene expression analysis.

To quantitate expression of genes of interest listed in Appendix iv, cell pellets were collected and RNA purification was performed using AllPrep DNA/RNA Mini Kit following manufacturer instructions (Qiagen, Valencia, CA, USA). The RNA concentration of the samples was determined by Nanodrop 2000c spectrophotometer (Thermo scientific, Wilmington, DE, USA) and 1µg of RNA across all the sorted cell lines was converted into cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY, USA). Q-PCR reaction was setup using iTaq Universal SYBR Green supermix (BioRad, Hercules, CA, USA) with appropriate concentration of primer and cDNA. The primer efficiencies of the internal control (GAPDH) and genes of interest were determined by a standard curve. Gene expression data (in triplicates) were acquired using CFX 96 real time system (C1000 touch). Details of the q-PCR setup and protocol are described in
Appendix v). GAPDH was run as a housekeeping reference gene and analysis was performed by normalizing the expression quantity of gene of interest to the expression quantity of internal control gene using the relative standard curve method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Statistical significance was determined by Student t-test, with p-value <0.05 being considered as significantly different.

3.4 Results

3.4.1 Lung comprises of subpopulation of cells that differentially express Sca-1 and PDGFR-α

In a single marker strategy to identify stem cells in the lung, Sca-1pos and linneg (CD31, CD45) multipotent lung stem cells constitute subpopulations of cells that are heterogeneous (Hegab et al., 2010). Various studies support the existence of more than one population of cells in tissues with high cell turnover or regeneration (Barker et al., 2010). More importantly, as widely elucidated in the hematopoietic system, distinguishing somatic stem and progenitor cells is important to understand their role in tissue homeostasis and repair. Here, we evaluated the characteristics of lung cells that differentially express Sca-1 and PDGFR-α post depletion of CD45 and CD31 expressing cells. Although a previous study has reported expression of PDGFR-α in lung cells (McQualter et al., 2009), isolation and characterization of lung cells differentially Sca-1 and PDGFR-α has not been studied.

Our results indicate that lung cells comprise of subpopulations of cells that differentially express Sca-1 & PDGFR-α. We noted that after depleting the CD45posCD31pos cells, the percentage of cells expressing Sca-1High PDGFR-αHigh constitute around 23.2% of the total population, Sca-
High PDGFR-αLow constitute 5%, while Sca-1Low PDGFR-αHigh and Sca-1Low PDGFR-αLow constitute 28.5% and 26.3% respectively (Figure 3-2). In sorting based on single cell surface marker Sca-1, Sca-1High expressing lung subpopulations consists of small spindle shaped cells mixed with large elongated cells. While sorting based on both Sca-1& PDGFR-α, lung subpopulations are more homogenous. Subpopulations expressing Sca-1High and either high or low expression of PDGFR-α constitute small spindle shaped cells. On the other hand, subpopulations expressing in Sca-1Low and either high or low expression of PDGFR-α constitute large elongated cells (Figure 3-3), additional qPCR analysis of the sorted subpopulations indicated representative expression of Sca-1 and PDGFR-α mRNA transcript levels (Figure 3-4).

We further evaluated the percentage of subpopulations expressing Sca-1 and PDGFR-α in unsorted lung cells from p53 knock-in mice and compared them to those isolated from p53+/+ wild type mice. Our results from flow cytometry analysis indicate that lung cells from p53 knock-in mutants, p53R172H/+ and p53QSH/+ , possess significant higher percentage (~3 fold increase) of subpopulations expressing Sca-1High PDGFR-αHigh compared to those from p53+/+ wild type mice (Figure 3-5). Interestingly, Sca-1High PDGFR-αLow, Sca-1Low PDGFR-αLow subpopulations are significantly lower in lung cells isolated from p53 mutant knock-in mice. These results demonstrate the potential for use of PDGFR-α as an important cell surface marker to identify novel subpopulations in the lung.
Figure 3-2: FACS sorting of lung cells differentially expressing Sca-1 & PDGFR-α

FACS sorting is performed with appropriate gating to deplete lineage positive cells (CD45 and CD31) from single cell suspension prepared from the whole lung harvested from normal mice. The remaining lung cell subpopulations were sorted for cells expressing both Sca-1, PDGFR-α cell surface marker and plated in tissue culture dish for propagation.
Figure 3-3: Subpopulations established based on both Sca-1& PDGFR-α expression consists of homogenous cells.

Sorted lung subpopulations were plated in tissue culture dish and images were captured (scale bar 100 µm). (A) In sorting based on single cell surface marker Sca-1, Sca-1\textsuperscript{High} expressing lung subpopulations consists of small spindle shaped cells mixed with large elongated cells. (B) In sorting based on both Sca-1 & PDGFR-α, Sca-1\textsuperscript{High} PDGFR-α\textsuperscript{High} expressing (left) lung subpopulations are more homogenous with spindle shaped cells while and Sca-1\textsuperscript{Low} PDGFR-α expressing are large elongated cells.
Figure 3-4: Lung subpopulations established based on Sca-1 & PDGFR-α expression show representative Sca-1 and PDGFR-α mRNA transcript levels

Q-PCR analysis of relative transcript levels of Sca-1 and PDGFR-α across the lung subpopulations established based on Sca-1, PDGFR-α cell surface marker expression. Results are presented together with standard deviation from experiment conducted in triplicates.
Figure 3-5: Sca-1<sup>High</sup> PDGFR-α<sup>High</sup> expressing lung subpopulations are significantly increased in mice carrying p53 point mutations.

Unsorted lung cells established across different p53 background were propagated in culture and cells differentially expressing both Sca-1, PDGFR-α (CD140-a) were analyzed by flow cytometry. (A) Scatter plots of unsorted cells stained with Sca-1-FITC (x-axis) and PDGFR-α (y-axis) (B) Bar plots show significant differences in percentage of cells expressing Sca-1, PDGFR-α between the p53 backgrounds. (* = p < 0.05). Results are presented together with standard deviation from 3 independent cultures.
3.4.2 Sca-1\textsuperscript{Low} PDGFR-\(\alpha\)\textsuperscript{High} expressing lung subpopulation show higher proliferation

Under the \textit{in vitro} culture conditions adopted in this study, we noted that these subpopulations exhibit proliferation differences, with the Sca-1\textsuperscript{Low}PDGFR-\(\alpha\)\textsuperscript{High} subpopulation exhibiting higher proliferation capability (Figure 3-8). Given that PDGFR-\(\alpha\) is a mesenchymal lineage marker, it is expected that the subpopulations possess higher proliferation ability. Similarly, Sca-1\textsuperscript{High}PDGFR-\(\alpha\)\textsuperscript{High} exhibit similar proliferation capabilities. We subsequently evaluated the expression levels of N-cadherin mRNA levels, a mesenchymal marker, in the different subpopulations. Our results indicate that the Sca-1\textsuperscript{Low}PDGFR-\(\alpha\)\textsuperscript{High} subpopulation exhibit significantly higher levels of N-cadherin when compared to all other sorted subpopulations (Figure 3-7). Additionally, the Sca-1\textsuperscript{Low}PDGFR-\(\alpha\)\textsuperscript{High} subpopulation expressed higher levels of BMI-1 transcript levels, indicating the higher self-renewal potential of these cells (Figure 3-6).
Figure 3-6: Sca-1<sup>Low</sup> PDGFR-α<sup>High</sup> expressing lung subpopulation show higher BMI-1 transcript levels

Q-PCR analysis of relative transcript levels of BMI-1 across the lung subpopulations sorted based on Sca-1 and PDGFR-α cell surface marker expression. Sca-1<sup>Low</sup>PDGFR-α<sup>High</sup> subpopulations exhibit higher BMI-1 transcript levels, indicative of higher proliferation and self-renewal potential. Results are presented together with standard deviation from experiment conducted in triplicates.
Figure 3-7: Sca-1^{Low}PDGFR-α^{High} expressing lung subpopulation exhibit higher N-cadherin mRNA expression

Q-PCR analysis of relative transcript levels of N-cadherin across the lung subpopulations established based on Sca-1 and PDGFR-α cell surface marker expression. Sca-1^{Low}PDGFR-α^{High} subpopulation exhibits higher N-cadherin transcript levels indicating its mesenchymal nature. Results are presented together with standard deviation from experiment conducted in triplicates.
Figure 3-8 PDGFR-α expressing lung subpopulation exhibit higher proliferation potential

The sorted cells are plated at 500 cells per well in 96 well plate, and the percentage reduction of Alamar blue was measured by absorbance at 570 nm. Sca-1$^{\text{Low}}$PDGFR-α$^{\text{High}}$ subpopulation exhibit higher proliferation rate when compared with the other sorted subpopulations. Results are presented together with standard deviation from two independent experiments conducted in quadruplicates.
3.4.3 Sca-1\textsuperscript{Low}PDGFR-α\textsuperscript{High} expressing lung subpopulation exhibit no differentiation ability

Stem cells are characterized by their dual ability of self-renewal and differentiation into specific lineages. Demonstrating the differentiation capability after repeated passages in culture is critical in order to fully validate the stem cell characteristics of any derived cell line. In our study, after repeated passages in culture, we evaluated the differentiation potential of early and late passage cells to form adipocytes and osteocytes. Our results suggest that except for Sca-1\textsuperscript{Low}PDGFR-α\textsuperscript{High} subpopulation, all the sorted subpopulations possess adipogenic differentiation capabilities at early and late passages, demonstrated by positive Oil Red O staining (Figure 3-9 & Figure 3-10).

In establishing lung stem/progenitor cells by use of Sca-1 alone, we noted that Sca-1\textsuperscript{High} cells readily differentiate to adipocytes while Sca-1\textsuperscript{Low} cells do not. In the 2-way sorting strategy, with Sca-1 & PDGFR-α, we did not observe any differentiation potential in Sca-1\textsuperscript{Low}PDGFR-α\textsuperscript{High} cells, while Sca-1\textsuperscript{High}PDGFR-α\textsuperscript{Low} and Sca-1\textsuperscript{Low}PDGFR-α\textsuperscript{Low} cells exhibited robust differentiation.

Based on calcium deposition by Alizarin Red S staining, we observed a passage number effect with early passage cells exhibiting osteogenic differentiation potential, while late passage cells do not (Figure 3-11 and Figure 3-12). It is useful to note that the sorted subpopulations exhibit similar osteogenic and adipogenic differentiation potentials. In single marker isolation using Sca-1, Sca-1\textsuperscript{High} cells readily differentiated when compared to Sca-1\textsuperscript{Low} cells. In the 2-way sorting strategy, with Sca-1 & PDGFR-α, we did not observe any osteogenic differentiation potential in Sca-1\textsuperscript{Low}PDGFR-α\textsuperscript{High} lung subpopulation, while Sca-1\textsuperscript{High}PDGFR-α\textsuperscript{Low} and Sca-1\textsuperscript{Low}PDGFR-α\textsuperscript{Low} cells exhibited robust differentiation.
Figure 3-9: Sca-1\textsuperscript{High} expressing lung subpopulation show higher adipogenic differentiation

The sorted cell lines established based on differential Sca-1 expression from normal mice were propagated in adipogenic differentiation medium for 10 days. Adipogenesis was indicated by formation of lipid vacuoles that stain with oil red o stain at the end of differentiation period. The Sca-1\textsuperscript{High} wild type lines show distinct oil red droplet formation compared to the Sca-1\textsuperscript{Low} lung subpopulation.
The sorted cell lines established based on differential expression of both Sca-1 and PDGFR-\(\alpha\) were propagated in adipogenic differentiation medium for 10 days. Adipogenesis was indicated by formation of lipid vacuoles that stain with Oil red O stain at the end of differentiation period. The Sca-1\(^{\text{Low}}\)PDGFR-\(\alpha^{\text{High}}\) subpopulation exhibit robust adipogenic differentiation capacity, while the Sca-1\(^{\text{Low}}\)PDGFR-\(\alpha^{\text{High}}\) lung subpopulation lack adipogenic differentiation potential.

**Figure 3-10:** Sca-1\(^{\text{Low}}\)PDGFR-\(\alpha^{\text{High}}\) expressing lung subpopulation lack adipogenic differentiation potential
Figure 3-11: Sca-1\textsuperscript{High} expressing lung subpopulations demonstrate higher osteogenic differentiation potential

The sorted cell lines established based on differential Sca-1 expression from normal mice were propagated in osteogenic differentiation medium for 21 days. Osteogenesis was indicated by deposition of calcium that stain with Alizarin red S at the end of the differentiation period. The Sca-1\textsuperscript{High} wild type lines show distinct Alizarin red S staining compared to Sca-1\textsuperscript{Low} lung subpopulation.
Figure 3-12: Sca-1\textsuperscript{Low} PDGFR-\(\alpha\textsuperscript{High}\) expressing lung subpopulation lack osteogenic differentiation potential

The sorted cell lines established based on differential expression of both Sca-1 and PDGFR-\(\alpha\) were propagated in osteogenic differentiation medium for 21 days. Osteogenesis was indicated by deposition of calcium that stain with Alizarin red S at the end of differentiation period. The Sca-1\textsuperscript{High} PDGFR-\(\alpha\textsuperscript{Low}\) and Sca-1\textsuperscript{High} PDGFR-\(\alpha\textsuperscript{High}\) subpopulations exhibit robust osteogenic
differentiation potential, while the Sca-1^{Low}PDGFR-α^{High} lung subpopulation lacks osteogenic differentiation potential.
3.5 Discussion

In this study, we adopted a two-marker strategy for isolation and characterization of multipotent stem cells from the mouse lung. Our results support the existence of a heterogeneous lung cell population, validating the recent evidence of multiple stem cell niches in the lung (Otto, 2002; Raiser and Kim, 2009). Complex tissues like the lung, although primarily epithelial in nature are believed to encompass both epithelial and mesenchymal stem cells (Slack, 2000; Stripp BR, 2011). Currently, functional assays to distinguish subpopulations with stem cell characteristics in lungs is solely based on in vitro assays, that capture self-renewal and multipotent differentiation potential. For instance, Bronchioalveolar Stem Cells (BASCs) identified by expression of Sca-1\textsuperscript{pos}CD34\textsuperscript{pos}, were characterized as epithelial lung stem cells (Kim et al., 2005), but another systematic analysis of the same subpopulation classify them as fibroblastic given that these cells also co-express Thy-1 and platelet-derived growth factor receptor α (PDGFR-α) (McQualter et al., 2009). The discrepancy in the results between independent research groups was ascribed to isolation methods, gating strategies, and culture conditions (Hegab et al., 2010; McQualter et al., 2009; Raiser and Kim, 2009; Teisanu et al., 2009). Existence of heterogeneous stem cell populations is not limited to the lung tissue alone with studies indicating co-existence of hematopoietic and mesenchymal stem cells in the bone marrow (Morikawa et al., 2009; Wilson and Trumpp, 2006). Identification of pure population of mesenchymal stem cells in bone marrow was achieved by employing Sca-1 and PDGFR-α cell surface markers, where Sca-1\textsuperscript{pos}PDGFR-α\textsuperscript{pos} subpopulation was characterized as a homogenous population of mesenchymal stem cells (Houlihan et al., 2012; Morikawa et al., 2009). Our analysis of differential expression of Sca-1 and PDGFR-α shows the potential role of PDGFR-α as a stem cell marker in the lung tissue. Interestingly, our results indicate that the Sca-1\textsuperscript{Low}PDGFR-α\textsuperscript{High} lung cell subpopulation lack...
Vast majority of human cancers carry mutations in p53, most commonly point mutations in one (p53^{mt/+}) or both (p53^{mt/mt}) p53 alleles (Lane, 1992; Lundin and Driscoll, 2012). These mutations result in expression of mutant p53 proteins that contribute to tumorigenesis either by inhibition of the wild type p53 activity or gain of function activities (Muller et al., 2011; Olive et al., 2004; Vaughan et al., 2012a). Mutation in p53 results in selective effects on the function of p53 protein thereby changing the phenotype of normal cells into cancerous nature. Advances in study of cancer stem cells (CSCs) across different tissues has allowed to identify putative cell surface markers that these cells express that contributes to distinguishing cancer stem cells from normal
cells (Klonisch et al., 2008). In our study, we investigated the expression of Sca-1 and PDGFR-α in lung population established from p53 mutants and p53\(^{+/+}\) wild type knock-in mice. The mice carrying point mutants p53\(^{R172H/+}\) and p53\(^{Q56H/+}\), exhibited a ~3 fold increase in subpopulation of cells expressing Sca-1\(^{High}\)PDGFR-α\(^{High}\) when compared with those isolated and propagated from p53\(^{+/+}\) wild type mice. Interestingly, Sca-1\(^{High}\)PDGFR-α\(^{Low}\) subpopulation is higher in unsorted cells established from p53\(^{+/+}\) wild type mice and significantly lower in cells isolated from p53 mutant mice. This shift in the subpopulations expressing putative stem cells markers, Sca-1 and PDGFR-α, indicates the potential effect of the p53 point mutations on the phenotype. Further characterization of Sca-1 and PDGFR-α expressing lung subpopulation in these cancer sensitized p53 mutants is thus required to establish PDGFR-α as a possible cancer stem cell marker.

In summary, our results presented here suggest the importance of PDGFR-α as an important marker to sort out putative stem cells and progenitor cells from a heterogeneous lung tissue that comprises of multiple stem cell niches. Moreover, our results demonstrate an increase in Sca-1\(^{High}\)PDGFR-α\(^{High}\) subpopulation in lungs isolated from cancer sensitized p53 mutants, indicating the potential use of PDGFR- α in sorting for subpopulations with defined stem cell characteristics and to further determining its importance in the context of lung cancer.
4.1 Conclusions

4.1.1 Chapter 2

Identifying somatic stem cells in various tissues has significantly progressed in the past decade, with use of specific cell surface markers to isolate resident stem cells that contribute to regeneration. Similar advancements in understanding lung biology have paved the way to identify and characterize lung stem/progenitor cells with multipotent differentiation potential. Unlike rapidly self-renewing tissues/organs like intestine, skin, and bone marrow, lung tissue does not exhibit high regenerative ability. However, identification and isolation of prospective lung stem/progenitor cells is of enormous interest given the increasing evidence of cancer stem cells in numerous tissue types. Given that lung cancer is the leading cause of cancer related mortality, identification and characterization of somatic lung stem cells not only aides in understanding the biology of the lung, but also in treating lung related disorders.

In this study, we aimed to investigate the characteristics of Sca-1 expressing subpopulations isolated from cancer sensitized p53 mutant (p53$^{R172H+}$ & p53$^{QSH+}$) mice and compared with their
counterparts isolated from p53\(^{+/+}\) wild type mice. Based on different experimental outcomes, we arrived at the following conclusions:

- Direct comparisons of phase contrast images demonstrate that the Sca-1\(^{\text{High}}\) and Sca-1\(^{\text{Low}}\) subpopulations from p53\(^{+/+}\) wild type comprise of a heterogeneous population. However, the same subpopulations isolated from cancer sensitized p53\(^{R172H/+}\) & p53\(^{QSH/+}\) mutant mice possess homogenous cells with prominent mesenchymal characteristics.

- Cancer sensitized p53\(^{R172H/+}\) & p53\(^{QSH/+}\) mutant mice possess a significantly higher percentage of Sca-1\(^{\text{High}}\) lung subpopulations, when compared with cell isolated from p53\(^{+/+}\) wild type mice. Comparisons within the two mutants indicate that the cells isolated from the p53\(^{R172H/+}\) knock-in mice possess a higher percentage of Sca-1\(^{\text{High}}\) cells.

- Cell lines isolated from p53\(^{R172H/+}\) & p53\(^{QSH/+}\) knock-in mice possess a higher colony forming ability at low seeding density and higher BMI-1 expression indicating their self-renewal potential.

- Cell lines generated from cancer sensitized p53 backgrounds exhibited a higher proliferation and self-renewal potential than those isolated from p53\(^{+/+}\) wild type mice.

- Cell lines isolated from p53\(^{R172H/+}\) & p53\(^{QSH/+}\) knock-in mice demonstrated tumorigenic characteristics when compared to cell lines isolated from p53\(^{+/+}\) wild type mice as determined by:
  
  - Sca-1\(^{\text{High}}\) and Sca-1\(^{\text{Low}}\) subpopulations from p53\(^{R172H/+}\) background show a higher migration rate.
  
  - Sca-1\(^{\text{High}}\) subpopulations from both p53\(^{R172H/+}\) & p53\(^{QSH/+}\) mutant backgrounds exhibited a significant colony formation in anchorage-independent growth conditions.
Sca-1\textsuperscript{Low} subpopulations from p53\textsuperscript{R172H/+} mice exhibited a significant colony formation in anchorage-independent growth conditions. However, Sca-1\textsuperscript{Low} subpopulations from p53\textsuperscript{QSH/+} mice did not exhibit significant colony formation in anchorage-independent growth conditions.

Sca-1\textsuperscript{High} and Sca-1\textsuperscript{Low} subpopulations from p53\textsuperscript{QSH/+} mice express an accumulation of p53 protein, indicating that aberrant p53 protein is expressed in these cells.

Subpopulations from p53\textsuperscript{R172H/+} knock-in mice showed significant tumorigenic properties but did not show any p53 accumulation.

- Both Sca-1\textsuperscript{High} and Sca-1\textsuperscript{Low} subpopulations from the p53\textsuperscript{+/+} wild type mice exhibit differentiation potential when differentiated into osteogenic and adipogenic lineage, while Sca-1\textsuperscript{High} subpopulations showed more homogenous differentiation ability compared to the Sca-1\textsuperscript{Low} subpopulation.

- Subpopulations isolated from cancer sensitized p53 mutants did not show any adipogenic differentiation. However, Sca-1\textsuperscript{Low} subpopulation from mutant mice showed scarce osteogenic differentiation.

- To further understand the potential role of p53 in regulating Sca-1 and PDGFR-\(\alpha\) we performed Ingenuity pathway analysis, based on peer reviewed published interaction data. We observed that, p53 regulates the expression of Sca-1 and PDGFR-\(\alpha\), which probably, explains the increase in Sca-1 and PDGFR-\(\alpha\) expression in lung cell lines that carry p53 point mutations. Our analysis also shows a direct interaction between p53 and BMI-1 inside the nucleus (Figure 4-1). Based on the Ingenuity pathway interaction data,
it is reasonable to expect that point mutations in p53 resulted in increased expression of
Sca-1, PDGFR-α and BMI-1 genes.

• Our current data supports low differentiation potential of lung sub-populations carrying
p53 point mutations. Hence, we investigated if p53 regulates any genes that are involved
in mesenchymal lineage differentiation. The process of adipocyte differentiation is tightly
regulated by a number of transcription factors, hormones and signaling pathway
molecules(Rosen and MacDougald, 2006). Adipogenesis from pre-adipocytes into mature
adipocyte is precisely coordinated by transcription factors such as CCAAT-enhancer-
binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPARγ),
cytokines, and hormones(Wu et al., 1999). Based on ingenuity analysis, we noted that
p53 inhibits the expression of PPARγ, an important transcription factor in adipogenesis.
We hypothesize that as a result of p53 point mutations, cellular accumulation of p53
could occur and inhibit PPARγ, thereby resulting in loss of cells during the differentiation
process and lack of differentiation further (Figure 4-1).
Using ingenuity pathway analysis, the role of p53 in regulating gene expression of Sca-1, PDGFR-α, BMI-1, and PPARG were analyzed. Known relationships based on ingenuity database are designated on line connectors. (A) Activation, (E) Expression, (I) Inhibition, (P) Phosphorylation, (PP) Protein-Protein interaction, (UB) Ubiquitination, (L) Molecular cleavage.

Figure 4-1: Possible role of p53 in regulating expression of genes investigated in this study
4.1.2 Chapter 3

Our current understanding of resident lung stem cells is not comprehensive. The distinction between adult stem cells and progenitor cells is still an area of active research. The lung is a complex organ comprised of different types of cells with distinct functionalities and it is thus important to tease apart these differences both *in vitro* and *in vivo*. Various research studies have indicated the existence of mesenchymal and epithelial stem cells in the lung, but identifying cell surface markers that homogenously identify these populations is still a research question. Here, for the first time we have isolated and characterized lung cells differentially expressing two cell surface markers Sca-1 and PDGFR-α in p53<sup>+/+</sup> wild type mice with the goal to identify and characterize a pure population of mesenchymal lung stem cells. We compared the characteristics of these subpopulations with those lung cells that were isolated based on Sca-1<sup>High</sup> and Sca-1<sup>Low</sup> only expression. Our results obtained from different experiments conducted in this study support the following conclusions:

- Existence of different subpopulations in the lung that express Sca-1 and PDGFR-α.
- Lung cells expressing Sca-1<sup>Low</sup>PDGFR-α<sup>High</sup> and Sca-1<sup>High</sup>PDGFR-α<sup>High</sup> subpopulations constitute cells with higher proliferation capabilities.
- Sca-1<sup>Low</sup>PDGFR-α<sup>High</sup> subpopulation exhibit a higher BMI-1 expression and a higher N-cadherin expression, indicating their proliferating and mesenchymal nature.
- Sca-1<sup>Low</sup>PDGFR-α<sup>High</sup> subpopulation lack adipogenic and osteogenic differentiation potential.
- Even though all other subpopulations show adipogenic and osteogenic differentiation potential, Sca-1<sup>High</sup>PDGFR-α<sup>Low</sup> and Sca-1<sup>High</sup>PDGFR-α<sup>High</sup> subpopulations exhibit more robust differentiation capabilities.
• Increase in Sca-1$^{\text{High}}$PDGFR-α$^{\text{High}}$ subpopulation in lungs isolated from cancer sensitized p53 mutants.

• Based on our preliminary results, PDGFR-α is a potential marker in sorting for subpopulations with defined stem cell characteristics.

4.2 Future directions

4.2.1 Chapter 2

Our results suggest that subpopulations isolated and propagated from cancer-sensitized p53 mutants show distinct tumorigenic properties such as a high migration rate, high proliferation, and colony formation in non-adherent culture conditions. Our preliminary results suggests that these cells show limited mesenchymal differentiation potential compared to those cells derived from wild type mice. We propose to further test the differentiation potential of these cells into different lung lineages and hypothesize that Sca-1$^{\text{High}}$ cells from p53 mutant mice possess unipotent rather than multipotent differentiation potential. Based on the outcome of this studies, we will also test the ability of Sca-1$^{\text{High}}$ cancer-sensitized cells to induce tumor formation in immune compromised mice. This could be achieved by the following specific aims:

**Specific aim 1:** Characterize Sca-1 expressing lung cells from p53 mutant mice for their differentiation ability to other lung lineages.

The goal of this specific aim is to characterize and compare the lineage specific differentiation capability of Sca-1 expressing lung cells. We will first investigate the expression of lung lineage specific genes in the sorted lungs by Q-PCR and immuno-staining. Based on these results, we will further subject the subpopulations to differentiation medium for lung endothelial and epithelial cell types, specifically into alveolar type I, II, and clara cells. We will compare and
quantitate the differentiation capability and assess the self-renewal ability by assaying lineage-specific marker expression with Q-PCR and Western blot analysis. We expect that results from this specific aim will assist in further evaluating the stem cell characteristics of specific subpopulations from cancer sensitized mice.

**Specific aim 2:** Determining the tumor-propagating ability of Sca-1 sorted cells across cancer-sensitized p53 mutant genotypes.

The goal of this specific aim is to establish the cancerous nature of putative lung stem cells *in vivo*. For this purpose, we will first investigate the expression of known cancer stem cell markers in these subpopulations by flow cytometry and conduct gene expression studies by Q-PCR. We will further transplant these Sca-1 expressing cells into NOD/SCID immune-deficient mice to investigate their proliferation and self-renewal nature *in vivo* by lineage labelling strategy. We expect that Sca-1 cells isolated from the p53 mutant mice will form a tumor and metastasize.

### 4.2.2 Chapter 3

The two-marker strategy to identify lung stem cells in this study supports the existence of Sca-1^{Low}PDGFR-\(\alpha^{High}\) subpopulation with no differentiation capability. Our preliminary results points to the potential role of PDGFR-\(\alpha\) as a stem cell marker in the lung. We propose to test the significant role of PDGFR-\(\alpha\) expressing cells by characterizing the sorted cells for their ability to differentiate into various lung lineages. We will further characterize the ability of these subpopulations to maintain long-term self-renewal and assess their role in lung repair. We speculate that results from the following specific aims will assist in enhancing our knowledge of lung biology, by delineating long-term self-renewing lung stem cells from the short term self-renewing progenitor cells.
Specific aim 1: To evaluate long-term self-renewal potential of putative lung stem cells identified by PDGFR-α and Sca-1 expression

It is important to understand the long-term self-renewal nature of lung cells differentially expressing Sca-1 and PDGFR-α. For this purpose, we will test the differentiation potential of these subpopulations over a time period of early and late passages. To ascertain both self-renewal and differentiation potential of these cells, we will select colonies obtained from a single cell and induce differentiation. We will test the long-term differentiation potential of these cells into clara cells (epithelial), adipocytes (mesenchymal), and bronchiolar (endothelial) lineages across early and late passages. We will further quantitate the expression of respective lineage markers at the end of the differentiation period. The results from this specific aim will help to distinguish differences in long-term self-renewal potential of these subpopulations, which will shed some light on uni- and multi-potent differentiation capabilities of these subpopulations.

Specific aim 2: To evaluate the regeneration ability of the Sca-1 and PDGFR-α cells in mice models of lung injury

In order to identify the role of Sca-1 and PDGFR-α expressing lung subpopulation in turnover and repair after injury, we propose to study mouse models of lung injury (naphthalene) that specifically depletes clara cell population. This will be achieved by a lineage labelling strategy that can track the subpopulations in vivo. The results from these in vitro and in vivo studies that investigate the regeneration potential of sorted subpopulations will aide us in better understanding the putative lung stem cell populations expressing Sca-1 and PDGFR-α.


APPENDIX

Appendix i: Conjugated antibodies list

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Sca-1-FITC</th>
<th>FITC Isotype Ctrl</th>
<th>PDGFR-α-APC</th>
<th>APC Isotype Ctrl</th>
<th>CD 31-PE</th>
<th>CD-45RA-PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Company</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BD Pharmigen</td>
<td></td>
</tr>
<tr>
<td>Catalog #</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Stock Conc. (µg)</td>
<td>50</td>
</tr>
<tr>
<td>Stock Conc. (mg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total volume (µl)</td>
<td>100</td>
</tr>
<tr>
<td>Stock Conc. (µg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Stock Conc. (mg/ml)</td>
<td>0.5</td>
</tr>
<tr>
<td>Working Conc.</td>
<td>1 µg per Million cells in 100 µl volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume required from stock</td>
<td>2 µl</td>
<td>2 µl</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

Appendix ii: Cell Counting with BD Accuri™ C6

1. Trypsin passage cells at confluence
2. Centrifuge and discard the trypsin medium. Re-suspend the pellet in 1 ml of fresh growth medium.
3. Aliquot 100 µl of the cell suspension into 0.5 ml centrifuge tubes and run through BD Accuri™ C6 flow cytometer.

4. Flow run settings: Run 50 µl of respective sample, at a Medium fluidics speed. Plot FSC-Area vs SSC-Area (as in example image) to gate the healthy cell population. Note the number of events in the healthy gate.

5. Divide the number events by volume run on flow (Ex: 10000 events/50 µl i.e. 200 events/ cells per µl) to obtain events or cell number per µl. Extrapolate to the total volume the cells are suspended in to get the total cell number. (900 µl * 200 = 180,000 cells)

6. Prepare a working cell suspension of 50,000 cells per 1 ml. (Ex: 250 µl of cell suspension in 750 µl of fresh growth medium.

7. Add required number of cells for respective experiment.

8. NOTE: For larger pellets increase the re-suspension volume to avoid clumping.

9. Triturate the cell suspension repeatedly before running through the flow cytometer. Avoid bubbles.

**Appendix iii: Differentiation and staining protocol**

**Adipogenic differentiation medium:** DMEM High Glucose 1x (Gibco Cat# 11965-092), 10% FBS (Hyclone Cat# SH30070.03), 1x Penicillin/Streptomycin (Life Technologies Cat# 15140-
122), 1:50,000 dilution of 1mM insulin (Sigma Cat#I2643) and 1:10,000 dilution of 10 µM T3 (Sigma Cat# T-2877).

**Adipogenic induction medium:** To the complete adipogenic differentiation medium, add 0.125M Indomethacin (Sigma Cat# I-7378), 2mg/ml dexamethasone (Sigma cat# D2915), 0.25M IBMX (Sigma Cat# I-5879) along with 1mM insulin (Sigma Cat#I2643) and 10 µM T3 (Sigma Cat# T-2877). (Prepare it fresh for every use). **Note:** For proper dissolution indomethacin and IBMX should be heated to 75 C before adding to medium.

**Osteogenic differentiation medium:** MEM 1x (Gibco Cat# 11090-081), 10% FBS (Hyclone Cat# SH30070.03), 1x Penicillin/Streptomycin (Life Technologies Cat# 15140-122), L-Glutamine (Gibco Cat# 25030081) with 10nM Dexamethasone (Sigma Cat# D2915), 50 µM L-Ascorbic acid 2-phosphate (Sigma Cat# A8960) and 20mM β-Glycerophosphate disodium salt hydrate (Sigma Cat# G9422).

**Oil Red O Stain:** To prepare stock solution, dissolve 0.5 g of Oil Red O powder (Santa Cruz Biotech, Cat# SC-203749) in 100 ml of isopropanol. The solution can be stored up to 3 months. For working solution, dilute 3 ml of stock solution in 2 ml of de-ionized water and filter it through Whatman Grade 1 filter paper. Prepare working solution fresh as per need.

**Alizarin Red S Stain:** Dissolve 1.369 g of Alizarin Red S (Santa Cruz Biotech, Cat# 205998) in 100 ml of distilled water. After complete dissolution the pH should be around 4.2. If not, adjust the pH using Ammonium hydroxide. The solution could be stored up to 3 months.

**Adipogenic differentiation and staining**

**Day 1:** Plate cells on a tissue culture plate at 5000 cells per cm² in adipogenic differentiation medium.

**Day 2:** Allow cells to grow 100 % confluence.
Day 3: Allow cells to grow 100 % confluence.

Day 4: If the cells are 100 % confluent, then aspirate the medium and replace fresh adipogenic induction medium.

Day 5: Do not disturb the cells.

Day 6: Gently replace the induction medium with adipogenic differentiation medium.

Day 7: Do not disturb the cells.

Day 8: Gently replace medium with fresh Adipogenic differentiation medium.

Day 9: Do not disturb the cells.

Day 10: Visible oil droplets should be noted at this point. Capture bright field images of the oil droplets.

NOTE: As the adipogenic differentiation progress the cells tend to detach from the surface. Hence, be gentle in movement, medium changes and washes.

Oil Red O Staining: Replace growth medium and wash the cells with PBS++

1. Fix the cells with 10% (v/v) formaldehyde (Sigma Cat# F8775) prepared in PBS++ and incubate at room temperature for 20 minutes.

2. Replace the fixative agent with freshly prepared Oil Red O working solution. Make sure the working solution is filtered properly. Incubate at room temperature for 1 hr.

3. Rinse the plates 2 to 3 times with di H2O to remove excess stain or precipitate.

4. Dry the plates. Visualize and capture images of the stained monolayers.

Osteogenic differentiation and staining

1. Plate cells on a tissue culture plate at 2500 cells per cm² in MLSC growth medium.

2. Allow cells to grow 70 % confluence.

3. Replace the medium with osteogenic differentiation medium.
4. Allow the cells to propagate for 14-21 days with medium replacement on every alternate day.

5. Monitor the cells for calcification mineral deposition. Capture images using an inverted microscope.

**Membrane Alkaline Phosphatase (AP) staining:** After 7 days in osteogenic differentiation medium the cells can be tested for expression of membrane alkaline phosphatase. For this purpose, wash the cells with PBS++, fix them in 4% (w/v) PFA for 10 minutes and appropriate volume of BCIP/NPT staining solution (Sigma B3804) is added. The plates are covered and incubated for 20-30 minutes at room temperature. Visible blue (low AP expression) to dark purple (high AP expression) color staining is observed at the end of incubation.

**Alizarin Red S (ARS) Stain:** After the 14 days of differentiation period

1. Wash the cells in di H2O.

2. Fix the cells in 10% (v/v) formaldehyde (Sigma Cat# F8775) prepared in di H2O.

3. Wash twice in di H2O, add appropriate volume of ARS stain and incubate the plates at room temperature for 1 hour.

4. Wash the plates 2-3 times with di H2O until the stain precipitate is aspirated out.

5. Visualize and capture images of the stained monolayers.
### Appendix iv: Primer list

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene Name</th>
<th>Direction</th>
<th>Sequence</th>
<th>GC %</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>12250</td>
<td>Mouse-E-cadherin</td>
<td>Forward</td>
<td>caggtctcctcatgctttgc</td>
<td>57.1</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>cttccgaaaagaaggctgtcc</td>
<td>52.4</td>
<td></td>
</tr>
<tr>
<td>12258</td>
<td>Mouse N-cadherin</td>
<td>Forward</td>
<td>atagcccggtttcaacttgaga</td>
<td>47.6</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>ccaggtttgtacccctctgga</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>12151</td>
<td>Mouse BMI-1</td>
<td>Forward</td>
<td>cttgcggtgtctcaagtaacg</td>
<td>52.4</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>aaatccccacttaatgtgtgcc</td>
<td>43.5</td>
<td></td>
</tr>
<tr>
<td>14433</td>
<td>Mouse GAPDH</td>
<td>Forward</td>
<td>agtgctggtgaacggatttg</td>
<td>52.4</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>ggggtcgtgtgatgcaaca</td>
<td>57.9</td>
<td></td>
</tr>
<tr>
<td>71950</td>
<td>Mouse-Nanog</td>
<td>Forward</td>
<td>caagtttgcctagtcttgagg</td>
<td>50</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>gcaagaatagttctcggatgaa</td>
<td>43.5</td>
<td></td>
</tr>
<tr>
<td>110454</td>
<td>Mouse Sca-1</td>
<td>Forward</td>
<td>gaggcagcagttatgctgat</td>
<td>47.6</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>cgttgacccatgacccagga</td>
<td>52.4</td>
<td></td>
</tr>
<tr>
<td>18595</td>
<td>Mouse PDGFR-α</td>
<td>Forward</td>
<td>atgagatgtagatgacag</td>
<td>47.6</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>eggcaagatgtagtgcagag</td>
<td>57.1</td>
<td></td>
</tr>
<tr>
<td>648</td>
<td>Human BMI-1</td>
<td>Forward</td>
<td>ccaacctgtggtgcttgg</td>
<td>52.4</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>ttcagatgtagtctgtccaggg</td>
<td>45.5</td>
<td></td>
</tr>
<tr>
<td>5460</td>
<td>Human-Oct4</td>
<td>Forward</td>
<td>gggagattgataactgtggtt</td>
<td>43.5</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>gtgtatatccccaggtgacctc</td>
<td>52.2</td>
<td></td>
</tr>
<tr>
<td>2597</td>
<td>Human-GAPDH</td>
<td>Forward</td>
<td>tgccgatcataagcccttca</td>
<td>47.6</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>cgcccacttgatttgga</td>
<td>52.6</td>
<td></td>
</tr>
</tbody>
</table>

### Appendix v: RNA extraction, cDNA and Q-PCR reaction setup

#### RNA/ DNA Extraction

1. Plate 5000 cells per cm$^2$ in 60 mm dish and propagate until confluence.

2. Collect the cells in trypsin, quench trypsin action in growth medium and wash the cells with PBS$^{++}$.

3. Pellet the cells by centrifuging at 1000 rpm for 4 minutes and aspirate PBS$^{++}$, without disturbing the pellet.
4. Snap freeze the pelleted cells in liquid nitrogen for 10 seconds. The cells could be stored at -80 °C until ready for RNA extraction.

5. Extract RNA and DNA using the AllPrep DNA/RNA Mini Kit (Qiagen Cat# 80204).

6. After extraction measure the quantity of RNA and DNA using nano drop 2000.

7. Dilute RNA to 100 ng/µl and proceed to cDNA synthesis step.

8. Dilute DNA to 10 ng/ul and proceed to Genotyping step.

**cDNA synthesis reaction setup**

<table>
<thead>
<tr>
<th>PCR Reaction Setup</th>
<th>Stock Conc.</th>
<th>per 20 µl Rxn</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x RT Buffer</td>
<td>10</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>25x dNTP Mix 100 mM</td>
<td>100</td>
<td>0.8</td>
<td>4</td>
</tr>
<tr>
<td>10xRT Random Primer</td>
<td>10</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>10</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Nuclease Free H20</td>
<td></td>
<td></td>
<td>4.2</td>
</tr>
<tr>
<td>100 ng/ul RNA</td>
<td>100</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td></td>
<td></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cycling Step</th>
<th>Temperature &amp; Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation</td>
<td>10 min at 25</td>
</tr>
<tr>
<td>RT reaction</td>
<td>120 min at 37 C</td>
</tr>
<tr>
<td>Enzyme Inactivation</td>
<td>5 min at 85</td>
</tr>
<tr>
<td>Storage</td>
<td>End at 4 C</td>
</tr>
</tbody>
</table>
### Q-PCR reaction setup

<table>
<thead>
<tr>
<th>PCR Reaction Setup</th>
<th>Stock Conc.</th>
<th>per 25 µl Rxn</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x SybrGreen Mix</td>
<td>2</td>
<td>12.5</td>
<td>1</td>
</tr>
<tr>
<td>dd H2O</td>
<td>5.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td></td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>5uM Forward primer</td>
<td>5</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>5uM Reverse primer</td>
<td>5</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>1 ng/µl cDNA</td>
<td>1</td>
<td>5</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td></td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cycling Step</th>
<th>Temperature &amp; Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymerase Activation</td>
<td>10 min at 95 C</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>15 sec at 95 C</td>
<td>40 Cycles</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>30 sec at 58.5</td>
<td></td>
</tr>
</tbody>
</table>

### Standard PCR reaction setup

<table>
<thead>
<tr>
<th>PCR Reaction Setup</th>
<th>Stock Conc.</th>
<th>per 20 µl Rxn</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR buffer</td>
<td>10</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>10uM Forward primer</td>
<td>10</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>10uM Reverse primer</td>
<td>10</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>500 Units Taq Pol</td>
<td>500</td>
<td>0.4</td>
<td>10</td>
</tr>
<tr>
<td>dd H2O</td>
<td>13.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 ng/µl DNA</td>
<td>50</td>
<td>3</td>
<td>7.5</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td></td>
<td><strong>20</strong></td>
<td></td>
</tr>
<tr>
<td>Cycling Step</td>
<td>Temperature &amp; Time</td>
<td>Cycles</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>10 min at 95 C</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>30 sec at 95 C</td>
<td>34 Cycles</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>30 sec at 56.5 C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>45 sec at 72 C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>4 min at 72 C</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**Agarose gel preparation**

- Dissolve 2g of ultra-pure agarose in 100 ml of TAE buffer. Heat until agarose dissolves into solution. Let it cool to about 50 C.
- Add 6 µl of ethidium bromide carefully and stir under the exhaust hood. Pour the solution into a gel tray and let it cool. Once gel is formed, carefully pull the comb without breaking the gel.
- Mix 20 µl of PCR end-product with 4 µl loading dye and add it to wells. Load appropriate gel ladder in one well. Run the gel at 100 V for about 1 -2 hours.
- Capture images of the bands under a gel dock reader.
Appendix vi: BCIP-AP staining in negative control

<table>
<thead>
<tr>
<th>Wild type</th>
<th>Sca-1&lt;sup&gt;High&lt;/sup&gt;</th>
<th>Sca-1&lt;sup&gt;Low&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53 R172H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53 QSH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix vii: Migration assay on Sca-1\textsuperscript{High} and Sca-1\textsuperscript{Low} subpopulations across different p53 backgrounds

**0 hrs**

<table>
<thead>
<tr>
<th>p53 Wild type Sca-1\textsuperscript{Low}</th>
<th>p53 R172H Sca-1\textsuperscript{Low}</th>
<th>p53 QSH Sca-1\textsuperscript{Low}</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**6 hrs**

<table>
<thead>
<tr>
<th>p53 Wild type Sca-1\textsuperscript{High}</th>
<th>p53 R172H Sca-1\textsuperscript{High}</th>
<th>p53 QSH Sca-1\textsuperscript{High}</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

124

Back To Table of Contents
Appendix viii Immunoblotting (Western Blot)

1. Sample preparation

- Cells must be confluent and ready to be harvested from a 60mm cell culture plate.
- Use the total protein extraction kit which has 50x Protease inhibitor cocktail + TM lysis buffer (with NP40). Make 1x Protease inhibitor by diluting it from 50x using lysis buffer.
- Wash the cell plate with PBS after aspirating the media out. Be careful not to leave any traces of media in the plate as this will obstruct viewing the protein of interest on SDS PAGE later on.
- Use ice cold PBS and cold tubes for the rest of the sample preparation.
- Trypsinize the cells and spin them down.
- Wash the cell pellet with PBS.
- Add 100 μl of lysis buffer to 1 million cells.
- Pipette it up and down.
- Tape the tubes down to a vortex and keep it on constant agitation at 4°C for 30 minutes.
- Centrifuge at 13000 g at 4°C for 15-20 minutes.
- Collect the supernatant and dump the pellet. The supernatant is the protein.

2. Protein concentration: BSA assay

- Dye reagent (5x)
- BSA lyophilized powder
- Add 20 ml water to do the BSA powder and mix it to get a 1.44 mg/ml protein solution.
- Make three dilutions of protein solution (0.2 mg/ml, 0.4 mg/ml, 0.8 mg/ml).
• 0.8 mg/ml: 1.44 mg – 1ml
• 0.8 mg – 0.8/1.44 = 0.556 ml
• Add 0.444 ml dd H2O
• 109
• 1ml of 0.8 mg/ml solution
• 0.4 mg/ml: Take 0.5 ml of 0.8 mg/ml and add 0.5 ml of dd H2O to get 1 ml of 0.4 mg/ml solution.
• 0.2 mg/ml: Make 1ml of protein solution in the same way as above.
• Dye reagent: 1 part of 5x dye + 4 parts water = 25 ml dye (1x)
• Pour the dye reagent through a Whatman filter paper.
• Take 20 μl of protein (standards/sample) for 1ml of dye reagent and vortex it properly and incubate it for 5min at room temperature.
• Perform spectrophotometric analysis at 595nm to get the OD values.
• Only 30 μg of protein per well in gel. Do the calculations and take the required amount of protein sample and make up the volume to 30 μl using laemmli buffer (1:1 or 1:2).
• Before using the laemmli buffer, it is required to add β-mercaptoethanol to the laemmli buffer.
• Add 50 μl β-mercaptoethanol to 950 μl Laemmli buffer and mix it well.
• Add 1:1 ratio of Laemmli buffer to protein sample.
• Boil water on a burner, parafilm-seal the lids of the eppendorf tubes that contain sample and place the samples in boiling water for around 10 minutes.
• Spin them down to the bottom of the tube.
3. Running SDS PAGE

- Making running buffer: Take 900ml ddH2O in a glass bottle and add 100ml of Tris-Glycine-SDS and mix it well.
- Once the samples and buffers are ready, take them near the running apparatus.
- Get the gel ready from 4°C and take the tape off the bottom of the gel.
- Place it carefully in the gel holder and make sure to place the gel in a way that the wells are facing inside. Balance the other side using another gel if you are running two gels at the same time or use a dam to seal the other end.
- Pour running buffer into the gel holder to check if it is sealed well.
- Once it is established that there are no leaks, go ahead and take the comb out of the wells and saturate it with running buffer.
- Load ladder and samples carefully into the respective wells without releasing air bubbles into the well from the pipette.
- Run at 20mA for around 45min or till the protein reaches the bottom of the gel. Be careful not to let the protein run out from the bottom of the gel. If the gel has run well, the ladder is well separated.

4. Transfer onto nitrocellulose membrane

- Preparation: While the SDS PAGE is still running, get the transfer buffer ready and store it at 4°C.
- Take 700 ml water, 100 ml tris-glycine, 200 ml methanol and mix it well.
- Take ice in white ice-holder and fill it with a little water and keep it at -20°C until use.
• Cut the required size of the nitrocellulose and be careful not to touch without gloves and hold it using forceps. Place it in some transfer buffer.

• Wet the sponges and filter paper in the transfer buffer as well.
• Once the gel is run, carefully break open the gel case without breaking the gel.
• Place the nitrocellulose on gel supported by a filter paper.
• Let the gel come onto the membrane and then place another filter paper on gel.
• Press them smoothly and now place sponges on either side and roll a Pasteur pipette on this sandwich tightly to get rid of bubbles. (IMPORTANT)
• Do not forget the gel side and the membrane side.
• Black side: Gel
• Clear side: Nitrocellulose membrane
• Clear side should go next to red and black side onto black and back goes back. Place the other sandwich with another set of gel-membrane or plain sponges.
• Place the ice pack in the apparatus and place the whole apparatus in a trough containing ice.
• Now pour the cold transfer buffer into the apparatus and run it at 90V for around 1 hour 15 min-30min.
• You know the transfer has begun once you notice bubbles rising up from the bottom.
• The transfer is successful when the ladder is perfectly visible on the nitrocellulose membrane.

5. Blocking and washes:
- Use 3% block solution or make 5% block using nonfat casein powder in TBS-Tween solution
- TBS-Tween: 900ml water, 100ml TBS, 1ml Tween-20
- Do a 10 min wash with TBS-Tween.
- Primary and Secondary Antibodies:
  - Make the primary antibody solution in 0.1% TBS-Tween solution (15ml). It can be reused when stored at 4°C.
  - Add it to the washed membrane and leave it overnight at 4°C with constant agitation or upto 2 hrs. at room temperature with stirring.
  - Do 3x 10 min washes with TBS-Tween.
  - Make secondary antibody in TBS-Tween (10ml).
  - Add it onto membrane and leave it on stirring for 1 hour.
  - Do not reuse the secondary antibody solution. Do 3x 10 min washes with TBS-Tween and detect using the Odyssey Infrared Imaging System (Li-Cor Biosciences, NE).
VITAE

Venkat Sundar Gadepalli was born in Visakhapatnam, India on June 17, 1982. He attended and graduated from St Francis de Sales High School, Visakhapatnam, India, in 1997. He then began his undergraduate studies at Andhra University in Visakhapatnam and received Bachelor of Science degree in Biotechnology, in the year 2003. In 2005, Venkat earned his Master’s degree in Biotechnology from Devi Ahilya Vishwavidyalaya, Indore, India. He received a fellowship to undergo training in Bioinformatics from University of Pune, Pune, India and worked at National Chemical laboratory, Pune as a research assistant. In 2007, he moved to United States of America to pursue graduate studies. In 2009, Venkat graduated in Master of Science in Bioinformatics (Biology track) from Virginia Commonwealth University (VCU). Since, 2009 Venkat has been pursuing his Doctoral degree in Integrative Life Sciences at VCU. His future plan is to work as a Data Scientist in Research & Development division of companies related to Healthcare.