

**BONE MARROW STEM CELL-MEDIATED
AIRWAY EPITHELIAL REGENERATION**

By

Amy P. Wong

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of the Institute of Medical Sciences
University of Toronto

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Bone Marrow Stem Cell-mediated Airway Epithelial Regeneration

Amy Paisan Wong

Doctor of Philosophy, 2008

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Abstract

It has been suggested that some adult bone marrow cells (BMC) can localize to the injured tissues and develop tissue-specific characteristics including those of the pulmonary epithelium. In Chapter 2 we show that the combination of mild airway injury as a conditioning regimen to direct the site of BMC localization and transtracheal delivery of short-term cultured BMC enhances airway localization and adoption of an epithelial-like phenotype expressing Clara cell secretory protein (CCSP) and pro-surfactant protein-C. Bone marrow cells from transgenic mice expressing green fluorescent protein driven by the epithelial-specific cytokeratin-18 promoter were injected transtracheally into airway-injured wild-type recipients. BMC retention in the lung was observed to be at least 120 days following cell delivery with increasing transgene expression over time. The results indicate that targeted delivery of BMC can promote airway regeneration.

Although bone marrow stem/progenitor cells can develop into lung epithelial cells, the specific subpopulation remains unknown. In Chapter 3 we identify a newly discovered population of murine and human BMC that express CCSP. These CCSP⁺ cells increase in the bone marrow and blood after airway injury and can be expanded in culture. CCSP⁺ cells are unique in that they express both hematopoietic and

mesenchymal stromal cell markers and can give rise to various lung epithelial lineages *in vitro*. Importantly, bone marrow transplant of CCSP⁺ cells to CCSP knockout recipients confirms that bone marrow CCSP⁺ cells contribute to airway epithelium after airway injury.

In Chapter 4 we enrich for a stem/progenitor cell population within the CCSP⁺ using the stem cell antigen (Sca)-1 as a marker. Here we identified a putative epithelial stem/progenitor cell that can be induced to differentiate into various lung epithelial cell lineages expressing markers exclusive to airway or alveolar epithelial cells when cultured under an air liquid interface. These cells also have self-renewal potential *in vitro* that can proliferate *in vivo* and repopulate the injured airway epithelium. This newly discovered epithelial-like cells may play a central role in the bone marrow contribution to lung repair and are exciting candidates for cell-based targeted therapy for treatment of lung diseases.

To
Mom and Dad

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List of Abbreviations

Ab	Antibody
ALI	Acute lung injury
ANOVA	One-way analysis of variance
AQP	Aquaporin
BADJ	Bronchiole-alveolar duct junction
BASC	Bronchiole-alveolar stem cells
BMC	Bone marrow cell
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CEBP	CCAAT-enhancer binding protein
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU-F	Colony forming unit-fibroblast
CMTMR	(5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine
CCSP	Clara cell secretory protein (aka. Secretoglobin, uteroglobin, CC10)
CT	Cross-point threshold
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EM	Electron microscopy
ENaC	Epithelial sodium channel
ESC	Embryonic stem cells
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FoxA1	Forkhead box A1 (aka. Hepatocyte nuclear factor alpha)
FoxA2	Forkhead box A2 (aka. Hepatocyte nuclear factor beta)
GFP	Green fluorescent protein
HGF	Hepatocyte growth factor
HKG	House-keeping gene
HSC	Hematopoietic stem cells
HSVtk	Herpes simplex virus thymidine kinase
IGF	Insulin growth factor
IL	Interleukin
IV	Intravenous
K5	Cytokeratin-5
K8	Cytokeratin-8
K14	Cytokeratin-14
K18	Cytokeratin-18
K19	Cytokeratin-19
KGF	Keratinocyte growth factor

LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
MAPC	Multipotent adult progenitor cells
MEF	Mouse embryonic fibroblasts
MHC	Major histocompatibility chain
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
MTEC	Murine tracheal epithelial cells
NGF	Neuronal growth factor
NGS	Normal goat serum
NHS	Normal horse serum
OCT4	Octomer-4
panCK	Pan-cytokeratin
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PECAM	Platelet-endothelial cell adhesion molecule
Pen/strep	Penicillin-streptomycin
PGC	Primordial germ cells
SP-C	Pro-surfactant protein-C
REST	Relative expression software tool
RT-PCR	Reverse transcription polymerase chain reaction
SABM	Small airway basal medium
SAGM	Small airway growth medium
Sca-1	Stem cell antigen-1
SDF-1	Stromal-derived growth factor-1
SEM	Standard error of mean
SP	Side population
SSEA	Stage-specific embryonic antigen
TA	Transient amplifying
TE	Translational enhancer
TEC	Tracheal epithelial cell
TEM	Transmission electron microscopy
TNF	Tumor necrosis factor
TT	Transtracheal
TTF-1	Transcription factor-1
VSEL	Very small embryonic-like
vWF	Von Willebrand factor
WT	Wild-type

Dissemination of Thesis Content

Publications

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Contents of Chapter 2 can be found at

<http://ajplung.physiology.org/cgi/content/full/293/3/L740>

Introduction

1.1 Airway Diseases

1.1.1 Epidemiology of pulmonary diseases

Based on 1998 Canadian Institute of Health Research survey¹, 10.1% of men and 10% of women died from respiratory disease while 5.9% and 6% respectively died from lung cancer. Approximately 3.2% of Canadians suffer from Chronic Obstructive Pulmonary Disease (COPD) and 8.4% from asthma. These numbers are estimated to have risen since the last survey due to the aging population. The Lung Association of Canada estimates that by 2010, respiratory diseases will be the 3rd cause of deaths in Canada².

1.1.2 *Chronic obstructive pulmonary disease (COPD)*

Chronic obstructive pulmonary disease (COPD) is a category of chronic lung diseases characterized by the pathological limitation of airflow in the airway that is not fully reversible. COPD is common among seniors in Canada. The most common forms of COPD are chronic bronchitis and emphysema. Cigarette smoking is the principal cause of COPD, however chronic exposures to pollutants can also contribute to the development or the exacerbation of COPD. In rare cases, people with a hereditary disease such as α 1-antitrypsin deficiency are predisposed to COPD development. Individuals with COPD experience shortness of breath, increased sputum and coughing and are prone to serious

¹ From the Public Health Agency of Canada “Respiratory Diseases in Canada” report downloaded from www.phac-aspc.gc.ca/publicat/rdc-mrc01/index.html

² From the Lung Association of Canada website www.lung.ca

conditions such as recurring chest infections, respiratory failure, pulmonary hypertension and heart failure.

1.1.2.1 Chronic Bronchitis

Chronic bronchitis is defined as a persistent cough with sputum production. Pathologically, hyperplasia and hypertrophy of goblet cells of the airways are observed resulting in an increase in mucus secretion and airway obstruction. In addition, neutrophil infiltration can be found in the airway wall resulting in fibrosis and thickening of the wall and narrowing of the airway lumen.

1.1.2.2 Emphysema

Emphysema is characterized by enlarged alveolar sacs causing a reduction in gas exchange surface area as well as a loss in elasticity of the lungs. The overall enlargement of the lungs burdens the thoracic cavity and clinically manifests as difficulty in breathing. This disease is commonly seen in smokers. However, emphysema can occur in people with the congenital genetic disorder α 1-antitrypsin deficiency which leads to increased protease-mediated tissue destruction. In 2000-2001, 4.3% of Canadians were diagnosed with emphysema (National Population Health Survey, Statistics Canada, September 2001 Canadian Institute for Health Information-Respiratory Diseases in Canada)¹.

1.1.3 Bronchiolitis obliterans

Bronchiolitis obliterans (BO) is a rare and life-threatening form of obstructive lung disease characterized by inflammation and obliteration of the smaller airways.

Causes of BO include bacterial or viral infections, complications of prematurity (bronchopulmonary dysplasia) and exposure to toxic fumes such as sulfur dioxide, ammonia, and nitrogen dioxide. BO may also be idiopathic (cause is unknown). It is also observed in about 30% of lung transplant recipients (based on >3-5 year post-transplant survival)³. BO is manifested as progressive blockage of the smaller airways with extracellular matrix and inflammatory cell deposits in the lumen. Symptoms include severe shortness of breath and dry cough.

1.1.4 Asthma

Asthma is a chronic disease that affect both children and adults. In the 1989-1999 National Population Health Survey, asthma was diagnosed in 7.5% of adults (1.629 million Canadians) and 10.7% of children and teens (0.845 million)¹. Asthma is characterized by chronic inflammation and narrowing of the airways resulting in clinical symptoms including cough, shortness of breath, wheeze and chest tightness. Various stimuli can induce “episodes” or attacks such as allergens, viral infections and exercise.

1.1.5 Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder that afflicts up to 1 in 2,500 people in the western world. It is a debilitating incurable disease that is characterized by abnormal salt and water transport leading to abnormal airway secretions, impaired mucociliary clearance and chronic inflammation (Smith et al., 1996). As a result, chronic bacterial infections (most commonly with *Pseudomonas aeruginosa*) and

³ According to the International Society for Heart & Lung Transplantation “Transplant Registry” available at www.isHLT.org

premature death often ensues (Quinton, 1990a; Quinton, 1990b). Although CF affects a variety of other epithelial tissues such as the pancreas, sweat ductal glands and the gastrointestinal ducts, the major cause of morbidity and mortality is the disease in the lungs. The pathogenesis of the disease remains unclear. Fifteen years ago, scientists identified the CF gene which codes for the CF transmembrane conductance regulator (CFTR) protein (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989) and since then around 1000 mutations have been identified associated with various classes of CFTR defects (details can be found at www.genet.sickkids.on.ca/cftr/). The most common mutation involves a deletion of phenylalanine at position 508 of the protein resulting in abnormal intracellular trafficking of the CFTR protein and ultimately leading to deficient membrane chloride (Cl⁻) conductance in epithelial cells (Cheng et al., 1990). Studies have shown that CFTR is a cyclic adenosine monophosphate (cAMP)-mediated Cl⁻ channel (Kartner et al., 1991; Rommens et al., 1991). During lung development CFTR modulates lung secretory cell differentiation and alveolar epithelial cell proliferation (Larson et al., 2000). In the airways, a defective CFTR protein results in changes in the salt and water composition of the periciliary fluid causing isotonic dehydration and impaired ability to clear infections and hyperinflammation. It remains unclear whether infection precedes inflammation or vice versa. In vitro evidence suggests that a defect in CFTR can induce the production of proinflammatory cytokines such as IL-8 (Perez et al., 2007) while others have demonstrated an increase in the cytokines only after exposure to a pathogen (Kube et al., 2001). In vivo evidence demonstrates an exacerbated inflammatory response upon infection with *Pseudomonas aeruginosa* (Heeckeren et al., 1997). The proinflammatory response following bacterial challenge in mice carrying

various CFTR mutations appear to be comparable (van Heeckeren et al., 2004) suggesting proper functional activity of the CFTR protein is essential in regulating airway inflammation.

1.1.6 Acute respiratory distress syndrome (ARDS)

Acute respiratory distress syndrome (ARDS) is a serious and potentially fatal condition characterized by inflammation in the airways, fluid in the alveolar sacs (pulmonary edema), hypoxemia and is frequently associated with multiple organ failure. ARDS is usually caused by an insult to the lung parenchyma such as septic shock, pneumonia, aspiration of vomit, chemical inhalation or trauma. The less severe form of ARDS is acute lung injury (ALI). Inflammation causes endothelial dysfunction which results in inflammatory cell and proteinaceous fluid to enter the alveoli, hyaline membrane development in the interstitium and impairment of the gas exchange surface. Type II cell dysfunction can also occur resulting in a reduction of surfactant production and collapse of the alveoli. Patients with ARDS are sustained with mechanical ventilation and critical care within the intensive care unit. The mortality rate of patients with ARDS is about 40% (Ware, 2006). There are currently no specific treatments for ARDS. Therefore many studies explore novel therapies aimed at treating ARDS or ALI. A recent publication by Lam showed that intravenous delivery of autologous circulating endothelial progenitor cells (EPC) into an oleic acid-induced rabbit model of ALI at the time of ALI-induction could preserve endothelial function and maintain the alveolar-capillary barrier (Lam et al., 2008). Higher levels of circulating EPC appears to be associated with an improved survival rate of patients with ALI and ARDS (Burnham et

al., 2005). Bone marrow-derived EPC also have preventative abilities in maintaining the integrity of the endothelium when delivered shortly after injury in a left-side rat lung transplant model of severe ALI (Kahler et al., 2007). Infusion of mesenchymal stem cells (MSC) as will be discussed later, have also been shown to ameliorate experimentally induced ALI (Gupta et al., 2007; Mei et al., 2007; Xu et al., 2008). These latter findings highlights the role of the bone marrow in ALI and ARDS repair and the potential for developing progenitor cell-based therapies in treating ALI and ARDS.

1.1.7 Infant respiratory distress syndrome

This condition is different from ARDS and is caused by a deficiency in lung surfactant production and poor structural development of the lung (low numbers of alveoli). The result is severe difficulty in breathing. Infant RDS affects about 1% of newborn infants and is more frequent in babies with diabetic mothers.

1.2 Regenerative medicine

Regenerative medicine is an innovative scientific field that focuses on new approaches to repairing and replacing cells, tissues and organs. It offers an integration of various fields of science and medicine including tissue engineering, stem cell biology and gene therapy. Regenerative medicine offers great potential for future medical care. People with end-stage lung diseases could potentially benefit from regenerative medicine since the current best treatment available is lung transplantation. The shortage of lung donations however remains an increasing challenge with the aging population. New treatments are desperately needed.

1.2.1 Tissue engineering

Tissue engineering is an innovative technology employing biomedically engineered cells and scaffolds to create artificial organs and tissues such as skin, bone and heart valves. The most challenging aspect of tissue engineering is the creation of an artificial organ/tissue that has similar biological composition and physiological functions to the native organ/tissue. This may be why tissue engineering remains a colossal challenge in the lung. Recapitulating the cellular components involved in immunity, the mechanical functions of breathing and the intricate physiology of gas exchanges are factors that overwhelm tissue engineering efforts in the lung. Although no one has ever been able to generate an entire lung through tissue engineering approaches, a few studies have shown some success in creating parts of the large airways (Choe et al., 2006; Paquette et al., 2004; Yang et al., 2003) or lung parenchyma (Chen et al., 2005; Shigemura et al., 2006). The combination of stem/progenitor cells with bioengineering has shown promise in generating “lung equivalents” comprised of lung-like cells (Andrade et al., 2007; Coraux et al., 2005; Cortiella et al., 2006) since stem cells have great potential to differentiate into most if not all cell types. Bioengineering of lung cells *in vitro* with embryonic stem cells (ESC) (Rippon et al., 2006) is another promising effort for cell-based therapies. Although tissue engineering is a highly desirable long-term goal for lung regeneration, it remains rudimentary.

1.2.2 Gene therapy

Gene therapy involves the insertion of a therapeutic gene into cells to treat diseases, especially hereditary diseases (such as CF) that involve replacing a mutant

allele with a normal one. The discovery of CFTR has stimulated intensive research into therapeutic gene therapy approaches to replace the defective gene in CF. The first clinical trial for CF began in 1992 where adenovirus-mediated gene transfer effectively restored Cl⁻ conductance in the nasal epithelium of cystic fibrotic patients (Zabner et al., 1993). Since then, much progress has been made in terms of using gene therapy as a technique to replace the defective gene in affected cells (Alton et al., 1993; Chow et al., 1997; Drumm et al., 1990; Flotte et al., 1993; Flotte et al., 2003; Koehler et al., 2003; Moss et al., 2004). Many animal models of cystic fibrosis have also been created to evaluate and delineate the pathogenesis of the disease. The first and most common is the CFTR knockout mice model which most closely resembles certain features of the disease in humans (Snouwaert et al., 1992).

Various gene delivery methods such as adenovirus (Johnson et al., 1996; Zabner et al., 1993), retrovirus (Olsen et al., 1992) and adeno-associated viruses (Flotte et al., 1993; Flotte et al., 2003; Moss et al., 2004) have been studied extensively for treatment of CF. These methods of gene delivery have proven to be somewhat effective, yet concerns remain over the long-term efficacy and safety of using viral-based vectors in humans. Liposomes have shown limited success in gene delivery (Alton et al., 1993; Noone et al., 2000; Yatvin, 2002). These vectors are unstable and have poor transfection efficiencies *in vivo* (McLachlan et al., 1995).

Extracellular and intracellular barriers hinder successful gene transfer. The lack of viral receptors on the apical surface of the airway epithelium for example limits viral transfection with the transgene to the target cell when delivered topically through the airways. Systemic delivery increases risks of multiorgan toxicity and most often results in

transfection of the respiratory epithelium than the airway epithelium. Furthermore, the inability of vectors to evade the host immune response limits the efficiency of successful transfection. Retroviral and adenoviral methods of gene delivery are often accompanied by significant inflammatory responses due to these vectors. The random delivery of the transgene by viruses poses another risk of insertional mutagenesis which can affect the expression of other important genes. In addition, a study has shown the presence of viral genetic material incorporated into host genome (Eissa et al., 1994). In this study, about 13% of the E1 genes (responsible for viral replication) were found in the affected epithelium of patients that received adenovirus-mediated gene transfer of CFTR. Intracellular barriers include cytoplasmic clearance mechanisms (eg. Ubiquitination and cytoplasmic protease digestion) that can digest the transgene preventing it from entering the nuclei (Kitson et al., 1999). Uptake of DNA into the nuclear compartment may also be an important barrier in terminally differentiated, non-dividing cells. Although attempts to increase nuclear uptake of oligonucleotides and plasmids by adding nuclear localization signals to the DNA (Zanta et al., 1999) have shown some improvements, sustained gene expression may remain a problem in slow-cycling cells.

1.2.3 Cell-based gene therapy

Cell-based gene therapy is another field of regenerative medicine. It promises the use of a host's own cells, after genetic manipulations, to deliver the therapeutic protein/DNA to the site of injury for repair and/or regeneration. Since it uses the host's own cells as vehicles for gene delivery, it avoids the immunological barriers that are often associated with viral-based vectors. In addition, this cell-based approach eliminates

the risk of DNA degradation as observed in naked-DNA delivery. Success has been shown in using endothelial-cell based gene delivery of endostatin, an angiogenic inhibitor, to prevent tumor angiogenesis (Dudek et al., 2007). Similarly, delivery of nitric oxide synthase via smooth muscle cells to attenuate monocrotaline-induced hypertension (Campbell et al., 2001) showed significant success and led to the first clinical trial in North America led by Dr. Duncan Stewart using cell-based therapy for treatment of pulmonary hypertension (PHACeT). Gene delivery of angiopoietin-1 using fibroblasts (McCarter et al., 2007) or mesenchymal stem cells (Mei et al., 2007) has shown promise in preventing LPS-induced ALI in rodent models.

1.2.4 Stem cells

Stem cells (see section 1.4) have received incredible attention within the past decade. Numerous studies have suggested the potential of stem cells to regenerate tissue/organs. The mechanism of stem cell-mediated regeneration remains controversial. While some studies suggest stem cells contribute to tissue cell lineages upon homing and lodging in the injured site by transdifferentiation and/or fusion (Kotton et al., 2001; Krause et al., 2001; Nygren et al., 2004; Reinecke et al., 2004; Rizvi et al., 2006; Shi et al., 2004; Wang et al., 2005), others suggest that stem cells do not contribute to tissue cell lineages but provide a pro-regenerative milieu (Fazel et al., 2006) for repair of injured tissue with local progenitor cells or by attenuation of immune/inflammatory responses to injury (Ayach et al., 2006a; Ortiz et al., 2007; Xu et al., 2007b). Functional tissue have been created through the use of stem cell manipulation/transplantation such as repair of the infarcted myocardium (Fazel et al., 2005), axonal regeneration following spinal cord

injury (Kim et al., 2007), alveolar regeneration (Ishizawa et al., 2004a) and the intestinal epithelium (Bruscia et al., 2006).

1.2.5 Rationale for stem cell-mediated regenerative medicine

The best current treatment for lung diseases such as end-stage lung diseases or CF is lung transplantation. Not only is a lack of organ donation a hindrance to treatment, but BO and graft rejection/failure are major limitations for long-term success in lung transplantation⁴. Stem cell-mediated regenerative medicine offers great promise for treatment of diseases that involves cell-based gene correction and replacement of a particular cell such as in cystic fibrosis or immunomodulation to prevent graft rejection such as in BO. Successful long-term restoration of continuously self-renewing tissues such as the lung depends on the use of self-renewing stem cells. In addition, the pluripotent/multipotent differentiation capabilities of stem cells allows for potential functional replacement and restoration of tissue components. Therefore stem cells provide appropriate targets for prospective cell-based gene therapies and cell replacement strategies.

1.3 The Lung

1.3.1 Structure of the adult lung

During embryonic lung development, the trachea and the primary lung buds are derived from different morphogenetic processes from adjacent regions of the embryonic foregut. Heterogeneity of the epithelial-mesenchymal interactions along the developing

⁴ According to the International Society for Heart & Lung Transplantation www.ishlt.org

respiratory tract results in the activation of distinct networks of signaling molecules (fibroblast growth factors, retinoids, Sonic hedgehog, bone morphogenic protein, and transforming growth factors) along the proximal-distal axis and determine branching and differentiation of the respiratory system (Cardoso, 2001).

The lung is a unique organ made up of conducting airways and a gas-exchange system. The adult mouse cartilaginous airways include the trachea and primary bronchi. The pseudostratified epithelium contains two main columnar cells types: ciliated cells and Clara-like cells as well as basal cells. Clara-like cells produce Clara cell secretory protein (CCSP or CC10, Clara cell antigen, secretoglobin) which is the most abundant secretory protein produced. A few pulmonary neuroendocrine cells also exist in the upper airway epithelium. Small submucosal glands are also found in the upper airways and harbour mucus-producing cells (goblet cells), ciliated cells and basal cells (and parabasal cells in human lung). Basal cells are unspecialized cells scattered along the upper airways distinguishable by cytokeratin-5 and cytokeratin-14 expression and do not appear in the large airways until around birth and after differentiation of ciliated and secretory cells (Rawlins and Hogan, 2006). In the more distal airways which include the small bronchi and bronchioles, the epithelium is predominately composed of Clara cells and some ciliated cells. More pulmonary neuroendocrine are also found but no basal cells. The junction between the terminal bronchioles and the gas exchange region is the bronchioalveolar duct junction (BADJ). The gas exchange region of the lung is comprised of two types of epithelial cells: Type I and Type II. Type I cells are thin-walled cells that provide a gas exchange surface. Type II cells are cuboidal in morphology and secrete surfactants which decreases surface tension during breathing.

1.3.2 Evidence for lung stem cells

Although cell turnover in the adult lung is normally slow, with an estimated number of cells cycling at any one time is about 1% (Boers et al., 1998), it is capable of repair and regeneration after injury (Warburton et al., 2001). This suggests that the lung may have resident stem cells. To date, a single lung stem cell capable of differentiating into all components of the lung has not been identified. This may reflect the complex composition of the lung. Several strategies have been used to study the steady-state maintenance of the lung epithelium and the lineage relationship of the epithelial cells. Studies have employed label retention/label dilution relationships over time to identify parent/progeny status of the epithelial cells (Borthwick et al., 2001; Chan and Gargett, 2006; Wei et al., 1995). The drawback of these methods is that they cannot ascertain the real cell origin of the progenies as there may be multiple progenitor cells that retain the label. This method also cannot distinguish whether the parent to progeny relationship is the same in different regions of the lung. The best method of ascertaining lineage relationships is to use genetic labeling methods to follow the descendants of specific cells. Cre-lox is a robust strategy that can be used in cell fate-mapping studies. Crossing transgenic mice carrying lox P sites flanking a reporter gene with mice expressing Cre under the control of a specific promoter can result in tissue-specific activation of the transgene and labeling of the progeny of all cells following promoter activation.

Regional stem cells or progenitor cells have been identified that can respond to local injuries. Since cell turnover is quite slow under normal homeostasis, to study putative stem cell populations in the airways, most investigators induce some sort of

lung-specific injury in animal models to promote higher cell turnover. Markers of proliferation such as BrdU and tritiated thymidine which marks cells that retain the label (label-retaining cells) for an extended period of time have allowed researchers to identify several putative lung stem cells and their niches. These cells include the bronchioalveolar stem cells of the BADJ (Kim et al., 2005), Clara cells (Barth et al., 2000; Hong et al., 2001) of the bronchioles, basal and parabasal cells of the trachea (Boers et al., 1998; Hong et al., 2004a; Hong et al., 2004b), ciliated cells (Park et al., 2006) of the airways and Type II pneumocytes of the alveoli (Adamson and Bowden, 1975).

The contribution of these putative lung stem cells in normal homeostasis remains unknown. At best, these cells are “facultative stem cells” (Rawlins and Hogan, 2006) in that they only self-renew and give rise to progeny upon insult or injury.

1.3.2.1 Basal cells

There is evidence to support the role of basal cells in airway epithelial regeneration (Barth et al., 2000; Boers et al., 1998; Hong et al., 2004b; Liu et al., 1994). Borthwick showed that epithelial damage in mice receiving weekly intratracheal detergent or inhaled sulphur dioxide had BrdU-labelled cytokeratin-14 and -18 positive LRC that repopulated the tracheal epithelium after injury (Borthwick et al., 2001). Based on their location visualized by immunostaining methods, these authors found 80% of the LRC were basal cells and 20% were columnar cells mostly in the upper trachea and the tracheal gland ducts. Other studies suggests that basal cells do not contribute to columnar cells during repair but rather form an epithelial barrier by flattening along the lumen of

the airways to establish contact with each other and may serve as a defense mechanism against further insult to the basement membrane (Erjefalt et al., 1997).

The first robust *in vivo* evidence of basal cells as putative stem cells of the trachea and main bronchi was performed using *in vivo* genetic lineage labeling (Hong et al., 2004a; Hong et al., 2004b). Tamoxifen-inducible cytokeratin-14 promoter-driven Cre recombinase was crossed with mice harbouring the Cre recombination substrate composed of ubiquitous Rosa26-promoter-driven Flox-lacZ mice. Administration of tamoxifen to these bitransgenic mice led to the activation of Cre recombinase resulting in lacZ expression in cytokeratin-14-expressing cells. Naphthalene injury allowed assessment of cytokeratin-14-expressing cell proliferation and the subsequent derivation of progeny during repair of the airway epithelium. Following completion of epithelial regeneration, patches of β -galactosidase-positive epithelial cells were identified which contained cytokeratin-14-positive cells, CCSP-positive cells and ciliated cells suggesting basal cells have self-renewal and multidifferentiation potential. However, there is evidence to suggest that other epithelial cells, not derived from basal cells, can activate cytokeratin-14 *de novo* during repair of the tracheal epithelium (Liu et al., 1994) and therefore these other cells may also be contributing to airway epithelial regeneration.

1.3.2.2 Pulmonary neuroendocrine cells

A small fraction of pulmonary neuroendocrine cells (about 0.5% total lung cells) do proliferate in uninjured human airways (Boers et al., 1998). These cells are the first differentiated cells to appear in the developing lungs which would suggest that they may be putative stem cells. Animal models have shown that these cells can regenerate the

adult lung epithelium following Clara cell depletion (Peake et al., 2000; Reynolds et al., 2000a; Reynolds et al., 2000b). Caltonin-gene related peptide, a neuroendocrine marker, was used to identify pulmonary neuroendocrine cells proliferation in neuroepithelial bodies during naphthalene-induced lung injury.

1.3.2.3 Clara cells

Clara cells are secretory cells that secrete mainly CCSP which may have a role in immunity and inflammation. In humans, Clara cells comprise 11% and 22% of the cells in the terminal and respiratory bronchioles and account for 15% and 44% of proliferating cells in these regions (Boers et al., 1999).

In the bronchiolar system of mice, Hong et al (Hong et al., 2001) found BrdU label-retaining, CCSP-expressing cells near the neuroepithelial bodies which suggested they were able to regenerate the airway epithelium after naphthalene-induced lung injury. These cells termed variant Clara cells were rare cells that survived naphthalene injury because they did not express the enzyme CYP2F2, a member of the cytochrome P450 family that converts naphthalene into toxic dihydrodiol metabolites (Hong et al., 2001; Stripp et al., 1995). Some of these CCSP-expressing label-retaining cells were also found in the BADJ (Giangreco et al., 2002) and may be bronchiolalveolar stem cells. In another lung injury model also developed by this group, CCSP-Herpes simplex virus thymidine kinase (HSVtk) transgenic mice that received gancyclovir to kill all CCSP-expressing cells including the putative stem cells due to the CCSP-promoter-driven thymidine kinase activity, 46% of the PNEC proliferated but were unable to give rise to variant Clara cells, specifically cytochrome P-450 negative cells, or regenerate the airway epithelium (Hong

et al., 2001). This further suggests that these variant Clara cells are another source of lung stem cells or at least are critical mediators of stem cell maintenance.

1.3.2.4 Bronchioalveolar stem cells

Bronchioalveolar stem cells have recently been identified and are localized to the BADJ of the mouse lung (Kim et al., 2005). These rare cells (1-2 cells per BADJ) are cuboidal in morphology and coexpress CCSP and surfactant protein-C (SP-C), a secretory product of Type II alveolar cells. These cells also express the surface glycoprotein, CD34, and the stem cell antigen (Sca)-1 both markers commonly seen in hematopoietic stem cells. After induction with airway-specific injury using naphthalene toxin and alveolar injury using bleomycin, bronchioalveolar stem cells were found to proliferate and give rise to daughter cells capable of regenerating the airway and alveolar epithelium. Furthermore, these cells were also found to contribute to cells in adenocarcinomas of the BADJ after transformed with oncogenic K-ras mutation. Surface expression of specific markers such as CD34 and Sca-1 and negative expression of the hematopoietic marker CD45 and platelet-endothelial cell adhesion molecule (PECAM) allows a method to isolate these cells by cell sorting techniques and study these cells *in vitro*. Single cells cultured on mouse embryonic fibroblasts (MEF) feeder layer demonstrated clonal expansion of these cells. Matrigel cultures of these bronchioalveolar stem cells revealed differentiation of these cells into Clara cells, Type I and Type II cells suggesting their multi-epithelial differentiation potential.

1.3.2.5 Ciliated cells

The role of ciliated cells in epithelial regeneration remains controversial. A study by Reynolds showed that when conditionally killing all the CCSP-expressing cells in the airways by giving ganciclovir to CCSP-HSVtk mice, the ciliated cells did not give rise to Clara cells or contribute to epithelial regeneration (Reynolds et al., 2000a). However, a study by Park showed that flattened ciliated cells can spread beneath Clara cells early during naphthalene injury to maintain the integrity of the epithelium (Park et al., 2006). These cells later underwent morphological changes from squamous (flattened) to cuboidal to columnar and expressed markers of both ciliated and Clara cells. Recently, this possibility was challenged by Rawlins who used a transgenic mouse strain in which the forkhead domain transcription factor promoter FOXJ1 (involved in late stage of ciliogenesis) directed expression of a tamoxifen-inducible allele of the Cre recombinase and found no involvement (cell proliferation or transdifferentiation) of ciliated cells in repair of lung injury (Rawlins et al., 2007).

1.3.2.6 Type II alveolar cells

In adults, of the roughly 70 m² of gas exchange surface 96% is occupied by Type I cells and 4% by Type II cells that constitute 8% and 15% of the total lung cells respectively (Griffiths et al., 2005). Type II cells have long been thought to be the local stem cells of the alveolar epithelium giving rise to both Type I and Type II cells. In 1969, Kapanai (Kapanai et al., 1969) was the first to describe Type II cells as progenitor cells of Type I cells. Using hyperoxia to destroy Type I cells in monkey lungs, they found that Type II cells replaced Type I cells 4 days after exposure. Evans and colleagues later

confirmed transformation of Type II to Type I cells in a rat model of alveolar injury using NO₂ and tritiated thymidine labeling of cells (Evans et al., 1975). In this study, mainly Type II cells were labeled in the alveoli 1 hour after delivery of the label. By 24 hours, Type I labeled cells appeared and predominated by 48 hours with a dramatic reduction in Type II cells. The key discovery was the identification of cells that were morphologically similar to both cell types suggesting they were transitional cells of Type II to Type I. Adamson and Bowden (Adamson and Bowden, 1975) found similar Type II to Type I transitional cells in the developing rat lungs. Reddy (Reddy et al., 2004) isolated a subset of Type II cells that was highly proliferative and expressed high telomerase activity. These putative progenitor cells were E-cadherin-negative and were resistant to DNA damage.

1.3.2.7 Side population cells

Side population (SP) cells are a rare subset of cells that are characterized by their ability to efflux Hoechst 33342 dye via the ATP binding cassette membrane transporter breast cancer resistance protein-1. When stained with Hoechst and excited by a UV laser (350-365 nm), SP cells exhibit a low blue (440-460nm) and low red (>675nm) fluorescent staining pattern. SP cells have been identified in many tissues (Asakura et al., 2002; Hussain et al., 2005; Kotton et al., 2005a) including the lung (Giangreco et al., 2004; Majka et al., 2005; Summer et al., 2004; Summer et al., 2003) and are thought to contain a local tissue stem cell population. Bone marrow-derived SP cells have also been found to contribute to lung cell lineages (Abe et al., 2003; Macpherson et al., 2005b). SP cells are enriched with putative stem cells because they express Sca-1 but not lineage

markers (lin⁻). Summer (Summer et al., 2003) reported that SP cells in the adult mice comprise 0.03-0.07% of total lung cells and were evenly distributed in the proximal and distal regions of the lung. These cells were heterogenous for the hematopoietic marker CD45. CD45⁺ SP cells were found to coexpress CD31 suggesting these SP cells may be progenitors of the pulmonary vasculature. These CD45⁺CD31⁺ cells were later found to be bone marrow-derived (Summer et al., 2004). CD45⁺Sca-1⁺ and CD34⁺ SP cells comprise 60-70% of the total lung SP cells while the CD45⁻Sca-1⁺CD34⁻ comprise of 30-40%.

CD45 negative SP cells have epithelial and mesenchymal potential in the mouse lung. Giangreco (Giangreco et al., 2004) found that CD45⁻ SP cells were phenotypically similar to the neuroepithelial body-associated variant Clara cells that expressed CCSP and vimentin mRNA. In contrast, a recent study by the same group showed that SP cells isolated from various regions of the lung are enriched for clonogenic precursors that are vimentin positive but negative for epithelial markers (Reynolds et al., 2007). Furthermore, the authors show that the clonogenic potential of these SP cells was compromised in bleomycin-induced lung fibrosis suggesting that these cells may play a role in wound repair. Based on Hoechst efflux properties alone, SP cells from the different lung compartments were not distinguishable by surface marker expression suggesting that this method of isolating lung-specific stem cells may not be useful in isolating a single putative stem cell.

1.3.2.8 Others

A recent report identified an OCT-4-expressing stem/progenitor cell residing in the lungs of neonatal mice (Ling et al., 2006). These cells were isolated and characterized to be positive for other stem cell markers such as the stage-specific embryonic antigen-1 (SSEA1) protein, Sca-1 and CCSP. These cells were negative for c-kit, and CD34 which suggest that these are a rare subpopulation of Clara cells that may have been previously implicated as airway stem cells. Similar to bronchioalveolar stem cells, these cells reside in the BADJ of neonatal lung and are label-retaining cells that are susceptible to SARS-CoV infection *in vivo*. However the group provided no evidence *in vivo* or *in vitro* to show the multipotential nature of these cells.

Another report by Lama (Lama et al., 2007) provide evidence, in human adult lung transplants, of a pulmonary MSC population expressing classical markers of MSC (CD73, CD90, CD105) that could differentiate into adipocytes, chondrocytes and osteocytes *in vitro*. The role of these cells in lung regeneration or lung fibrosis remains unknown.

1.3.3 Lung stem cell niches

A few potential stem cell niches have been found in the lungs. Borthwick (Borthwick et al., 2001) found BrdU LRC clustered in the intercartilage regions of the large airways. More distally, the neuroepithelial body (NEB) appears to be the niche for the variant Clara cells (Hong et al., 2001) and the PNEC (Reynolds et al., 2000b). The BADJ may serve as the niche for putative BASC (Giangreco et al., 2002; Kim et al., 2005). All these regions are ideal since they are well supplied with blood vessels and

nerves and would serve as a good environment for stem cell maintenance and growth. These niche environments comprised of non-epithelial cells in the lungs is consistent with other tissue stem cell niche systems (Calvi et al., 2003; He et al., 2004; Lie et al., 2005). For example, some studies have shown the importance of niche endothelial cell signaling in controlling neuronal stem cell self-renewal (Li et al., 2006; Shen et al., 2004). In the marrow, the osteoblastic cell regulate HSC cell fate (Calvi et al., 2003).

A highly regulated network of genes are involved in controlling stem cell self-renewal and differentiation (Ivanova et al., 2002; Ramalho-Santos et al., 2002). Many of the genes active in intercellular crosstalk during lung development such as Sonic hedgehog (Shh), Wnt, bone morphogenic protein (Bmp) and fibroblast growth factor (FGF) are also active in some stem cell niches (see section 1.4) and the regenerating lung epithelium (Bellusci et al., 1997; Bhardwaj et al., 2001; Cardoso, 2001; Fisher et al., 2005; Shannon and Hyatt, 2004; Shu et al., 2005; Watkins et al., 2003; Weaver et al., 2000; Willert et al., 2003).

1.3.4 Homeostasis versus disease

A stimuli or mutation that may influence the intricate balance of genes involved in maintaining stem cell self-renewal and differentiation may also dictate the fate of the stem cell towards regeneration or disease initiation/progression. During injury and repair the fate of a stem cell may be dependent on the cross-talk among the stem cell niche, the stem cell and the inflammatory cells. Watkins (Watkins et al., 2003) found significant upregulation of Shh signaling during airway injury which preceded pulmonary neuroendocrine cell differentiation and epithelial regeneration suggesting a role in Shh in

inducing quiescent pulmonary neuroendocrine cell differentiation. Genes that are involved in lung development such as Sox-2, Sox-17, Foxa2, and beta catenin are also upregulated during epithelial regeneration that precedes transdifferentiation of ciliated epithelial cells to columnar epithelium (Park et al., 2006). On the flip side, mutations in the Wnt pathway resulting in transactivation of the Tcf/ β -catenin target genes can lead to transformations of epithelial progenitors into a malignant phenotype (van de Wetering et al., 2002). Similarly, mutations resulting in higher nuclear β -catenin in granulocyte-macrophage progenitors derived from chronic myeloid leukemic patients enhanced the self-renewal and leukemic potential of these cells (Jamieson et al., 2004). Disruption of BMP signaling in the intestinal intravillus mesenchyme results in the formation of numerous ectopic crypt units perpendicular to the crypt-villus axis, a histological hallmark seen in patients with the cancer predisposition syndrome juvenile polyposis (Haramis et al., 2004).

1.4 Lung injury

1.4.1 Models of lung injury for studying putative stem cells

1.4.1.1 Naphthalene injury

Naphthalene injury is a popular lung injury model that specifically depletes airway Clara cells. Naphthalene is a volatile, polycyclic aromatic hydrocarbon that can form a flammable vapor. A method of delivery is by intraperitoneal injection where it reaches the lungs through the blood stream. Sex, age and strain of mice can effect differences in the response to naphthalene injury (Fanucchi et al., 1997; Lawson et al., 2002; Van Winkle et al., 2002). Clara cells in the airways predominantly express the

cytochrome P450 enzyme CYP2F2 which metabolizes naphthalene into cytotoxic dihydrodiol intermediates leading to cell death (Shultz et al., 1999). Cell toxicity occurs within hours following naphthalene injection. The few Clara cells that do not express CYP2F2 (variant cells) are resistant to naphthalene injury and are thought to regenerate the airway epithelium (Hong et al., 2001). Ciliated cells have also been shown to dedifferentiate into a squamous phenotype, internalize and disassemble the cilia and spread under the dying Clara cells to protect and maintain the permeability barrier of the epithelium (Lawson et al., 2002; Park et al., 2006; Van Winkle et al., 1999). Cell proliferation can occur as early as 2 days after injury and by 3-4 weeks, the airway epithelium is completely regenerated (Stripp et al., 1995).

Although it appears that different epithelial cells may be involved in Clara cell renewal, the specific cell type may depend on the region in the lung. Basal cells give rise to Clara cells in the large airways (trachea and main bronchi) (Hong et al., 2004a; Hong et al., 2004b). In the distal airways, ciliated cells, bronchioalveolar stem cells (which may be related to the variant Clara cells) have all been associated with Clara cell renewal (Giangreco et al., 2002; Hong et al., 2001; Kim et al., 2005; Reynolds et al., 2000b).

1.4.1.2 Inhaled oxidants and bleomycin injury

Inhalation of oxidants are popular models to specifically destroy ciliated cells and Type I alveolar cells. Oxidants such as NO₂ and ozone are common oxidants used in lung injury models to destroy large airway cells and ciliated cells (Barth and Muller, 1999; Evans et al., 1975; Evans et al., 1986). Administration (transtracheal injection) of bleomycin, a chemotherapy agent, selectively depletes Type I cells and induce fibrosis

(Aso et al., 1976; Nozaki et al., 2000). Select populations of Type II cells are thought to be important in the repair of bleomycin injury (Aso et al., 1976; Daly et al., 1998).

1.4.1.3 Sulfur Oxide

Sulfur oxide (SO₂) is a non-specific injury model used to assess global repair of the large airway epithelium. Borthwick (Borthwick et al., 2001) used SO₂ inhalation in mice to destroy most of the pseudostratified epithelium in the trachea and saw complete regeneration within 7 days following injury.

1.4.1.4 Enzyme-induced emphysema

Elastase or trypsin delivered transtracheally is a potent inducer of emphysematous changes in rodents. An increase in lung volume as well as the appearance of larger and fewer alveoli resulting in diminished alveolar surface area are typical changes that occur. Studies have used this model to study alveolar regeneration by growth factors, hyaluronic acid, vitamin A and surfactants (Cantor et al., 1997; Hind and Maden, 2004; Massaro and Massaro, 1997; Otto-Verberne et al., 1992; Plantier et al., 2007; Shigemura et al., 2005).

1.4.1.5 Radiation injury models

Studies have suggested that bone marrow cells can contribute to epithelial cell lineages in the lung (Kotton et al., 2001; Krause et al., 2001; Macpherson et al., 2005b; Wong et al., 2007), although the mechanism remains controversial. Total body irradiation is a mode of injury often used to study the role of endogenous BMC to lung epithelial cell lineages (Grove et al., 2002; Theise et al., 2002). While there appears to be a correlation

between the extent of irradiation and pulmonary engraftment of bone marrow-derived cells (Herzog et al., 2006), others have shown lung engraftment and phenotypic conversion with minimal irradiation (Jiang et al., 2002a).

To study the role of bone marrow in lung repair, bone marrow-derived mesenchymal stem cells (MSC) from transgenic mice that constitutively express GFP driven by the beta-actin promoter were injected intravenously into bleomycin-injured mice. The GFP⁺ MSC could indeed engraft in the lung and give rise to Type I and II cells, endothelial cells, fibroblasts and bronchiolar epithelial cells (Rojas et al., 2005). Suppression of the host bone marrow with busulfan resulted in worsening of the lung repair process further strengthening the role of endogenous marrow in limiting lung injury. Stem cell transplantation of a single highly purified HSC (Fr25lin⁻) after lethal irradiation gave rise to bronchiolar and alveolar epithelial cells (Krause et al., 2001). Transplantation of a subtype of MSC termed multipotent adult progenitor cell (MAPC) were also able to differentiate into alveolar cells following minimal whole body irradiation (Jiang et al., 2002a).

Bone marrow cell contributions to the lung have also been identified in human allograft studies. Suratt (Suratt et al., 2003) analyzed lung samples from diagnostic lung biopsy or autopsy from two female subjects who had previously received sex-mismatched hematopoietic stem cell transplantation and found donor-derived epithelium (2.5-8.0%) and endothelium (37.5-42.3%). Similarly, Mattson (Mattsson et al., 2004) found donor-derived epithelial cells (2% and 6%) from autopsy specimens of two female subjects after allogeneic hematopoietic stem cell transplantation. Another study by Albera echoed these results (Albera et al., 2005). However, not all studies found bone

marrow-derived lung cells. Davies (Davies et al., 2002) analyzed nasal brush samples from female subjects who had received male bone marrow transplant 15 years prior and found a median of 2.5% donor derived (Y-chromosome positive) cells however none were cytokeratin positive.

1.4.1.6 Other models of lung injury

Like the liver, the lung has the capacity to undergo compensatory growth following resection therefore pneumonectomy is a classical model employed to assess the mechanism of regeneration (Park et al., 2006; Sakamaki et al., 2002; Shigemura et al., 2006). Heterotopic tracheal transplantation is another useful model to study the cellular and extracellular matrix changes involved in BO syndrome (Kallio et al., 2000; Neuringer et al., 2002; Suga et al., 2000). This model has also been used to study stem cell-mediated airway regeneration (Gomperts et al., 2006; Macpherson et al., 2005b; Neuringer et al., 2002).

1.5 Stem cells

1.5.1 Developmental hierarchy of the stem cell compartment

1.5.1.1 Stem cells

A stem cell is defined as an undifferentiated cell capable of long-term self-renewal and multi-lineage differentiation potential. Under homeostatic conditions, stem cells are slow-cycling and/or quiescent. Upon stimulation such as during tissue injury, stem cells can give rise to daughter cells called transient amplifying (TA) cells that generates sufficient specialized progenies for tissue maintenance.

Stem cells can undergo symmetric cell division whereby a stem cell divides and gives rise to 2 daughter stem cells identical to itself. Stem cells can also undergo asymmetric cell division in which one of the daughter cell loses some of the stem cell characteristics of the parent cell and begins the process of cell lineage commitment.

1.5.1.2 Progenitor cells and transient amplifying cells

A progenitor cell has more limited differentiation potential but may still be multipotent. Progenitor cells may have self-renewal capacity but, unlike stem cells, progenitor cells do not have unlimited self-renewal capabilities.

Transient amplifying cells are intermediary cells between a stem cell and its final differentiated progeny. These cells are highly proliferative and can give rise to one or more differentiated cell type. It remains unclear whether TA cells are the same as progenitor cells.

1.5.2 Stem cell characteristics

1.5.2.1 Self-renewal

Self-renewal is defined as the ability of a cell to give rise to daughter cells that retain the properties of the parent cell. The process has been rigorously studied in HSC and involves several intrinsic signals such as the proto-oncogene Bmi-1 (Park et al., 2003) which encodes for polycomb group complex proteins that repress genes involved in differentiation, the transcription factor HoxB4 (Sauvageau et al., 1995), and signaling molecules Notch (Varnum-Finney et al., 2000), Sonic hedgehog (Bhardwaj et al., 2001) and Wnt (Austin et al., 1997; Nemeth and Bodine, 2007; Reya et al., 2003). Activation

of Wnt signaling is also important in MSC proliferation and mesenchymal lineage specification (Etheridge et al., 2004).

Telomerase activity is associated with increased cell proliferation. Increased telomerase activity has been associated with HSC self-renewal potential (Morrison et al., 1996; Schuller et al., 2007). Telomeres are regions of highly repetitive DNA found at the end of linear chromosomes that can not be replicated by the DNA polymerase complex. Therefore, with every replication of the chromosome, it will lose a small portion of the genetic information. Telomerase is a subgroup of reverse transcriptase enzymes known as TERT that can extend telomeres. The decrease in telomerase activity has been linked with aging (Hornsby, 2007) while an increase in telomerase activity has been associated with cancer (Deng and Chang, 2007).

1.5.2.2 Development of diverse phenotypes

Cells can develop and mature through a lineage program through many pathways. The most common pathway is differentiation of an immature cell such as a progenitor cell into a cell with more specialized function(s) and greater level of complexity. The process may require several rounds of cell division going through several intermediary cell types before becoming the fully mature cell type. Transdifferentiation occurs when a cell becomes another cell type outside of its already established differentiation pathway. One interesting example of transdifferentiation is from a study in which corneal epithelial cells, after transplanting onto the dermis of embryonic skin, resulted in the generation of hair follicles and associated stem cells (Pearson et al., 2005). Another example is the generation of functional cardiomyocytes from germline stem cells (Guan et al., 2007).

Dedifferentiation is the process by which a terminally differentiated cell reverts to a cell of an earlier developmental stage. An example of this is the conversion of mature vascular smooth muscle cells into an immature cell phenotype capable of self-renewal (Wong et al., 2005). Cell fusion may be another pathway by which a cell acquires the phenotype of another cell. Bone marrow cells have been shown to give rise to lung epithelial, cardiomyocyte, and muscle cells by cell fusion with resident tissue cells (Nygren et al., 2004; Shi et al., 2004; Spees et al., 2003; Terada et al., 2002; Wong et al., 2007).

Epigenetic regulation plays a role in stem cell differentiation. There are three major epigenetic mechanisms that have been shown to control stem cell differentiation: 1. DNA methylation, 2. Histone modification, 3. Non-coding RNA-mediated regulatory events. DNA methylation occurs mainly at CpG dinucleotides where DNA methyltransferases Dnmt3a and Dnmt5b adds methyl groups to the cytosine residue just 5' to the guanine residue. Hypermethylation is associated with gene silencing whereas hypomethylation is associated with gene expression. DNA methylation is primarily associated with parental-specific imprinting during gametogenesis (Kaneda et al., 2004) and silencing of genes on the inactivated X-chromosome (Maatouk et al., 2006) but it also involved in cell differentiation (Takizawa et al., 2001).

Post-translational modification of histones by acetylation of lysine residues on the amino end of the chromatin is another mechanism that controls cells differentiation (Cheung et al., 2000). Lysine acetylation by histone acetyltransferases (HATs) is associated with gene activation, whereas deacetylation with histone deacetylases (HDACs) is associated with gene inactivation. Histone methylation however is associated

with transcriptional repression whereas demethylation is associated with transcriptional activation.

Finally, post-transcriptional gene regulation by small non-coding RNAs or microRNAs is a newly emerging epigenetic mechanism. These RNAs are distinct from siRNA (small interfering RNA) in that they are transcribed products produced from RNA-coding genes. Like siRNAs, microRNAs are processed in the nucleus into hairpin RNAs of 70-100 nucleotides by double stranded RNA-specific ribonuclease called Droscha (He and Hannon, 2004). These hairpin RNAs are transported to the cytoplasm and digested by another ribonuclease called Dicer resulting in a 19-23mer microRNA bound to a complex resembling the RNA-induced Silencing Complex (RISC). These microRNAs can then bind to mRNA sequences that share some complementary sequences to the mRNA preventing the mRNA from being translated. The outcome is reduced expression of a particular group of genes. MicroRNAs have been shown to be involved in ESC and HSC development (Chen et al., 2004; Hatfield et al., 2005; Houbaviy et al., 2003).

1.5.2.3 Plasticity

Plasticity is defined as the ability of a cell to produce progenies that cross lineage boundaries. There is a hierarchy of stem cell plasticity. Totipotent cells can give rise to all cells in the body as well as the placenta and embryonic cells (ESC and germ cells). These cells are considered to be the most primitive stem cells and exists in the first few cell divisions of the fertilized egg. Pluripotent stem cells are descendents of totipotent stem cells and can give rise to cells of all germ layers. These cells are found in the inner

cell mass of the blastocyte and while ESC are the best known, there are others such as primordial germ cells, and possibly certain adult stem cells such as bone marrow-derived very small embryonic-like stem cells. Multipotent stem cells have more restricted differentiation potential than pluripotent stem cells. As the progeny become more lineage-committed or determined, the ability of the cell to give rise to various cell lineages becomes restricted down to the unipotent progenitor cell which can only give rise to one particular cell phenotype e.g. Type II alveolar cell can become Type I alveolar cell.

It has long been felt that multipotent stem cells can only give rise to cells within a lineage eg. HSC can only give rise to blood cell lineages. However, there is growing evidence that cells that were once thought to be multipotent such as HSC and MSC can give rise to cells outside of their traditional lineage commitment. For example, MSC can give rise to endodermal (lung), mesodermal (heart) and ectodermal (neural) tissue.

1.5.3 Types of stem cells

1.5.3.1 Embryonic stem cells, primordial germ cells

Embryonic stem cells (ESC) are pluripotent cells derived from the inner cell mass of the blastocyst within the first 5-7 days after an egg is fertilized by sperm (Thomson et al., 1998). In other words, they can produce cells from all three germ layers: endoderm, ectoderm and mesoderm. These cells transiently exist in the developing embryo. These cells can be isolated and cultured ex vivo as cell aggregates called embryoid bodies and maintained in an undifferentiated state indefinitely through artificial conditions using leukemia inhibitory factor containing media and on feeder layers. A recent study showed

that ESC can also be cultured as single cell suspensions in an undifferentiated state using a Rho-associated kinase inhibitor (Watanabe et al., 2007). ESC express several markers associated with self-renewal and pluripotency namely OCT-4, Rex-1, Nanog, SSEA-1 (mouse) and SSEA3/4 (humans) and the presence of alkaline phosphatase and telomerase activity (Armstrong et al., 2005; Babaie et al., 2007; Pan and Thomson, 2007; Wobus et al., 1984). ESC has been shown to differentiate into cells of neuronal (Bibel et al., 2004), cardiac (Guo et al., 2006; Reppel et al., 2004), retinal (Tabata et al., 2004), hepatic (Shirahashi et al., 2004), and lung epithelial (Samadikuchaksaraei et al., 2006) cells.

Primordial germ cells (PGC) are derived from the epiblast stem cells and give rise to oocyte and sperm (Molyneaux and Wylie, 2004). These cells are pluripotent stem cells that can also give rise to cells of all three germ layers (Turnpenny et al., 2003) and give rise to embryonic stem cells with in vitro manipulation of growth conditions (Matsui et al., 1992). PGC-like cells have been found in the mouse bone marrow and shown to give rise to oocyte (Johnson et al., 2005) and spermatogonia-like cells (Nayernia et al., 2006).

1.5.3.2 Adult stem cells

Adult stem cells are multipotent in that they have less self-renewal capacity and their potential for differentiation was believed to be limited to their tissue of origin. However, recent evidence have suggested that certain adult stem cells may have pluripotent characteristics (Devine et al., 2003; Jiang et al., 2002a; Krause et al., 2001; Kucia et al., 2006a).

(i) Hematopoietic stem cells

Drs. James Till and Ernest McCulloch found that the bone marrow contained cells that gave rise to multiple colonies of cells which manifested as nodules in the spleen after bone marrow transplantation. The number of the nodules were proportional to the number of bone marrow cells injected. The cells in the spleen were coined the “spleen-colony-forming cells” (McCulloch and Till, 1964; Till and Mc, 1961; Till et al., 1964) and each colony was believed to have arisen from a single stem cell. Later discoveries characterized this stem cell as the hematopoietic stem cell (HSC). HSC have since been the most rigorously studied stem cells. These cells can differentiate into all blood cells including the lymphoid, myeloid, platelet and red blood cell lineages. HSC express specific markers such as c-kit, Sca-1, Flk2, Thy1.1^{low} and CD34^{low} and represent 1 in 10⁴ to 1 in 10⁵ cells of the total bone marrow. HSC are found in the bone marrow and to a lesser extent in the peripheral blood. They are also found in the fetal liver and these HSC have been shown to have greater long-term reconstitution potential than bone marrow-derived HSC (Morrison et al., 1995). Stimulation with granulocyte-colony stimulating factor (GCSF) can promote the proliferation of these cells in the marrow and their mobilization from the marrow into the blood (de Kruijf et al., 2007; Lowenthal et al., 2007). HSC are believed to be derived from a CD133-positive precursor cell (Gehling et al., 2000). HSC have been classified into two types: short-term and long-term repopulating cells (Lanzkron et al., 1999) and can be distinguished by their surface markers $\text{ckit}^+\text{lin}^-\text{Sca-1}^+\text{CD150}^-\text{CD34}^-\text{Flt3}^+$ and $\text{ckit}^+\text{lin}^-\text{Sca-1}^+\text{CD150}^+\text{CD34}^-\text{Flt3}^-$ respectively. Short-term repopulating cells are fast cycling cells with limited marrow reconstitution potential whereas long-term repopulating cells are quiescent cells once

they engraft in the marrow. HSC are closely associated with their bone marrow mesenchymal counterparts and it has been shown that HSC proliferation and mobilization may be regulated by several signaling molecules expressed by the mesenchymal cells such as SDF-1 (Avecilla et al., 2004; Hattori et al., 2001) and ckit ligand (Heissig et al., 2002). Studies have suggested that HSC have the potential to differentiate into various types of tissue cells (Camargo et al., 2003; Lagasse et al., 2000) including those of the lung (Krause et al., 2001; Macpherson et al., 2005b) and thus their potential use in stem cell mediated-tissue regeneration is promising.

(ii) Mesenchymal stem cells

The other type of bone marrow-derived stem cell is the mesenchymal stem cell (MSC). MSC were first discovered as bone marrow plastic-adherent cells with the ability to form bone and cartilage (Friedenstein et al., 1974). Since then, two morphologically distinct MSC populations have been described in early-passaged cells maintained at low density (~3 cells/cm): A large, flat population that is slowly replicating and a small, spindle-like population that rapidly self-renew (RS cells) (Colter et al., 2000). These RS cells were very small (~7 µm in diameter) and had greater capacity for multilineage differentiation (Prockop et al., 2001). In late passages, RS cells cease proliferating while the large flat cells predominate. Serum deprivation of MSC also selects for an Oct-4-expressing progenitor cell population with ESC genetic makeup. However these cells proliferate more slowly than RS cells (Pochampally et al., 2004). MSC have been shown to divide in vitro indefinitely and can differentiate into various somatic cell types such as osteoblasts, chondrocytes, adipocytes (Majumdar et al., 1998; Meirelles Lda and Nardi,

2003), cardiomyocytes (Kawada et al., 2004) and lung (Jiang et al., 2002a; Popov et al., 2007; Rojas et al., 2005).

The recent clarification of the term “mesenchymal stem cells” by the International Society for Cellular Therapy (Horwitz et al., 2005) articulated the definition of MSC as cells that: 1. are fibroblast-like plastic-adherent cells; 2. have the ability to give rise to chondrocytes, osteocytes and adipocytes; 3. express three fundamental surface markers - CD73, CD90 and CD105. MSC have also been shown to express other markers including CD29, CD49, CD44, CD81, CD106, vimentin, STRO-1 (human), VCAM-1 and Type IV collagen (Baddoo et al., 2003; Meirelles Lda and Nardi, 2003; Peister et al., 2004; Tropel et al., 2004).

Multipotent adult progenitor cells (MAPC) were initially discovered as a subpopulation of bone marrow MSC through culturing artifact that morphologically resemble RS cells (8-10 μm in diameter) and have pluripotent characteristics (Jiang et al., 2002a). These cells can give rise to cells of all three germ layer in vitro and contribute to most somatic cells following injection into early blastocysts. The phenotype of cultured MAPC is CD34⁻CD45⁻CD44⁻c-kit⁻ and MHC class I and II negative, but they do express SSEA-1, Oct4, Rex-1 and low levels of Sca-1 and CD90. These cells also have enhanced telomerase activity. MAPC have recently been found in the hair follicle (Yu et al., 2006), muscle and brain (Jiang et al., 2002b).

(iii) Very small embryonic-like stem cells

Recently, a novel embryonic-like stem cell has been isolated from the adult mouse bone marrow and termed very small embryonic-like stem cell (VSEL) (Kucia et

al., 2006a). As the name implies, these cells are very small, about 2-4 μm , and have a large nuclei with very little cytoplasm. Within the bone marrow mononuclear fraction, only $\sim 0.02\%$ of these cells are VSEL. Phenotypically, VSEL express Sca-1 but are negative for lineage markers and CD45. These cells also express SSEA-1, OCT-4, Nanog and Rex-1, all markers of pluripotent ESC and can be induced to differentiate into cardiomyocyte, neuronal and pancreatic cells in vitro. Similarly, these VSEL cells were also found in human cord blood and were highly enriched in the $\text{CXCR4}^+\text{CD133}^+\text{lin}^-$ $\text{CD34}^+\text{CD45}^-$ fraction, 0.01% of mononuclear cells (Kucia et al., 2007a). Like their bone marrow counterpart, these cord-blood-derived VSEL also express pluripotent stem cell markers SSEA-4, OCT-4, Nanog and Rex-1. The authors speculate that these VSEL may be directly derived from primordial germ cells that are deposited in developing organs during gastrulation and persists into adulthood (Ratajczak et al., 2007). These cells may serve as a source or “mother lineage” for all somatic cells in the adult body during cell/tissue repair (Kucia et al., 2007b).

(iv) Bone marrow-derived tissue-committed stem cells

Several groups have identified tissue-specific stem/progenitor cells in the bone marrow. Most of these cells express a common chemokine receptor CXCR4 and seem to respond to elevated levels of SDF-1 induced by remote tissue injury (Gomperts et al., 2006; Kucia et al., 2005a). Cells enriched in early neural marker genes (β -III-tubulin, Nestin, GFAP and NeuN) are present in the marrow and are mobilized to the peripheral blood following stroke (Kucia et al., 2006b). Similarly, bone marrow cells enriched for early cardiac markers (Nkx2.5/Csx, GATA-4, MEF2C) are mobilized to the peripheral

blood following myocardial infarction (Kucia et al., 2004a). Liver progenitor cells expressing cytokeratin-19 (K19) and fetoprotein as well as muscle progenitors expressing Myf-5 and Myo-D have also been found in the mouse and human marrow (Kucia et al., 2004b). These cells reside in the nonadherent, mononuclear fraction and are CXCR4⁺Sca-1⁺lin⁻CD45⁻ in mice and CXCR4⁺CD34⁺CD133⁺CD45⁻ in humans. Airway progenitor cells expressing cytokeratin-5 (K5) and CXCR4 have also been found in the bone marrow and peripheral blood of mice (Gomperts et al., 2006). These cells can respond to SDF-1 upregulation in the airway epithelium in a model of heterotopic tracheal transplantation and contribute to normal pseudostratified regeneration of the airway epithelium. Whether or not these tissue-committed cells that reside in the bone marrow are MSC or HSC-derived remains unknown; however micro-SAGE analysis of a single-cell derived colony of undifferentiated MSC show that these cells can express an array of tissue-specific genes (Tremain et al., 2001).

(v) Inducible pluripotent stem cells

Recently, independent laboratories have found a method to genetically reprogram somatic cells into pluripotent stem cells termed inducible pluripotent stem cells (iPS). Four transcription factors, Oct-4, Sox2, c-myc and klf4, were deemed essential to confer pluripotent status (Takahashi and Yamanaka, 2006; Wernig et al., 2007). Retroviral transduction of these four transcription factors induced fibroblasts into pluripotent embryonic stem cell-like cells that gave rise to cells of all three germ layers following injection into nude mice and contributed to embryonic development following injection into the blastocyst (Takahashi and Yamanaka, 2006). These four transcription factors

may induce epigenetic reversion in the somatic cell into an ESC-state (Wernig et al., 2007). Interestingly, Nanog, a transcription factor classically associated with ESC self-renewal (Ivanova et al., 2006) was suppressed and was not required for pluripotency of iPS cells.

(vi) Local tissue stem cells

Recent publications have identified several tissue-localized stem cells such as those of the heart (Laflamme et al., 2002), eye (Tropepe et al., 2000) and lung (Giangreco et al., 2002; Hong et al., 2001; Hong et al., 2004a; Kim et al., 2005; Majka et al., 2005) that play a role in local tissue repair. *See Section 1.2 for lung stem cells.*

1.5.3.3 Cord blood stem cells

Cord blood-derived stem cells have been identified and shown to have tissue regeneration potential. Cord blood stem cells can give rise to lung (Berger et al., 2006; Sueblinvong et al., 2007), gastrointestinal (Ishikawa et al., 2004) and skin (Dai et al., 2007; Kamolz et al., 2006) epithelial cells.

1.5.4 Stem cell niches

The microenvironment of a stem cell can have a profound impact on the fate of the cell. This microenvironment is called the stem cell niche. The niche is composed of extracellular matrix and niche cells that serve to anchor and nurture the stem cells. The niche is important in sequestering stem cells from stimuli and produces factors that protect against apoptosis and differentiation that might otherwise deplete the stem cell

reservoir (Moore and Lemischka, 2006). It also controls stem cell division, protecting against excessive proliferation and acquiring mutations that may lead to cancer. In several systems such as the hematopoietic (Zhang et al., 2003), intestinal (He et al., 2005), hair follicular (Blanpain et al., 2004; Ito et al., 2007) and lung epithelial (Borthwick et al., 2001; Giangreco et al., 2002), the niche maintains the stem cells in a quiescent state preventing cell proliferation as evidenced by label-retention (Chan and Gargett, 2006; Hong et al., 2001; Wei et al., 1995). Transient signals such as chemokines/cytokines upregulated during injury may stimulate stem cells to divide and produce transient amplifying (TA) cells that are committed to produce mature specialized cells. To illustrate the importance of the stem cell niche, an elegant study by Zhang (Zhang et al., 2003) showed that transgenic mice with conditional inactivation of the bone morphogenic protein (BMP) receptor *Bmpr1A* had increased HSC niche cells (osteoblasts) which in turn increased the number of HSC. On the contrary, depleting the niche cells resulted in the loss of HSC stores and hematopoiesis (Visnjic et al., 2004). Furthermore, deregulation of the mammary epithelial stem cell niche can result in abnormal proliferation of the stem cells that may lead to breast cancer (Chepko et al., 2005). Therefore the stem cell niche has an important functional role in maintaining stem cell homeostasis, allowing the stem cells to undergo self-renewal while supporting ongoing tissue regeneration (He et al., 2005).

Soluble mediators in the stem cell niche compartment have been well studied in the hematopoietic, intestinal crypt and hair follicle systems. Known signals involved in regulating stem cell homeostasis in the niches are Wnt, BMP and Sonic hedgehog. Wnt signaling has been shown to play a role in promoting stem cell proliferation in the skin,

intestines and HSC compartment in the marrow (Gregorieff et al., 2005; He et al., 2004; Ito et al., 2007; Reya et al., 2003). Wnt signaling involves binding of Wnt to its receptor Frizzled which ultimately inhibits β -catenin degradation. β -Catenin can translocate to the nucleus and bind with transcription factors Lef/TCF resulting in cell proliferation. The Wnt pathway inhibitors Dkk, Wif, sFRP are reportedly predominantly expressed and secreted by the hair follicular stem cell niche which inhibits stem cell growth (Tumbar et al., 2004). Abnormal accumulation of nuclear β -catenin in granulocyte-macrophage progenitor cells seen in some patients with chronic myeloid leukemia has been associated with enhanced self-renewal activity in these cells (Jamieson et al., 2004).

Bone morphogenic protein belongs to the TGF- β superfamily and is reported to inhibit stem cell growth by repressing nuclear β -catenin accumulation. BMP-2/4 binds to Bmpr1A and Bmpr1B which in turn activates the Smad proteins (1,5,8 and 4) to repress gene transcription. BMP-4 is expressed in mesenchymal cells in the intestinal stem cell niche and is involved in maintaining intestinal stem cell quiescence. Conditional knockout of the receptor for BMP, Bmpr1a in crypt cells leads to proliferation of intestinal stem cells (He et al., 2004). BMPs have also been shown to inhibit human brain tumour-initiating cell growth (Piccirillo et al., 2006). Noggin is potent inhibitor of BMP signaling known to sustain undifferentiated proliferation of human ESC (Xu et al., 2005) and is expressed by mesenchymal cells in the intestinal stem cell niche (Haramis et al., 2004).

Hedgehog signaling is involved in stem/progenitor cell proliferation (Bhardwaj et al., 2001; Watkins et al., 2003). Hedgehog signaling is important in branching morphogenesis in the developing lung (Bellusci et al., 1997) and is involved in airway

epithelial progenitor cell maintenance following injury and small-cell lung cancer development (Watkins et al., 2003). Elevated expression of Shh, a member of the hedgehog signaling ligand family and Gli, a transcriptional target of hedgehog signaling, was observed in the epithelial compartment 72 hours after naphthalene injury which diminished with the appearance of differentiated neuroendocrine cells. Similarly, elevated Shh ligand was found to be involved in inhibiting progenitor cell growth in small-cell lung carcinoma as blockade of the hedgehog pathway led to elevated expression of BMP4.

Proteins involved in anchoring stem cells to their niche are also important in maintaining stem cell fate. These proteins keep the stem cells close to their niche and within the range of niche signals. N-cadherin and β -catenin are two adherens junction molecules that have been shown to anchor HSC to in its niche (Zhang et al., 2003). While the monomeric form of β -catenin binds to Tcf in the nucleus (Gottardi and Gumbiner, 2004) to activate cell cycle-related genes such as c-Myc (He et al., 1998) and cyclin D1 (Tetsu and McCormick, 1999), the heterodimeric form of β -catenin is involved in anchoring stem cells in their niche (Li and Xie, 2005). Tie2 and angiopoietin-1 are involved in anchoring HSC to the marrow niche and maintain HSC quiescence (Taichman et al., 2000).

Mouse embryonic fibroblasts are often used to maintain the stem cell phenotype *in vitro*. Mouse embryonic fibroblasts produce fibroblast growth factor (FGF)-2 and large amounts of BMP4 which promotes ESC self-renewal and prevent ESC differentiation (Greber et al., 2007). Intestinal epithelial cells cocultured with mouse embryonic fibroblasts, were found to “reprogram” these cells into an “embryonic-like” stem cell

phenotype with proliferative and multilineage differentiation capabilities (Wiese et al., 2006).

1.5.5 Methods to enrich for, or identify, stem cells

There are many “stem cell” assays designed to identify putative stem/progenitor cells that vary depending on the isolated cells. For example, to study embryonic stem cell pluripotency in vitro, ES cells are induced to differentiate by removal/addition of growth factors into the culture media. To study the “stemness” of HSC, a single HSC is transplanted into a lethally irradiated recipient and assessed for chimeric bone marrow reconstitution and tissue repopulation. Some stem cell assays that are pertinent to the understanding of the contents of this thesis are discussed below.

1.5.5.1 Hoechst efflux dye Assay

The Hoechst 33342 efflux dye assay was found to be a method to isolate a population of hematopoietic cells termed side population (SP) cells. These SP cells were thought to be enriched for primitive stem cells (Goodell et al., 1996; Scharenberg et al., 2002; Zhou et al., 2001). SP cells express high levels of the ABCG2 multidrug transporter which can efflux the Hoechst 33342 dye. SP cells expressing low Hoechst fluorescence are characterized to have higher levels of self-renewal and plasticity. Furthermore, these cells have greater repopulation potential (Uchida et al., 2001). Although seemingly simple, this assay requires meticulous optimization of the staining procedure and dye concentration to capture the dim Hoechst red and blue emission tail of the SP (Sales-Pardo et al., 2006). Therefore isolation of SP cells by this method can be a challenge and vary from lab to lab.

1.5.5.2 Differentiation Assays

To ascertain the second requirement to meet the definition of a stem cell, that is multilineage differentiation potential, stem cells can be subjected to in vitro manipulation to induce differentiation along certain lineages. Since in vitro cultivation allows for expansion of these cells, differentiation culture media supplemented with specific growth factors are often used to assess the plasticity of these cells. For example, MSC have been shown to give rise to osteoblasts, chondrocytes and adipocytes. To determine osteogenic potential, MSC are cultured in media supplemented with ascorbic acid, β -glycerophosphate and dexamethasone for 2-3 weeks (Pereira et al., 1995; Phinney et al., 1999). Studies have developed protocols involving BMP-2 for rat and human MSC differentiation into an osteogenic lineage (Hanada et al., 1997; Sekiya et al., 2005). To determine chondrogenic and adipogenic differentiation in vitro, MSC are typically cultured in media containing TGF (Mackay et al., 1998; Sekiya et al., 2002) and a hormonal cocktail of dexamethasone, isobutyl methyl xanthine (IBMX) and indomethacin (Sekiya et al., 2004).

In vitro cultures have been used to test the differentiation potential of lung stem cells. The challenge has been to devise a culture condition that mimics the in vivo microenvironment of the cell that would allow reproducible differentiation of stem cells into secretory, ciliated and non-ciliated cells. Studies have shown using genetically marked tracheal cells isolated from *Rosa26* mice (these mice ubiquitously express *lacZ*) mixed with feeder cells resulted in colonies of cells composed of ciliated, goblet cells and columnar epithelial cells (Inayama et al., 1989; Schoch et al., 2004). Putative BASC in

the BADJ of the mouse lung (Kim et al., 2005) have also been tested through in vitro by specific media-induction for their multi-epithelial differentiation potential (as discussed above). Others have used air liquid interface models to assess the differentiation potential of stem cells into “epithelial-like” cells (Spees et al., 2003; Wang et al., 2005).

In vivo assessment of multilineage differentiation is the ideal proof that stem cells can differentiate along several cell lineages. This method avoids the possibility of a cultivation artifact that may render the cells susceptible to gene alterations in the presence of a stimuli/growth factor. In vivo the stem cell phenotype is highly regulated by its’ biological niche and therefore removing the cells from their niche, such as in vitro cultivation, may change the phenotype of the cells.

In this thesis, differentiation assays to induce epithelial differentiation, mesenchymal stem cell differentiation and hematopoietic stem cell differentiation will be discussed in further detail in Chapters 3 and 4.

1.5.5.3 In vitro colony-forming unit assay

There are various methods to study the process of “self-renewal” in vitro. The classical method of measuring self-renewal in adherent cells such as MSC is the colony formation unit (CFU)-fibroblast assay. In this assay cells are seeded at specific cell densities and assessed for the formation of colonies (identified as >50 cells per colony) after several weeks (Friedenstein et al., 1974; Peister et al., 2004; Pereira et al., 1995). The fraction of cells in the primary colony that can give rise to secondary or tertiary colonies suggests that there are at least a stem cell within the colony that has self-renewal potential. However, evidence suggests that the stem cell population can be quite

heterogenous (Bhatia et al., 1998; Colter et al., 2001) and therefore this assay can not distinguish the cell origin of the progenies. The best method of studying self-renewal and plasticity is the single cell clonal assay where 1 single cell is plated and assessed for colony formation (Franken et al., 2006; Kim et al., 2005). Any progeny that arise from a single cell is evidence of multi-potentiality if the progeny can give rise to multiple cell lineages in differentiation assays.

1.5.5.4 In vivo reconstitution assay

In vitro “self-renewal” or clonogenic assays are subject to artifactual predisposition as mentioned earlier. A more definitive way to study the self-renewing and repopulating potential of a stem/progenitor cell is to use an in vivo assay. The first evidence of self-renewal in vivo was discovered in 1967 by Drs. James Till and Ernest McCulloch where large mixed colonies of cells derived from injected bone marrow were identifiable in mouse spleen following irradiation (Till and Mc, 1961). These cells gave rise to multiple cell lineages (Wu et al., 1967) and can give rise to progeny that generated more colony forming units (CFU)-spleen (Siminovitch et al., 1963; Till et al., 1964). To date, bone marrow transplantation is the most commonly used in vivo reconstitution/repopulation assay to assess HSC characteristics. Transplantation of a single stem/progenitor cell (Ema et al., 2006; Smith et al., 1991) or a limiting number of cells (Bhatia et al., 1998; Larochelle et al., 1996; Spangrude et al., 1995) into a lethally irradiated recipient can determine whether the cell has self-renewal and reconstitution potential. To identify donor cells, the cells are usually marked with a commercially available dye (Krause et al., 2001) or genetically marked or transduced cells

(Gammaitoni et al., 2006; Hacein-Bey-Abina et al., 2002). Additional tests of the “stemness” of a cell can be assessed by serial transplantation experiments (Gammaitoni et al., 2006; Ito et al., 2003b) where isolation of the donor-derived progeny would theoretically have the same reconstituting potential as the parent cell.

1.6 Extrapulmonary stem cells and regeneration

A number of reports have suggested that several bone marrow cell populations may potentially localize to and acquire phenotypic and functional characteristics of mature tissue cells such as the heart, liver, brain, skin and lung (Abbott et al., 2004; Abe et al., 2003; Borue et al., 2004; Keilhoff et al., 2006; Kocher et al., 2001; Kotton et al., 2001; Krause et al., 2001; Okamoto et al., 2002b). These studies have challenged the view that tissue-specific stem cells are the sole cell types involved in tissue repair. The results have been conflicting in that different groups report different frequencies of engraftment and different cell types formed as a result of BMC delivery or transplantation. It is likely that differences in technical factors such as detection methods, injury models, or subpopulation of BMC delivered may explain the discrepant results. For example, the dose of irradiation may determine the level of marrow-derived cells in the lung epithelium in bone marrow transplant models (Herzog et al., 2006).

1.6.1 Supporting evidence of engraftment and “epithelialization”

Human and animal models of disease/injury have shown bone marrow-derived epithelium in various lung injuries such as bone marrow transplantation (Abe et al., 2003; Krause et al., 2001; Rojas et al., 2005; Suratt et al., 2003), bleomycin-induced fibrosis

(Hashimoto et al., 2004; Ishii et al., 2005; Phillips et al., 2004), radiation pneumonitis (Epperly et al., 2003; Theise et al., 2002), hypoxia-induced pulmonary hypertension (Davie et al., 2004), elastase-induced emphysema (Ishizawa et al., 2004a), and human lung transplants (Bittmann et al., 2001; Kleeberger et al., 2003). While most studies have used bone marrow transplant models, to study the contribution of circulating cells in repair of lung injury, an elegant experiment was done using parabiotic mice generated by surgically joining GFP mouse with a wild-type littermate (Abe et al., 2004). These mice developed a common circulation (~50% GFP cells in the blood of WT mouse) after 2 weeks of surgery. Injury to the lung in the wild-type littermate resulted in GFP+ monocytes/macrophages, fibroblasts, and Type I epithelial cells in the lungs.

There is evidence of repopulation of human pulmonary epithelium by bone marrow cells. Lung biopsies from female recipients that received lung or bone marrow transplantation from male donors showed bone marrow/circulation-derived lung epithelial cells up to 24% (Albera et al., 2005; Kleeberger et al., 2003; Mattsson et al., 2004; Suratt et al., 2003).

Hematopoietic stem cells (HSC) have been implicated in lung regeneration. In 2001, Krause (Krause et al., 2001) were the first to show that HSC transplantation of a single highly purified Fr25lin⁻ (fractionated and lineage depleted) cell resulted in multi-organ (liver, lung, GI and skin) engraftment of the progeny of these cells. Up to 20% of the engrafted donor cells became Type II alveolar cells and 4% were found in the bronchiolar epithelium (detected by Y-chromosome FISH and surfactant B mRNA).

Bone marrow-derived stromal cells have also been implicated in lung regeneration. Although no characterization was done to confirm stromal cell-phenotype

or purity of the cells, Kotton (Kotton et al., 2001) demonstrated that 7-day plastic-adherent BMC became Type I alveolar cells expressing both the genetically marked β -galactosidase protein and Type I marker T1 α in a bleomycin-induced lung injury model. No Type II cells were found. Ortiz (Ortiz et al., 2003) used negative selection methods (anti-CD11b, anti-CD34, anti-CD45) to isolate and inject “MSC” into bleomycin-injured mice and found these cells ameliorated fibrosis. Again, whether or not the immuno-depleted cells were truly MSC was not confirmed.

Bone marrow-derived circulating fibrocytes have been implicated in the pathogenesis of lung diseases. Epperly (Epperly et al., 2003) found donor-derived BMC (GFP⁺, Y⁺) in fibrotic lesions in a model of radiation pulmonary fibrosis. Similarly, Ishii (Ishii et al., 2005) found Collagen Type I⁺, CD45⁺ donor derived (GFP⁺) cells in fibrotic lesions after bleomycin administration. Phillips (Phillips et al., 2004) demonstrated the role of SDF-1 (CXCL12) in recruiting circulating fibrocytes to the lung. Neutralizing antibodies to SDF-1 were found to inhibit recruitment of fibrocytes and attenuated lung fibrosis.

Bone marrow side population (SP) cells also contribute to lung cell lineages in vivo. In a bone marrow transplant model, MacPherson (Macpherson et al., 2005a) found 0.6-1.5% engraftment of male donor SP cells in the airways of mice. While a large proportion of donor-derived cells expressed cytokeratin (60.2%), 32% of the donor cells expressed both cytokeratin and CD45 suggesting hematopoietic lineage-derived epithelium. Since SP are highly enriched in the CD45⁺ population, the authors speculated that CD45 expression may have been lost in the remaining proportion of the engrafted donor cells. Another group identified Type I cells, fibroblasts, monocytes and

macrophages in the lungs but not Type II or bronchiolar cells after transplanting whole marrow or SP cells (Abe et al., 2003).

ESC have been suggested as the Holy Grail for future regenerative medicine because these cells have pluripotent capabilities. ESC can differentiate into lung epithelial cells in vitro (Ali et al., 2002; Samadikuchaksaraei et al., 2006; Wang et al., 2007). However, ethical concerns that surround the use of these cells impedes further use of ESC for therapy. In addition, transplanting undifferentiated ESC can form teratomas because of their pluripotent nature.

Antagonizing evidence of transdifferentiation and engraftment

Not all studies have found chimerism or lung engraftment with adult BMC. In one human study, lung biopsies from sex-mismatched lung transplant recipients were analyzed and found to contain no recipient derived lung cells (Bittmann et al., 2001). Zander (Zander et al., 2005) found minimal engraftment of Type II alveolar cells (0%-0.553%) by bone marrow-derived stem cells in transplanted human lungs. Lower levels of Type II alveolar cells (1.75%) were found to be donor-derived in patients that had received allogeneic hematopoietic cell transplantation (Zander et al., 2006). Loi (Loi et al., 2006) performed bone marrow transplantation of wild-type marrow into CFTR knockout mice and found rare (0.025%) donor cell engraftment in the lung, of which 0.01% expressed CFTR. Assessment of nasal brushings from 6 patients who received bone marrow transplantation showed significant engraftment of donor-derived cells but none were epithelial-like cells (Davies et al., 2002).

Animal models using transgenic mice have also demonstrated lack or limited contribution of the marrow/circulation in lung epithelial cell lineages. Perhaps contrary to expectations raised by his earlier paper, Kotton (Kotton et al., 2005b), used transgenic animals expressing GFP under the control of the SP-C promoter, showed lack of SP-C-expressing marrow cells in the recipient lungs following BMC transplantation. In support of this evidence, another independent group used the same transgenic animal and also found no contribution of BMC in repopulating the alveolar epithelium (Chang et al., 2005). Studies have suggested that lung injury is necessary for BMC engraftment in the lung. Beckett (Beckett et al., 2005) found no significant increase in the number of donor BMC in the lungs after endotoxin and NO₂ injury.

These studies challenge the contribution of the bone marrow in lung cell lineages. There are possible explanations for these apparent discrepancies in the involvement of BMC in pulmonary epithelium. First, while the most recent publication by Kotton appears to refute his original finding that the bone marrow can contribute to alveolar epithelium, there are possible explanations why lack of donor bone marrow cells was observed in the lung epithelium in his second study. First, not all Type II cells may express SP-C-driven GFP. Second, not all the bone marrow cells may express the SPC-GFP transgene. And third, donor bone marrow cells may contribute to the lung epithelium by cell fusion with other epithelial cells without activation of SP-C-driven GFP transgene. Another possible explanation for the controversy and discrepancy in bone marrow cell contribution to lung epithelium is the type and extent of lung injury used to induce bone marrow cell engraftment. Injuries that may deplete/injure the local lung progenitor cell populations may affect the ability of bone marrow/circulating progenitor

cells to reconstitute/regenerate the epithelium. Indeed, epithelial regeneration does not occur in airway injury where the local naphthalene-resistant CCSP⁺ (putative airway stem cell) is depleted (Hong et al., 2001). Injuries that expose the appropriate niches may selectively allow for extrapulmonary progenitor cell engraftment. Is it possible that the local progenitor cell may have selective advantage for epithelial regeneration over extrapulmonary progenitors and thereby hinder the contribution of the bone marrow cells in lung repair.

1.6.2 Mechanism of phenotypic conversion

1.6.2.1 Transdifferentiation

Stem cell transdifferentiation into lung epithelial cells in vivo have been shown in several studies as discussed earlier. In vitro, stem cells can be induced to develop phenotypic characteristics of tissue-committed cells with exposure to growth factors and cytokines. Embryonic stem cells can develop into Type II alveolar cells and tracheal-bronchiolar cells using specific differentiating media when cultured under air liquid interface (Coraux et al., 2005). Human MSC have also been shown to acquire phenotypic and functional epithelial characteristics when cocultured with airway epithelial cells (Spees et al., 2003; Wang et al., 2005). This suggests that soluble factors released by epithelial cells or epithelial cell-MSc contact may be important in phenotypic and functional conversion of MSC to lung epithelial cells. Human MSC cultured in media containing specific growth factors (KGF, EGF, HGF and IGF) can induce differentiation into epithelial-like cells expressing cytokeratin-18 and -19 (Paunescu et al., 2007). Assessment of functional potential using this method of cultivation and differentiation

was not determined and therefore it remains unclear whether soluble factors alone can induce full-scale epithelial differentiation.

1.6.2.2 Cell fusion

Cell fusion of BMC, specifically monocytic cells, with mature tissue-specialized cells has been reported in the heart, liver, intestine and muscle (Nygren et al., 2004; Rizvi et al., 2006; Shi et al., 2004; Vassilopoulos et al., 2003; Wang et al., 2003b). In the lung, an in vitro study has suggested that human MSC can fuse with lung epithelial cells and adopt the epithelial phenotype (Spees et al., 2003). In vivo, some studies have reported low but detectable levels of cell fusion by FISH in the lung (Krause et al., 2001; Wong et al., 2007) while others have shown no evidence of cell fusion using Cre-lox recombination strategy (Harris et al., 2004). Some possible explanations for the negative fusion result in this study are 1. Not all donor cells from the b-actin Z/EG Cre-reporter strain expressed GFP reporter, 2. Pulmonary Cre-recombinase expression may be weak and therefore recombination may appear to be a rare event even if cell fusion had occurred. 3. Loss of the genomic DNA containing the transgene in a reductive division event. 4. Cell fusion may depend on the type of host cell and extent of cell/tissue injury. In this study, cell fusion was not assessed after lung injury.

1.6.2.3 Cell phagocytosis as a possible mechanism of “epithelialization”

Cell phagocytosis may be another explanation for the observation of what appears to be phenotypic conversion of bone marrow cells into mature tissue cells. Efferocytosis, meaning the removal of dead apoptotic cells by epithelial cells, is a regulatory checkpoint

for the innate and adaptive immune system important in lung repair (Vandivier et al., 2006). Alveolar Type II cells are known to phagocytose apoptotic Type II cells during repair (Fehrenbach, 2001). It is also likely that these cells phagocytose dead BMC in the lungs. Bone marrow dendritic cells are also known to phagocytose apoptotic cells (Nayak et al., 2006). In this thesis, we show BMC can phagocytose lung cells in vivo and may appear to be transdifferentiation of BMC into epithelial cells (Wong et al., 2007) – *See Chapter 2.*

1.6.3 Clinical potential of bone marrow stem cell therapy

Theoretically, BMC could be used as vehicles for targeted transgene expression in epithelial cells. Grove (Grove et al., 2002) demonstrated that marrow cells transduced with a retrovirus encoding eGFP transplanted into irradiated mice gave rise to transgene-expressing lung epithelial cells as late as 11 months after transplantation. This is exciting since long-term transgene expression remains unachievable in most other gene therapy approaches. This approach is also particularly promising in diseases where replacement of a protein and replacement of damaged tissue would be beneficial such as CF. Not only can stem cells deliver the corrected gene but these cells could also replenish destroyed tissue. Encouragingly, Johnson (Johnson et al., 1992) showed that only 6-10% of CFTR-corrected cells can restore normal chloride secretion in a CF epithelial sheet. This finding is significant since few stem cell studies show up to 4% donor bone marrow engraftment (Krause et al., 2001; Suratt et al., 2003; Wong et al., 2007). Overall, partial replacement of the defective epithelium may be achievable using stem cells as vectors for gene

therapy. In support of this, Bruscia (Bruscia et al., 2006) demonstrated modest restoration of CFTR activity in CCSP-null mice after bone marrow transplantation of CFTR⁺ BMC.

In addition to gene delivery, autologous stem cells are also attractive vectors for therapy because it avoids the need for immunosuppression which has been a major barrier in most gene therapy approaches. Autologous stem cells can be obtained from the patient manipulated *ex vivo* to carry the transgene and delivered back to the patient thereby avoiding immune responses. Bone marrow-derived mesenchymal stem cells (MSC) have immunomodulatory potential. The immunomodulatory role of stem cells is discussed below.

1.6.3.1 Immunomodulatory roles of stem cells

Recently it has been reported that bone marrow cells may facilitate tissue regeneration not by transdifferentiation into tissue-committed cells but by production of pro-angiogenic, anti-inflammatory mediators through paracrine mechanisms (Ayach et al., 2006a; Fazel et al., 2006; Ortiz et al., 2007; Xu et al., 2007b).

(i) CCSP protein

In *Chapter 3*, we discuss the identification of a CCSP-expressing bone marrow cell population. The significance of CCSP expression in the bone marrow cells remain unclear. The protein however has been associated with immunomodulation in the airways. CCSP or CC10 is a homodimeric protein secreted by airway Clara cells. This protein is a small (10-16 kDa) protein that is resistant to proteases and stable in extreme heat and pH (Pilon, 2000). CCSP is also produced in the uterine endometrium under

progesterone stimulation in females (Peri et al., 1993; Peri et al., 1994) and in the prostate in males (Rauch et al., 1985). In humans, the CC10 gene contains 3 short exons and 2 introns spanning 4.1 kb of DNA (Wolf et al., 1992; Zhang et al., 1997b). It is located on chromosome 11q12.3-13.1 clustered around genes associated with immunomodulation. The CCSP protein represents the most abundant secretory protein in the airways and is believed to play a role in regulating local inflammatory responses (Dierynck et al., 1996; Harrod et al., 1998; Hayashida et al., 2000; Yoshikawa et al., 2005). The CCSP protein also contains a hydrophobic pocket that serves as a binding site for lipophilic ligands such as polychlorinated biphenyls (Stripp et al., 1996). Although this protein is also produced in the kidney epithelium (known as secretoglobin, uteroglobin), CCSP produced in the lungs can leak into the serum and localize to the proximal tubule of the kidney through active reuptake from the glomerular filtrate (Stripp et al., 2000).

CCSP has been associated with various human lung diseases such as asthma (de Burbure et al., 2007; Martin et al., 2006; Sengler et al., 2003) and bronchiolitis obliterans (Mattsson et al., 2005; Nord et al., 2002) and may serve as a biomarker for distal airway damage (Broeckeaert et al., 2000; Gioldassi et al., 2004; Shijubo et al., 2000). Polymorphism in the CC10 gene appears to be associated with severity of airway responsiveness in asthmatics (Martin et al., 2006; Sengler et al., 2003).

The function of CCSP is becoming unveiled. A CCSP^{-/-} mouse line generated by Dr. Barry Stripp (and the strain used in this study) has provided some insight as to the function of the CCSP protein. CCSP is important for normal Clara cell secretory function and maintenance of normal airway lining fluid composition (Stripp et al., 2002). CCSP^{-/-} mice demonstrate an increased susceptibility to oxidative stress and experience more

severe lung injury compared to wild-type animals (Johnston et al., 1997; Mango et al., 1998; Plopper et al., 2006). When exposed to respiratory pathogens such as *Pseudomonas aeruginosa* CCSP^{-/-} mice exhibit elevated inflammatory responses increased production of cytokines (IL6, IL1 β and TNF- α) and chemokines (MIP1 α , MIP2 and MCP-1) (Harrod et al., 1998; Wang et al., 2003a). Temporary deficiency of CCSP appears to be important in enhanced bacterial killing after acute pulmonary infection by permitting early infiltration of inflammatory cells into the lungs (Hayashida et al., 2000). CCSP also plays a role in modulating acute ventilator-induced lung injury in mice by inhibiting the cytosolic phospholipase A₂ pathway (Yoshikawa et al., 2005) thereby decreasing pulmonary edema. CCSP^{-/-} mice demonstrate increased lung immunoglobulin A (IgA) and secretory protein W82219 (SCGB3A2) (Reynolds et al., 2002; Watson et al., 2001). CCSP also promotes epithelial remodeling in an ovalbumin model of allergic inflammation (Wang et al., 2001). In another independently generated CCSP^{-/-} mouse line, these mice exhibit a severe lethal phenotype with wasting, multiple organ damage and kidney failure caused by fibronectin deposition in the glomeruli of the kidney (Zhang et al., 1997a) which is not observed in the previous CCSP^{-/-} mouse line. This CCSP^{-/-} strain rarely survives beyond the first few months. The ones that do develop solid organ tumors suggesting a role of the CCSP protein in anti-tumor development. Indeed in vitro studies using 13 different tumor cell lines treated with recombinant CCSP protein inhibited these neoplastic cells from proliferating and invading through a matrigel (Pilon, 2000). Treatment of CCSP-null mice with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a potent carcinogen in tobacco smoke had significantly higher incidence of airway epithelial hyperplasia and lung adenomas compared with wild-type controls (Yang

et al., 2004). Higher BAL CC10 levels have been correlated with regression of bronchial dysplasia and improvement in sputum cytometry assessment in smokers with high lung cancer risk (Chen et al., 2008).

The anti-inflammatory/immunomodulatory role of CCSP⁺ BMC remains to be elucidated.

(ii) Leukocyte regulation

Studies have suggested that MSC may play a role in modulation of immune responses. These cells can evade allogeneic rejection because they do not express MHC class II allowing them to escape alloreactive CD4⁺ T-cells (Le Blanc et al., 2003). MSC are hypoimmunogenic and can modulate immune cell behaviour because they do not express costimulatory molecules (CD40, CD40L, CD80, CD86) which is required for effector T-cell activation (Tse et al., 2003). Lack of expression of costimulatory molecules suggests that residual engagement of T-cell receptors may result in anergy and contribute to tolerance. Indeed, HSC transplant patients that were treated with matched or mis-matched MSC showed no alloantibodies in their blood (Sundin et al., 2007). Koc showed no evidence of alloreactive T-cells or graft versus host disease in patients with metachromatic leukodystrophy and Hurler's syndrome that had received allogeneic MSC (Koc et al., 2002). Horwitz showed allogeneic bone marrow transplantation with MSC improved bone remodeling in 3 children with osteogenesis imperfecta (Horwitz et al., 1999). MSC can create an immunosuppressive local milieu as shown by inhibiting T-cell proliferation in mixed leukocyte reactions and induce apoptosis of activated T-cells by production of indoleamine 2,3-dioxygenase (IDO, an enzyme that converts tryptophan

into kynurenine thereby depleting the tryptophan stores in the culture medium of T-cells) (Krampera et al., 2003; Plumas et al., 2005; Tse et al., 2003). MSC also secrete prostaglandin E2 which in turn can suppress B cell activation and induce expansion of regulatory T cells (Aggarwal and Pittenger, 2005). In addition, MSC can prevent TNF- α secretion by mature Type I dendritic cell and promote interleukin-10 production by mature Type II dendritic cell. MSC can also inhibit Th1 cell production of IFN- γ , promote interleukin-4 production by Th2 cells, inhibit natural killer cell secretion of IFN- γ , and induce peripheral tolerance by interfering with dendritic cell maturation. Coculture experiments showed MSC can down-regulate CD40, CD80, CD86 and HLA-DR expression on dendritic cell during maturation (Jiang et al., 2005; Zhang et al., 2004).

(iii) Soluble factors

Bone marrow stem cells have been shown to secrete soluble factors that may ameliorate inflammation. Cultured MSC delivered directly into the airways of mice after endotoxin-induced ALI improved survival and decreased lung injury by local production of IL-10 and reducing TNF- α and MIP-2 production (Gupta et al., 2007). Recently, a new potential anti-inflammatory mediator, interleukin-1 (IL-1) receptor antagonist, was identified in a small population of cultured MSC. IL-1 receptor antagonist blocks IL-1-dependent T-cell proliferation and production of TNF- α . Sequestering IL-1 can reduce lymphocyte and neutrophil trafficking to the lung thereby reducing inflammation (Ortiz et al., 2007). Another group demonstrated bone marrow-derived MSC when delivered early before the onset of injury can prevent endotoxin-induced inflammation, injury, and

edema in the lungs by suppressing proinflammatory cytokine production with physical engraftment of the stem cells (Xu et al., 2007b).

1.6.4 Concerns of bone marrow stem cell therapy

Although the prospect of BMC-mediated tissue regeneration seems promising, the mechanisms that control stem cell-mediated regeneration as opposed to pathological development such as fibrosis remains unknown. It is also possible bone marrow-derived stem cells when mobilized at the wrong time and deposited in areas of chronic inflammation may contribute to the development of malignancies (Avital et al., 2007; Houghton et al., 2004). Many studies have identified stem cells in tumors (Ezeh et al., 2005; Gu et al., 2007; Hart et al., 2005; Houghton et al., 2004). Stem cells may acquire critical mutations that would promote cancer development (Jamieson et al., 2004; Kim et al., 2005) instead of tissue regeneration. In the presence of carcinogens, bone marrow-derived stem cells may form several sarcomas including teratomas (Liu et al., 2006).

1.7 Mechanism of stem cell mobilization and recruitment to the injured tissues

1.7.1 SDF-1/CXCR4

Stromal-derived factor (SDF)-1, also known as CXCL12, is a chemokine that chemoattracts cells expressing the cognate receptor CXCR4. SDF-1/CXCR4 signalling is involved in many biological processes including cell migration, proliferation, and survival (Aiuti et al., 1997; Broxmeyer et al., 2003; Cashman et al., 2002; Wang et al., 2000) and stem cell mobilization (Kucia et al., 2004b). Damaged tissues including the heart, neuronal tissue, lung, and kidney express upregulated levels of SDF-1 (Abbott et

al., 2004; Askari et al., 2003; Hoffmann et al., 2006; Kollet et al., 2003; Takeuchi et al., 2007; Xu et al., 2007a). Studies have suggested that SDF-1 is involved in MSC (Wynn et al., 2004) and hematopoietic progenitor cell homing and mobilization (Hattori et al., 2001; Jo et al., 2000). As evidence, blocking CXCR4 on human HSC prevented engraftment in nonobese diabetic (NOD)/SCID mice (Peled et al., 1999) while stimulation of CD34⁺ hematopoietic progenitor cells with SDF-1 promoted engraftment (Plett et al., 2002). Recently it has been suggested that SDF-1-induced chemotaxis can mobilize tissue-specific CXCR4⁺ progenitor/stem cells from the bone marrow (Kucia et al., 2004a; Kucia et al., 2004b; Kucia et al., 2006b; Pituch-Noworolska et al., 2003; Ratajczak et al., 2004) to injured tissue and may be involved in tissue regeneration. The origin of these tissue-committed bone marrow cells remain unknown although MSC derived from the bone marrow and cord blood have been shown to express mRNA for cardiac, muscle, neural and liver markers (Son et al., 2006). In addition to mobilizing tissue-committed cells, SDF-1/CXCR4 may also play a role in tumor stem cell metastasis (Kucia et al., 2005b; Tavor et al., 2004).

1.7.2 HGF/c-met

Hepatocyte growth factor (HGF) ligand, also known as scatter factor (Weidner et al., 1991), binds to its cognate receptor c-met, a transmembrane tyrosine kinase. It is a strong chemoattractant for various cells including stem cells and may play a role in MSC mobilization and wound repair (Neuss et al., 2004). In the bone marrow, HGF is produced by bone marrow stromal cells to regulate HSC proliferation, adhesion and survival (Weimar et al., 1998). In the lungs, HGF is a potent stimulator of angiogenesis

by mobilizing endothelial progenitor cells (Ishizawa et al., 2004b) and is a pulmotrophic factor for lung regeneration (Ohmichi et al., 1996; Yanagita et al., 1993). HGF plays a role in epithelial regeneration (Shigemura et al., 2005) and compensatory growth after pneumonectomy (Sakamaki et al., 2002) by acting as a motogen to enhance motility of epithelial cells (Ohmichi et al., 1998; Tajima et al., 1992). HGF is also important in inducing MSC conversion to an epithelial phenotype in vitro (Paunescu et al., 2007). Bone marrow cells can express HGF and may promote tissue regeneration through a paracrine effect on local tissue cell proliferation, migration and survival (Adamson and Bakowska, 1999; Izumida et al., 2005; Okada et al., 2004).

1.7.3 SCF/c-kit

The steel factor receptor (c-kit) is a member of the tyrosine kinase receptor family and is activated by stem cell factor (SCF or kit ligand). Binding of SCF to c-kit results in receptor dimerization and activation of signaling pathways involved in stem/progenitor cell mobilization, anti-apoptosis and cell proliferation (Jiang et al., 2000; Ronnstrand, 2004). Recently it has been demonstrated that c-kit signalling is important in cardiac regeneration following myocardial infarction by bone marrow progenitors (Ayach et al., 2006b; Fazel et al., 2005; Fazel et al., 2006). In the lung, elevated expression of SCF is associated in some lung diseases such as interstitial fibrosis (Fireman et al., 1999), asthma (Da Silva et al., 2006b) and induction of multidrug-resistant malignant mesothelioma cells (Catalano et al., 2004). Enhanced production of SCF in the lungs of patients early following lung transplantation has been observed (Da Silva et al., 2006a)

therefore early expression of SCF may be associated with critical tissue repair with progenitor cells.

Hypothesis and Objectives

Introduction

This thesis describes the role of bone marrow-derived stem/progenitor cells in airway epithelial regeneration. The goal of the research described in this thesis is 2-fold: 1. To elucidate a novel strategy using targeted cell replacement to enhance bone marrow cell engraftment in the airway epithelium and 2. To identify and characterize a newly discovered epithelial-like stem cell in the bone marrow and elucidate its contribution to recovery following lung injury.

Human and animal studies have shown that bone marrow-derived stem cells can engraft and develop into lung and airway epithelium. However, the reported frequencies of pulmonary engraftment have been shy of clinical relevance. Furthermore, the specific subpopulation of BMC that may have greater propensity for pulmonary engraftment remains unknown.

Hypothesis

1. Targeted cell delivery of short-term cultured BMC into selectively injured airways can promote BMC retention and adoption of an epithelial phenotype.
2. The bone marrow contains an epithelial-like stem/progenitor cell (CCSP⁺Sca-1⁺) population with greater propensity for airway engraftment and regeneration.

Objectives

1. To assess the feasibility of selective airway injury and targeted delivery for airway cell replacement therapy.
2. To isolate and analyze the self-renewal and multi-differentiation potential of bone marrow-derived epithelial-like stem/progenitor cell population.
3. To determine whether CCSP⁺ BMC have airway reconstitution and epithelial functional restoration potential

Chapter 2

Targeted cell replacement with bone marrow cells for airway epithelial regeneration

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A link to the published paper can be found at

<http://ajplung.physiology.org/cgi/content/full/293/3/L740>

2.1 Rationale

Human and animal studies have shown that bone marrow-derived stem cells can engraft and develop into lung and airway epithelium. We explored the potential of using a targeted cell replacement approach with short-term cultured BMC in regenerating the airway epithelium as a novel therapeutic modality.

Objective of Study: We examined the phenotype and potential of bone marrow cells (BMC) to reconstitute airway epithelium in an acute injury model that selectively depletes Clara cells.

Summary of Results: Here, we show that the combination of mild airway injury (naphthalene-induced) as a conditioning regimen to direct the site of BMC localization and transtracheal delivery of short-term cultured BMC enhances airway localization and adoption of an epithelial-like phenotype. Confocal analysis of airway and alveolar-localized BMC (fluorescently-labeled) with epithelial markers show expression of the pulmonary epithelial proteins, CCSP and surfactant protein-C (SP-C). To confirm epithelial gene expression by BMC, we generated transgenic mice expressing green fluorescent protein (GFP) driven by the epithelial-specific cytokeratin-18 promoter and injected BMC from these mice transtracheally into wild-type recipient after naphthalene-induced airway injury. BMC retention in the lung was observed to at least 120 days following cell delivery with increasing GFP transgene expression over time. Some BMC cultured in vitro over time also expressed GFP transgene suggesting epithelial transdifferentiation of the BMC.

Conclusion: The results indicate that targeted delivery of short-term cultured BMC can promote airway regeneration. Donor-derived BMC retained in the airway express the lung epithelial markers, CCSP, proSP-C and K18. The injured lung milieu appears to facilitate BMC engraftment and possibly, epithelial differentiation.

Please go to the journal's website to read the contents of Chapter 2.

<http://ajplung.physiology.org/cgi/content/full/293/3/L740>

Chapter 3

Identification of a novel epithelial-like bone marrow cell population that can repopulate the airway epithelium

3.1 Rationale

Bone marrow stem/progenitor cells can develop into lung epithelial cells but the specific subpopulation remains unknown. In Chapter 2, we found that BMC retained in the airway can express CCSP as early as 1 day following cell delivery. This generated the hypothesis that the bone marrow already contains “epithelial progenitor cells” that may have a greater propensity to become lung epithelial cells.

Objective of study: To determine the specific subpopulation of BMC that may contribute to lung regeneration.

Results: Here we identify a newly discovered population of murine and human bone marrow cells that express CCSP, a marker of airway progenitor cells. These CCSP⁺ cells increase in the bone marrow and blood after airway injury and can be expanded in culture. CCSP⁺ cells can give rise to various lung epithelial lineages *in vitro* and exhibit some (but not all) epithelial ion transport properties. Importantly, bone marrow transplant of CCSP⁺ cells to CCSP-knockout recipients suggests that bone marrow CCSP⁺ cells may contribute to lung regeneration after airway injury. The mechanism of homing to injured lung may involve SDF-1-CXCR4 signalling.

Conclusion: The present study identifies a newly discovered cell that has airway reconstitution potential and greater propensity to differentiate into lung epithelial cells. The CCSP⁺ BMC population expresses both hematopoietic and mesenchymal stem cell markers. This finding may reconcile previous controversies of the contribution of specific

subpopulation of bone marrow-derived stem cells in lung regeneration. These cells also have multilineage epithelial differentiation potential and exhibit epithelial sodium transport property suggesting the possibility of epithelial functional restoration. CCSP⁺ BMC may potentially be useful in cell replacement strategies for epithelial diseases such as cystic fibrosis.

3.2 Introduction

Adult bone marrow contains a variety of stem cell populations that can be recruited to injured tissues. Although controversial, studies have suggested the possible involvement of bone marrow-derived hematopoietic, mesenchymal or Hoechst-effluxing side population cells in lung repair following injury (Kotton et al., 2001; Krause et al., 2001; Macpherson et al., 2005a; Ortiz et al., 2007; Rojas et al., 2005; Wong et al., 2007; Xu et al., 2007b). Moreover, in vitro studies have shown that BMC cultured either alone or in co-culture with epithelium can express epithelial functional properties (tight junction formation and the cystic fibrosis transmembrane conductance regulator (CFTR)-mediated chloride ion transport) (Spees et al., 2003; Wang et al., 2005), which further suggests a potential therapeutic use of BMC in treatment of pulmonary diseases. More recent studies employing transgenic animal models and more stringent analytical methods to assess pulmonary engraftment of BMC have failed to demonstrate substantial involvement of bone marrow in lung regeneration (Chang et al., 2005; Kotton et al., 2005b; Loi et al., 2006). Although variations in technical and analytical methods used to assess BMC engraftment and/or transdifferentiation may explain the discrepant

observations, a likely explanation is the uncertainty of the specific subpopulation of BMC that has pulmonary engraftment and regenerative potential.

We have previously shown that targeted delivery into acutely injured airways of short-term cultured plastic-adherent BMC improved airway retention and promoted expression of epithelial proteins by retained BMC (Wong et al., 2007). We found BMC in the lung which expressed CCSP as early as 1 day following cell delivery. This prompted us to examine CCSP expression in our short-term cultured and freshly-isolated bone marrow cells. We report here a newly discovered population of cells in mouse and human bone marrow that express CCSP, a marker of airway progenitor cells (Giangreco et al., 2004; Hong et al., 2001; Reynolds et al., 2004) and stem cells (Kim et al., 2005; Ling et al., 2006). As CCSP expression is also found on the cell surface of these BMC, they can be easily isolated from whole marrow. These cells adhere to plastic and expand in culture. The proportion of CCSP⁺ cells is increased in bone marrow and peripheral blood in response to naphthalene-induced airway injury. Transtracheal and intravenous injection of cultured cells demonstrated the ability of these cells to home to the injured airways and contribute to lung cell lineages. Bone marrow transplantation of CCSP⁺ cells to CCSP-null recipients demonstrated restoration of pulmonary CCSP expression. The mechanism of homing to the injured airways may involve SDF-1-CXCR4 signalling. The majority of CCSP⁺ cells express CD45 and some mesenchymal markers CD73, CD90, CD105. Culturing of these cells under an air-liquid interface demonstrates the ability of these cells to differentiate into multiple epithelial lineages including “intermediary” cells co-expressing proteins specific to both airway and alveolar lineages. Collectively, these studies clarify previously conflicting data surrounding “transdifferentiation” of the

specific subtype of BMC into pulmonary epithelial cells in vivo. The identification of the CCSP⁺ cells challenges conventional dogma of a distinct hematopoietic and mesenchymal BMC populations that can be distinguished by surface marker expression and contribute to lung regeneration.

3.3 Materials and Methods

Animals

Adult male (4-6 weeks) C57Bl6, CD1 (Charles River Laboratories, Wilmington, MA), β -actin-GFP (C57Bl/6 mice constitutively expressing GFP under the control of the β -actin promoter, Jackson Laboratories) and transgenic K18GFP (GFP driven by the K18 promoter; CD1 background) mice were used as BMC donors (Wong et al., 2007). Age and strain-matched C57Bl/6 and CCSP-null (Stripp et al., 2000) females were used as recipients respectively. All animal procedures were approved by the University Health Network Animal Care Committee. All mice received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research, and the Guide for the Care and Use of Experimental Animals formulated by the Canadian Council on Animal Care. Human samples from volunteer healthy donors were collected under a protocol approved by University Health Network Research Ethics Board.

Animal procedures

Mouse whole bone marrow was harvested and cultured as previously described (Wong et al., 2007). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco-Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin (Gibco, Grand Island, NY). For lung injury studies, mice were given intraperitoneal injections of naphthalene (>99% pure; Sigma Chemical, St. Louis, MO), 200 mg per kg body weight as previously described (Wong et al., 2007). For marrow transplantation studies, female CCSP^{-/-} recipient mice were irradiated with 9.5 cGy. Within 2 hours of irradiation, a group of mice received 2×10^7 freshly prepared female whole bone marrow from CCSP-null mice mixed with either 3×10^5 male cultured and sorted CCSP⁺ or female CCSP-null cells via tail vein injection.

Bone marrow harvest and culture

BMC were harvested as previously described (Kotton et al., 2001). Briefly, whole bone marrow was harvested aseptically by flushing femurs and tibiae of donor mice with cold DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin (Gibco) using a 23 gauge needle. BMC were plated on standard plastic tissue culture flasks at a density of 5×10^5 cells/cm² and the medium was changed every 2-3 days. Non-adherent cells were gradually washed away with successive media changes. The remaining plastic-adherent BMC were cultured for 7 days in DMEM containing 10% FBS and 1% penicillin-streptomycin before use as BMC in vivo.

Fluorescence activated cell sorting and cell labelling

Freshly isolated BMC and cultured, plastic-adherent BMC from naphthalene-injured and non-injured mice (5×10^5 cells per sample) were stained with rabbit anti-mouse CCSP (1:1000, Upstate Labs) in FACS buffer containing 2% BSA in PBS for 30 minutes on ice after blocking with buffer containing 5% normal goat serum and 2% BSA to block non-specific binding. After primary antibody treatment, the cells were washed and incubated with AlexaFluor 488 secondary IgG (1:500, Molecular Probes) or streptavidin-conjugated AlexaFluor 488 (1:500) on ice for 30 minutes. Relative expression was compared to isotype controls for all staining. Dead cells were labeled with propidium iodine staining (1:100) on ice for 5 minutes. A FACScalibur flow cytometer and CXP Software (Becton-Dickinson, San Jose, CA) were used to assess expression of these markers. Similar results were obtained with primary anti-CCSP antibodies from Abnova (cat #H00007356-M01).

Plastic-adherent BMC cultured for 7 or 14 days were sorted by CCSP cell surface expression using the MoFlo Cell Sorter (Becton Dickinson, Franklin Lakes, NJ, USA). The staining protocol was followed as above. After sorting, the cells were plated and cultured separately on collagen-coated culture dishes. Sorted cells were cultured for 4 days prior to labelling with CMTMR (CellTracker™ Orange CMTMR Molecular Probes, Eugene, OR) according to the manufacturer's protocol. They were injected via either the transtracheal or intravenous route into naphthalene-injured recipients (10^6 cells/mice). In addition, a fraction (25%) of sorted CCSP⁺ cells were mixed with the CCSP⁻ population and co-injected as a “mixed” population.

Co-culture Study

Female CCSP⁻ BMC were sorted and plated on 35 mm² dishes for 24 hours. Male CCSP⁺ cells were then added to some cultures containing female CCSP⁻ cells in a 1:4 (CCSP⁺:CCSP⁻) ratio. “Day 0” cells were immediately harvested after mixing and analyzed using flow cytometry for CCSP and real time PCR for CCSP mRNA and SRY genomic DNA. “Day 14” cells were cultured for 14 days and harvested for flow cytometry and PCR.

Real-time PCR analysis

Total RNA was prepared from FACS-sorted BMC using the RNeasy Kit (Qiagen, Valencia, CA). For RNA, reverse transcription for first-strand cDNA was generated using Superscript II (Sigma) according to manufacturer’s protocol. Real-time PCR (SYBR green detection method; Applied Biosystems, Foster City, CA) was performed for amplification of the CCSP gene (Table 3-1). For characterization of the cells, various hematopoietic, mesenchymal and epithelial genes were assessed in CCSP-sorted cells (Table 3-1).

To quantify the number of BMC retained in the lung, genomic DNA from the left lung of each mice were extracted as previously described (Wong et al., 2007). Gene expression of the male sex-determining gene SRY (forward primer 5'-GGGATGCAGGTGGAAAAGC-3' and reverse primer 5'-GTGACACTTTAGCCCTCCGAT-3') was used to quantify level of donor cell in the host lung. A standard curve was generated by spiking female lungs with increasing numbers of male cells.

The real-time PCR conditions and instrumentation was as follows: 50°C for 2 min; 95°C for 10 min; 35 cycles at 94°C for 15 sec; 60°C for 1 min followed by dissociation using the ABI7900HT robot and analyzed with SDS 2.0 software. GAPDH was determined to be the most stable housekeeping gene by geNorm program therefore it was used as the housekeeping gene to normalize gene expression levels using REST-384 (relative expression software tool, available at www.Gene-Quantification.com) program. Normalized mRNA or gDNA levels are expressed as “relative” to the positive control samples (airway epithelial cells or male lung).

Western blot analysis

Cells (2×10^6 /ml) and homogenized lung tissue were lysed for western blot analysis using RIPA buffer (1X PBS, 1% NP-40 substitute, 0.5% SDS, one Complete Mini EDTA-free protease inhibitor tablets from Roche (Indianapolis, IN) per 10ml, 4 mM Na_3VO_4 , 4 mM NaF) for 1 hour on ice. The supernatants were resolved by 12.5% SDS-PAGE and assessed for CCSP (07-623, Upstate) protein levels.

Table 3-1: Primer sequences for phenotypic characterization of CCSP⁺ cells

Gene	Forward primer 5'→3'	Reverse primer 5'→3'	Notes
K18Intron	CACTCACCCACCCAT CC	CTCCTCGCCCTTGCT CAC	Detects transgenic DNA
K18GFP expression	CAAAGCCTGAGTCCTG TCCT	GAACTTCAGGGTCA GCTTGC	Detects K18-GFP expressing donor cells
GAPDH	TGTGTCCGTCGTGGAT CTGA	GATGCCTGCTTCACC ACCTT	House keeping gene
CD90	GCCTGACCCGAGAGAA GAG	GGACCTTGATATAG GGCTGGT	MSC marker
CD105	GGGTGAGGTGACGTTT ACCAC	AGCATTCCGGGAAA ATCCAGG	MSC marker
CD73	GGACATTTGACCTCGT CCAAT	GGGCACTCGACACT TGGTG	MSC marker
CD34	AAGGCTGGGTGAAGAC CCTTA	TGAATGGCCGTTTCT GGAAGT	HSC marker
CD45	CAGAAACGCCTAAGCC TAGTTG	ATGCAGGATCAGGT TTAGATGC	Hematopoietic marker
CC10	ATGAAGATCGCCATCA CAATCAC	GGATGCCACATAAC CAGACTCT	Aka CCSP
CD106	AGTTGGGGATTTCGGTT GTTCT	CCCCTCATTCTTAC CACCC	Mesenchymal marker
CD44	TCTGCCATCTAGCACT AAGAGC	GTCTGGGTATTGAAA GGTGTAGC	Mesenchymal marker
SP-C	GCTCATGGGCCTCCAC AT	TGGAGCCGATGGAA AAGG	Type II epithelial marker
CFTR	TCAAGCCGCGTTCTTG ATAA	AATGTGCCAAGGCA AGTCCT	Chloride channel
ENaC	TGCAGTGTGACCAACT ACAAG	TCTCGAAGATCCAAT CCTGGG	Sodium channel

Immunofluorescence

Assessment of epithelial marker expression

Immunofluorescence staining for proSP-C (AB3428, Chemicon or monoclonal version from Abnova, H-00006440-M01), cytokeratin 18 (K18: clone Ks 18.04), cytokeratin 14 (K14, Vector Labs), CD34 (553732, BD Pharmingen), CD45-PE (clone 30-F11, BD Pharmingen), collagen Type IV, vimentin, CD90, CD105, CD106, CD73-PE (all from BD Biosciences), collagen Type I (Rockland), CFTR (MAB1660, R&D Systems), ENaC (AB3532P, Chemicon), acetylated α -tubulin IV (ab24610, Abcam), and AQP-5 (178615, Calbiochem) was performed as previously described (Wong et al., 2007). Briefly, adherent cells were fixed with 4% paraformaldehyde (PFA), blocked with 5% goat serum and 2% BSA in PBS containing 0.5% Triton X-100 for 1 hour, and incubated with primary antibodies overnight at 4°C. Secondary antibodies Alexa Fluors 350, 488, 532, 633 or 647 (depending on the species in which the primary antibody was raised, see figures) were applied after 3 successive washes with PBS and incubated at room temperature for 1 hour. For CD90/105 and CD45 double staining, goat anti-rat secondary was added to the cells for 1 hour followed by extensive washing and staining with rat anti-CD45-PE. In some cases, nuclei were visualized with Hoechst dye (1:5000, Molecular Probes) for 5 minutes. Slides were mounted with immunofluorescent mounting medium. As controls for the antibodies, airway epithelial, CCSP⁺, and CCSP⁻ cells were stained with appropriate non-specific IgG isotypes. Images of representative fields were acquired using an Olympus FluoView 1000 Confocal Imaging System (Olympus, Melville, NY). Background noise was reduced using Kalman filter setting of 12. Resolution was improved with sequential imaging and an imaging speed of 8 μ m/us.

Mouse MSC were kindly provided by Dr. A. Keating.

Assessment of donor cells in recipient lungs-Immunofluorescence

Recipient lungs were fixed at constant pressure (20 cm H₂O) with 10% formalin. Paraffin-embedded sections (5 µm thick) were prepared and stained for CCSP using an antibody kindly provided by Dr. Barry Stripp, Duke University. Sections were deparaffinized through xylene and graded alcohol washes. Antigen retrieval was done by treating the slides with Digest-All pepsin (Zymed laboratories) for 10 minutes at 37°C. Non-specific binding were blocked with 5% BSA for 3 hours. Primary antibody mix (anti-CCSP: 1:10,000 and Alexa Fluor 488-conjugated anti-GFP (Molecular Probes): 1:500) was added and incubated overnight at 4°C followed by secondary antibody anti-goat Alexa Fluor 532 for double immunofluorescence microscopy. For triple immunofluorescence microscopy, goat anti-CCSP was incubated overnight at 4°C by secondary donkey anti-goat Alexa Fluor 532 for 1 hour at room temperature. After extensive washes with PBS and 0.3% Triton X-100, slides were incubated with Alexa Fluor 488-conjugated anti-GFP and anti-CK18 (RDI) overnight at 4°C followed by secondary anti-mouse Alexa Fluor 647. Nuclei were visualized with Hoechst stain (1:5000) and mounted with immunofluorescent mounting media (Dako). Isotype controls were used for non-specific binding. Images were acquired using an Olympus FluoView 1000 Confocal Imaging System (Olympus) with a pinhole diameter of 80 µm. Images were acquired using sequential mode to eliminate cross talk of the fluorescence with a sampling speed of 8 µs/pixel.

Assessment of donor cells in recipient lungs-Chromogenic

Recipient lungs were fixed at constant pressure (20 cm H₂O) with 10% formalin. Paraffin-embedded sections (5 µm thick) were prepared and stained for CCSP (07-623, Upstate Laboratories). Sections were deparaffinized through xylene and graded alcohol washes. Endogenous peroxidases were blocked with 1.5% H₂O₂ in methanol for 30 minutes and non-specific binding sites were blocked with 5% normal goat serum for 3 hours. Primary antibody (1:2000) was added and incubated overnight at 4°C followed by secondary antibody detection using Elite VectaStain kit (Vector Labs) according the manufacturers protocol. Positive stains were visualized with DAB chromogenic substrate (Vector Labs) and counterstained with hematoxylin. Sections were mounted with Permount medium and visualized with a Nikon Eclipse 80i microscope (Nikon instruments Inc, Melville, NY, USA) and ACT-2U Imaging software.

Epithelial differentiation assay

Air-liquid interface cultures

To determine whether CCSP⁺ cells could give rise to other epithelial cell lineages, plastic-adherent BMC were sorted into CCSP⁺ and CCSP⁻ cells after 7 days in culture. After an additional 2 weeks they were then cultured in air-liquid interface conditions for 4 weeks as previously described (You et al., 2002). Cell suspensions were prepared by cytopsin for immunofluorescence staining. TEC were used as positive control for the assay and staining. TEC were harvested by 0.15% pronase digestion overnight at 4°C followed by DNase I digestion on ice for 10 minutes. Cells were plated on plastic culture plates for 3 hours to allow fibroblasts to adhere. The non-adherent cells populations were

then seeded onto rat collagen type IV (BD Biosciences) -coated transwell membranes (Costar) and cultured for 2 weeks in MTEC medium containing 10 mg/ml insulin, 5 mg/ml transferrin, 0.1 mg/ml cholera toxin, 25 ng/ml epidermal growth factor, 0.03 mg/ml bovine pituitary extract and 5% FBS in DMEM-F12 mixture until confluency. After 2 weeks in submerged culture, only the bottom of the transwell membrane were exposed to media. CCSP⁺ or CCSP⁻ BMC were cultured in media containing 75% MTEC and 25% DMEM.

Perforated patch clamp recording

Perforated patch-clamp recordings were performed by Dr. Weiyang Lu (Sunnybrook Hospital). Recordings were carried out on CCSP⁻ and CCSP⁺ cells using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA, USA). Perforated patch recording was used because this approach would not alter intracellular metabolism, thus not interrupting metabolism-controlled ion conductance such as CFTR. BMC were rinsed with, and bathed in, the standard extracellular solution (ECS) containing (in mM) 145 NaCl, 1.3 CaCl₂, 5.4 KCl, 25 HEPES and 10 mM glucose (kept at 32°C). The solution filling the patch electrode consisted of (in mM) 150 Cs-gluconate, 10 HEPES, 2 MgCl₂, 1 CaCl₂, and the pore-forming agent gramicidin ($50 \mu\text{g ml}^{-1}$, Sigma-Aldrich Corp., Buchs, Switzerland). The pH of ECS and ICS was adjusted to 7.4 and 7.3 respectively, and the osmolarity was corrected to a range of 310–315 mOsm. Patch electrodes (3–5 M) were constructed from thin-walled glass (1.5 mm diameter; World Precision Instruments, Sarasota, FL, USA) using a two-stage puller (PP-830, Narishige, East Meadow, NY, USA). Recordings started under voltage-clamp mode and membrane perforation was

observed as a constant decrease in serial resistance after electrode seal. In most recordings, the resistance declined to a value ranging from 30 to 35 MΩ within 5–8 min after the electrode seal. Recordings showing a sudden change in the resistance were abandoned and not used for the study. The CFTR inhibitor glybenclamide and the ENaC inhibitor amiloride were focally applied to the recorded cell using a multibarreled perfusion system (SF-77B, Warner Instruments, Hamden, CT, USA). Total transmembrane conductance was revealed by a voltage-ramp (a steady voltage change from -100 to 100 mV within 1.5 s). All the electrical signals were digitized, filtered (1 kHz) and acquired online using Clampex software and analyzed offline using Clampfit software (Axon Instruments). Data analysis was performed using the Clampfit program (pClamp 8.1; Axon Instruments).

Assessment of CCSP⁺ cells in the developmental hierarchy of HSC compartment

cDNA from cells of various hematopoietic developmental stages (kindly provided by Dr. Norman Iscove, Ontario Cancer Institute) were assayed for CCSP and MSC markers (CD90, CD105, CD73) by real time PCR to determine the developmental pathway of CCSP⁺ cells. Primers were specifically designed to recognize sequences within 300 base pairs of the 3' untranslated region of the gene Table 3-2.

Table 3-2: Primer sequences

Gene	Forward primer 5'-->3'	Reverse primer 5'-->3'
CD34	AGCTTCCTCTCCTGGGGTTA	CCCAATCCTCTCATCTCTGG
CCSP	CATCATGAAGCTCACGGAGA	GGGCAGTGACAAGGCTTTAG
CD90	GTCTTGCAACCTGCCTCTTC	GGAGAGGATCCTTGGGAAAG
CD105	ACTCAGACATGCCAGCCTA	TCAGCTTCTGTTTCCCCAAC
CD73	AAGGGCGGATCAAGTTCTCT	AAGGATCATTGCCCAAATG

***In vitro* chemotaxis assay**

Chemotaxis analysis *in vitro* was performed with CCSP⁺ and CCSP⁻ BMC. Recombinant murine SDF-1 (CXCL12, Peprotech, Rocky Hill, NJ) was added to a 24-well tissue culture plate (100 ng/ml) in 600 μ l of RPMI + 0.1% BSA. Transwell inserts with 3 μ m pore size (Corning, Corning, NY) were placed in each wells and 100 μ l of stimulated cells (2.5×10^5) were added to the top chamber. After overnight incubation at 37°C, migrated cells in the bottom chamber were recovered and counted. SDF-1-induced migration was blocked with anti-CXCR4 antibody (MAB21651, R&D Systems) for 30 minutes on ice prior to addition of cells to the Transwell.

Transmission Electron Microscopy

BMC sorted by CCSP expression were rinsed in PBS and fixed in suspension with 2.5% (v/v) glutaraldehyde in 0.1M phosphate buffer for 30 minutes. Cells were then postfixed in 1% (v/v) osmium tetroxide in 0.1 M cacodylate buffer, centrifuged and the cell pellet was dehydrated through graded alcohols. Followed by propylene oxide and embedded in Araldite resin. Ultrathin sections were stained in saturated uranyl acetate in 50% (v/v) ethanol and Reynolds lead citrate and examined with a JEM-1011 transmission electron microscope (JEOL Ltd, Tokyo, Japan).

Statistical Analysis

Data are presented as mean \pm SEM. For comparison of freshly isolated BMC and cultured plastic-adherent BMC phenotype one-way analysis of variance (ANOVA)

followed by Tukey's post-hoc tests (Prism 4.0) were performed. Statistical significance was defined as $p < 0.05$.

For patch clamp analysis, means were compared with Student's paired *t*-test where appropriate using the SigmaStat software from Jandal Scientific Co. A *P*-value < 0.05 was considered as significant.

Acknowledgements

Thanks to Dr. Xinghua Wang (Dr. Keating's lab) for performing the bone marrow transplants and Yewmeng Hung for helping with the electron microscopy studies.

3.4 Results

A CCSP-expressing population exists in the bone marrow of mice and humans.

Previously, BMC-derived cells were identified in the lung expressing CCSP as early as 1 day after intratracheal administration (Wong et al., 2007). This led us to examine the starting BMC population. A significant proportion of the adherent cells expressed CCSP. Moreover, freshly isolated bone marrow also contained a small percentage of cells which expressed CCSP on the cell surface (Figure 3-1A, arrow). Multiple anti-CCSP primary antibodies showed similar results. No cytokeratin-expressing cells were detected in the bone marrow (Wong et al., 2007). The small population of freshly isolated BMC that expressed CCSP ($1.9 \pm 0.1\%$) expanded in culture up to $25.8 \pm 9.5\%$ after 7 days (Figure 3-1B). Intracellular staining showed even higher expression. Western blotting of freshly isolated and cultured BMC confirmed the existence of CCSP protein (11kD) in BMC (Figure 3-1C). To further validate the

existence of these cells, we assessed the bone marrow of CCSP-null mice by FACS and could not detect any CCSP⁺ cells (Figure 3-1D). Real-time PCR detected the expression of CCSP gene in freshly isolated and cultured BMC of wild-type but not CCSP-null animals (Figure 3-1E). Neither cell surface nor intracellular staining of cultured CCSP-null BMC showed detectable CCSP⁺ cells. Bone marrow of the outbred strain CD1 and a transgenic strain K18EGFP (Wong et al., 2007) also had CCSP⁺ populations (Figure 3-3). Because CCSP is supposed to be an intracellular or secreted protein, the finding of detectable surface staining was surprising. FACS-sorting confirmed that CCSP⁺ BMC have appreciable levels of CCSP mRNA while CCSP⁻ cells do not (Figure 3-1F).

The increased proportion of CCSP⁺ cells seen over time in culture could be due to conversion of CCSP⁻ to CCSP⁺ cells or differential survival or proliferation of these 2 populations. To assess this, cultured BMC were sorted by FACS and examined for CCSP expression immediately after sort and after 14 days in culture. CCSP⁻ cells did not give rise to CCSP⁺ cells. Female CCSP⁻ and male CCSP⁺ cells were isolated by FACS and mixed together in a 4:1 ratio. CCSP⁺ cells almost doubled after 14 days in culture (Figure 3-2A). Assessment of SRY genomic DNA and CCSP mRNA using real time PCR showed a 2.5-fold increase in SRY DNA (Figure 3-2B) with a concomitant increase in CCSP mRNA (Figure 3-2C) suggesting CCSP⁺ cell proliferation as the cause of the increase in the proportion of CCSP⁺ cells.

A small CCSP⁺ BMC population was also found in healthy human volunteers (Table 3-3). The number of CCSP⁺ cells in the freshly isolated bone marrow of donors show variability independent of age or sex. Real time PCR show detectable levels of CCSP mRNA in 4 of 5 of the human bone marrow samples (Figure 3-4).

Table 3-3: Identification of CCSP⁺ cells in human bone marrow. Human volunteer marrow were kindly provided by Dr. Keating with an approved REB from University Health Network.

Donor #	Sex	Age (yrs)	% CCSP⁺ cells
1	Male	24	2.8%
2	Male	67	4.34%
3	Female	45	2.3%
4	Male	60	0.6%
5	Female	20	1.2%

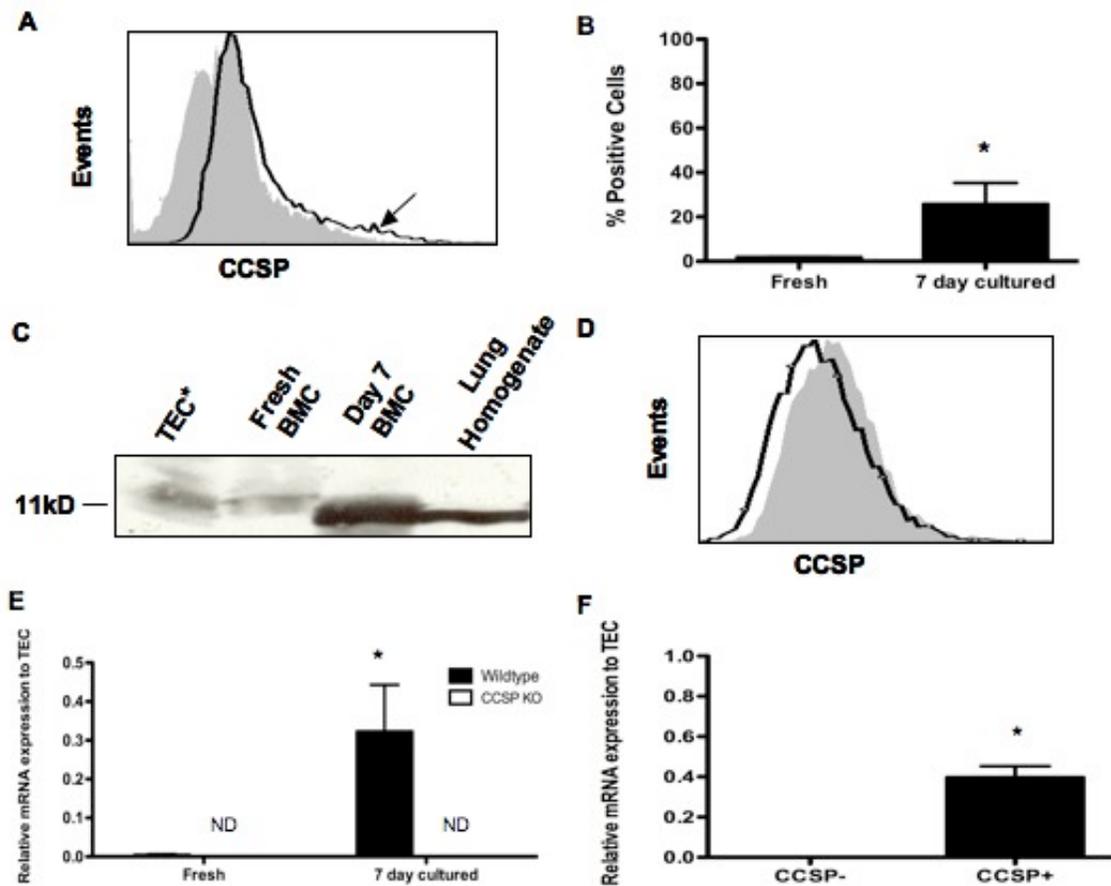


Figure 3-1 Identification of a bone marrow CCSP-expressing population.

(A) Representative flow cytometry histograms (solid black line) of freshly isolated BMC from C57Bl/6 mice compared to isotype staining (grey filled) show a small population (arrow) of BMC-expressing CCSP. (B) Freshly isolated bone marrow contain 2% CCSP⁺ cells which expand in culture after 7 days up to 25% (*P<0.001 compared to fresh BMC). (C) Western blot analysis confirms the presence of a specific 11kD band corresponding to CCSP protein in whole lung homogenate and tracheal epithelial cells (TEC, *10-times (15 µg total) less protein was loaded compared to BMC and lung tissue (150 µg)), freshly isolated, and 7-day cultured BMC. (D) No CCSP-expressing BMC population can be detected in CCSP-null mice. (E) Real time PCR confirms the presence of a CCSP gene transcript in freshly isolated and cultured BMC of wild-type but not CCSP-null animals (ND = not detectable). GAPDH was used as a housekeeping gene for normalization of expression levels. Each bar represents normalized levels relative to TEC. Each bar represents mean ± SEM (n≥3). *P<0.01 compared to “Fresh”. (F) Real time PCR detects CCSP gene expression in CCSP⁺ cells but not CCSP⁻ cells. *P<0.01 compared to CCSP⁻ cells.

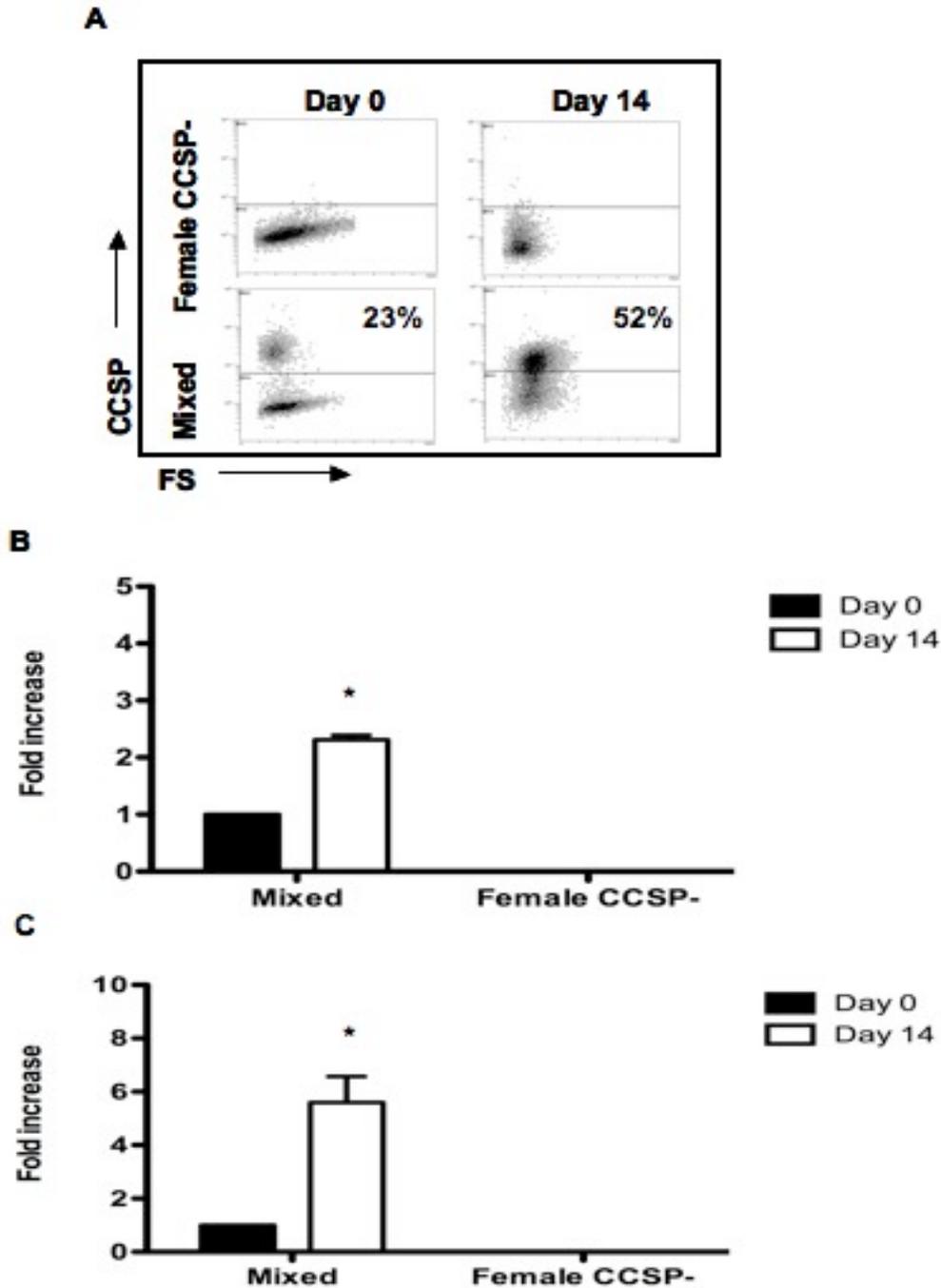


Figure 3-2 CCSP⁺ cells can proliferate in culture.

(A) Female CCSP⁻ cells cultured alone on plastic for 14 days show no detectable CCSP⁺ cell while a mixed population (Female CCSP⁻ and male CCSP⁺ cells, 1:4) show an increase in CCSP⁺ cells after 14 days in culture. (B) Real time PCR shows a 2.5-fold increase in SRY mRNA after 14 days in culture in the mixed population. (C) The increase in CCSP⁺ cell number corresponds to an increase in CCSP mRNA. Each bar represents mean \pm SEM ($n \geq 3$). * $P < 0.01$ compared to Day 0.

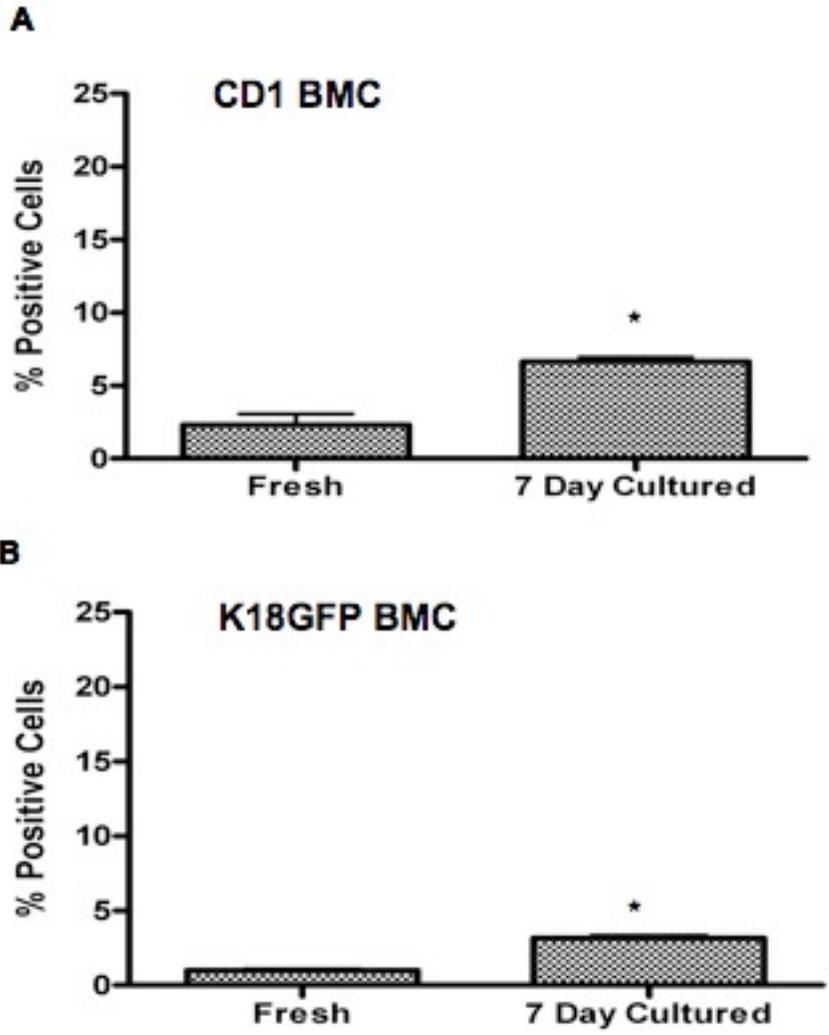


Figure 3-3 CCSP⁺ BMC are also found in other mouse strains

Culturing BMC on plastic cultures for 7-days results in an increase in CCSP⁺ cells.
 *P<0.05 compared to “Fresh” BMC.

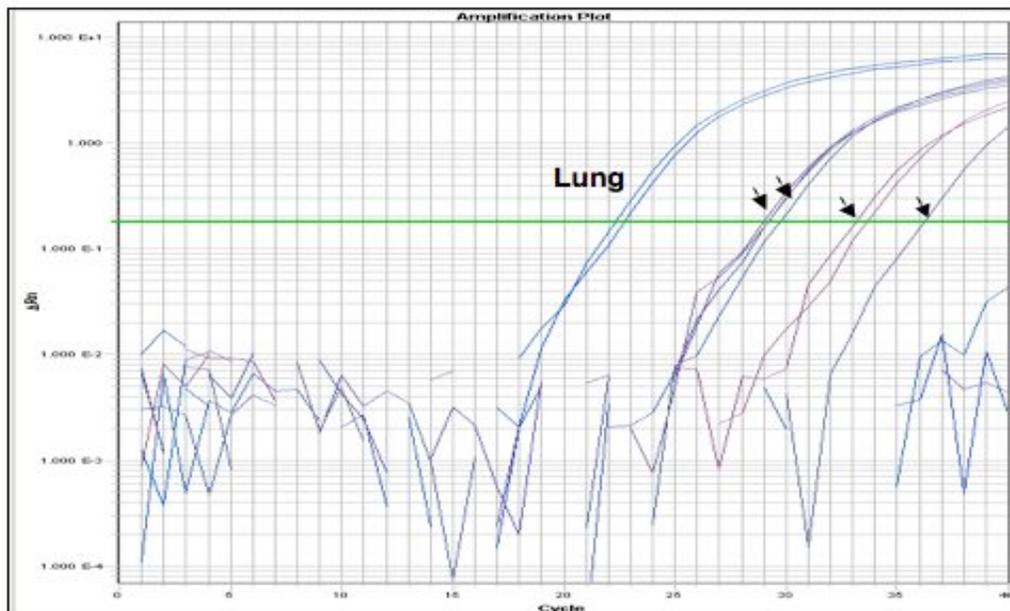


Figure 3-4 Human BMC express CCSP mRNA

Real time PCR demonstrate CCSP expression in 4 of 5 of the human BMC samples. Human lung epithelial cells were used as positive controls for PCR. GAPDH was used as housekeeping gene for normalization of expression levels. Each bar represents normalized relative levels compared to epithelial cells. Arrows point to amplification curve for 4/5 human bone marrow samples.

Characterization of CCSP⁺ BMC

Morphologically, CCSP⁺ cells were small (~5-10 μm), round cells with small cytoplasmic extensions. In contrast, CCSP⁻ cells (>10 μm) ranged from very large cells with a lot of cytoplasm to medium-sized cells with large cytoplasmic extensions (Figure 3-5).

To determine the hematopoietic or mesenchymal origin of CCSP⁺ cells, 7-day plastic-adherent CCSP⁺ and CCSP⁻ cells were isolated by FACS and plated on slide chambers for an additional 4 days to recover from post-sort stress and stained for various phenotypic markers. Phenotypic characterization of the CCSP⁺ and CCSP⁻ cells show that in contrast to CCSP⁻ cells and in spite of their ability to adhere, the majority of CCSP⁺ cells expressed CD45 and CD34 (Figure 3-5). Few CCSP⁺ cells expressed CD31 and CD133. CD117 (c-kit receptor) was expressed regularly by CCSP⁺ and occasionally by CCSP⁻ cells. The majority of the CCSP⁺ cells also expressed the mesenchymal markers CD73, CD90, and CD105 (Figure 3-6A) but not CD106, collagen Type IV or vimentin (Figure 3-7B). MSC markers were observed in some but not all CCSP⁻ cells. Appropriate isotype controls were used for each specific antibody (shown as an inset). Mouse MSC were used as positive controls for MSC markers (Figure 3-7A). To determine whether the same CCSP⁺ cells expressing mesenchymal cell markers were also expressing hematopoietic markers, double immunofluorescence staining for CD90 or CD105 and CD45 were performed. The same CD90 or CD105-expressing CCSP⁺ co-expressed CD45 (Figure 3-6B) but not CCSP⁻ cells (Figure 3-6C). As controls, MSC and a mouse fibroblast cell line NIH3T3 were double stained for CD90 or 105 and CD45 (Figure 3-8). Photomicrographs without an inset share the same isotype staining controls as the image

immediately to the left. As another staining control, the green AlexaFluor secondary antibody was added to anti-CD45-PE stained cells to eliminate possibility of cross-binding of the secondary to anti-CD45-PE. No green signal was found in these control slides (Figure 3-9). CCSP⁺ cells did not express von Willebrand factor (a marker of mature endothelial cells) or collagen Type I⁺ (Figure 3-7B). Real time PCR confirmed CD90, CD105, CD73, (Figure 3-10A) and CD34 and CD45 (Figure 3-10B) gene expression in CCSP⁺ cells. Assessment of the hematopoietic cell lineages at various developmental stages from early HSC stage to lineage-committed cells show no detectable levels of CCSP mRNA by real time PCR suggesting CCSP⁺ cells may not arise from any of the known hematopoietic cell lineage (Figure 3-11). Freshly isolated bone marrow cells were sorted by CCSP-expression and analyzed for mesenchymal and hematopoietic markers by flow cytometry immediately after sorting (Table 3-4). Subpopulations of freshly isolated CCSP⁺ cells expressed CD90 (14.4%), CD105 (22.3%), Sca-1 (12.3%), and CD31 (12.3%). A smaller percentage expressed CD73 (3.2%) and CD34 (1.3%). A greater proportion expressed CD45 (40%). However the number of CCSP⁺ cells that expressed both CD45 and CD90 or CD105 were small (2% and 13% respectively) but expanded over 2-fold after 7 days in culture (4% and 38.7% respectively). Both freshly isolated and cultured CCSP⁻ cells expressed the mesenchymal markers CD90, CD105, CD73 which expanded in culture but did not express any of the hematopoietic markers (Table 3-5).

CCSP is a secretory protein and to determine how BMC could be sorted by surface CCSP expression, CCSP⁺ and CCSP⁻ cells were stained for cubulin, a surface receptor for CCSP in the proximal tubules of the kidney involved in CCSP uptake and

catabolism (Burmeister et al., 2001). All CCSP⁺ cells and rare CCSP⁻ cells expressed cubulin (Figure 3-12).

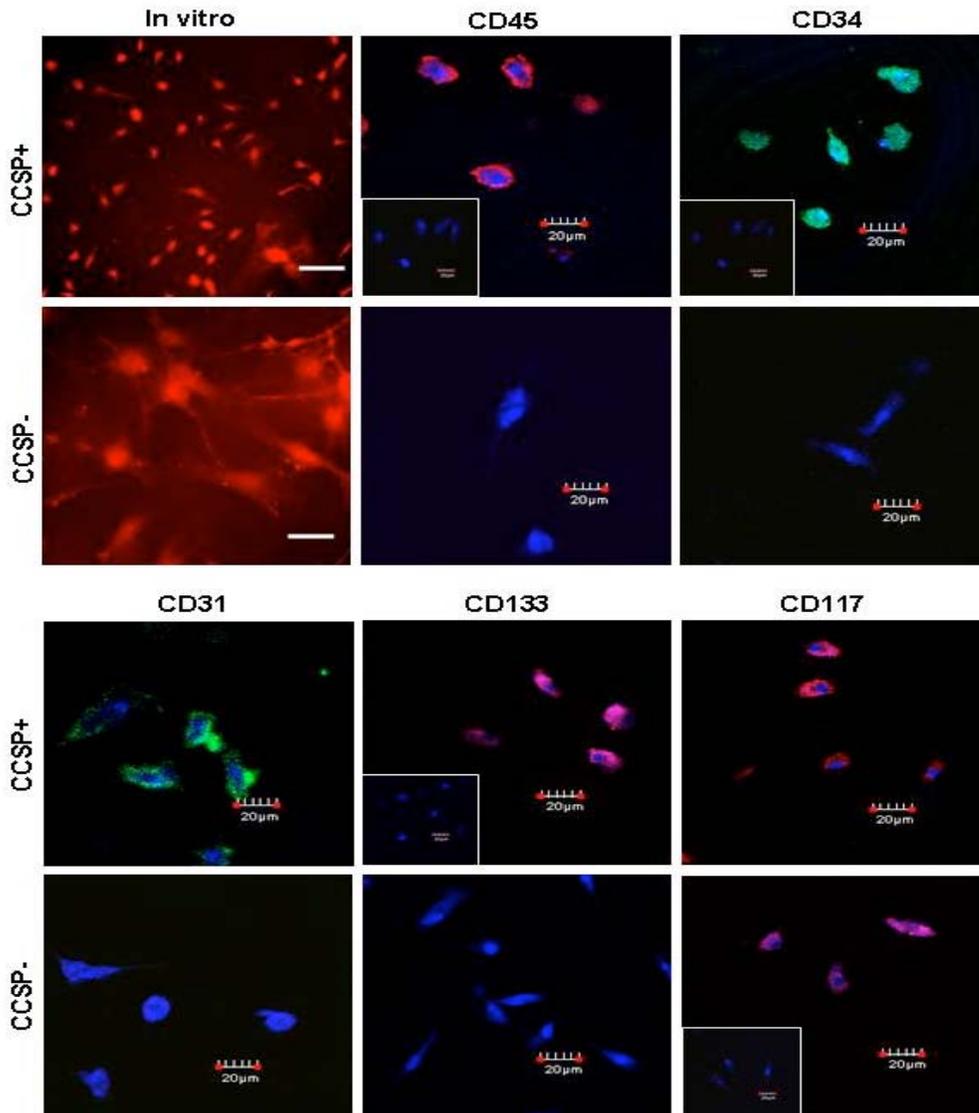


Figure 3-5 Hematopoietic characterization of the CCSP⁺ BMC population

Morphologically, CCSP⁺ and CCSP⁻ cells are distinguishable in vitro. CCSP⁺ cells are small rounder cells while CCSP⁻ cells are larger with more cytoplasmic extensions. CCSP⁺ cells express all hematopoietic markers including markers of well-differentiated cells, CD45 and CD34, and the stem/progenitor cell markers CD31, CD133, CD117. A few CCSP⁻ cells express CD117 but no other hematopoietic markers. White scale bars represents 50 microns. N_≥4 per group.

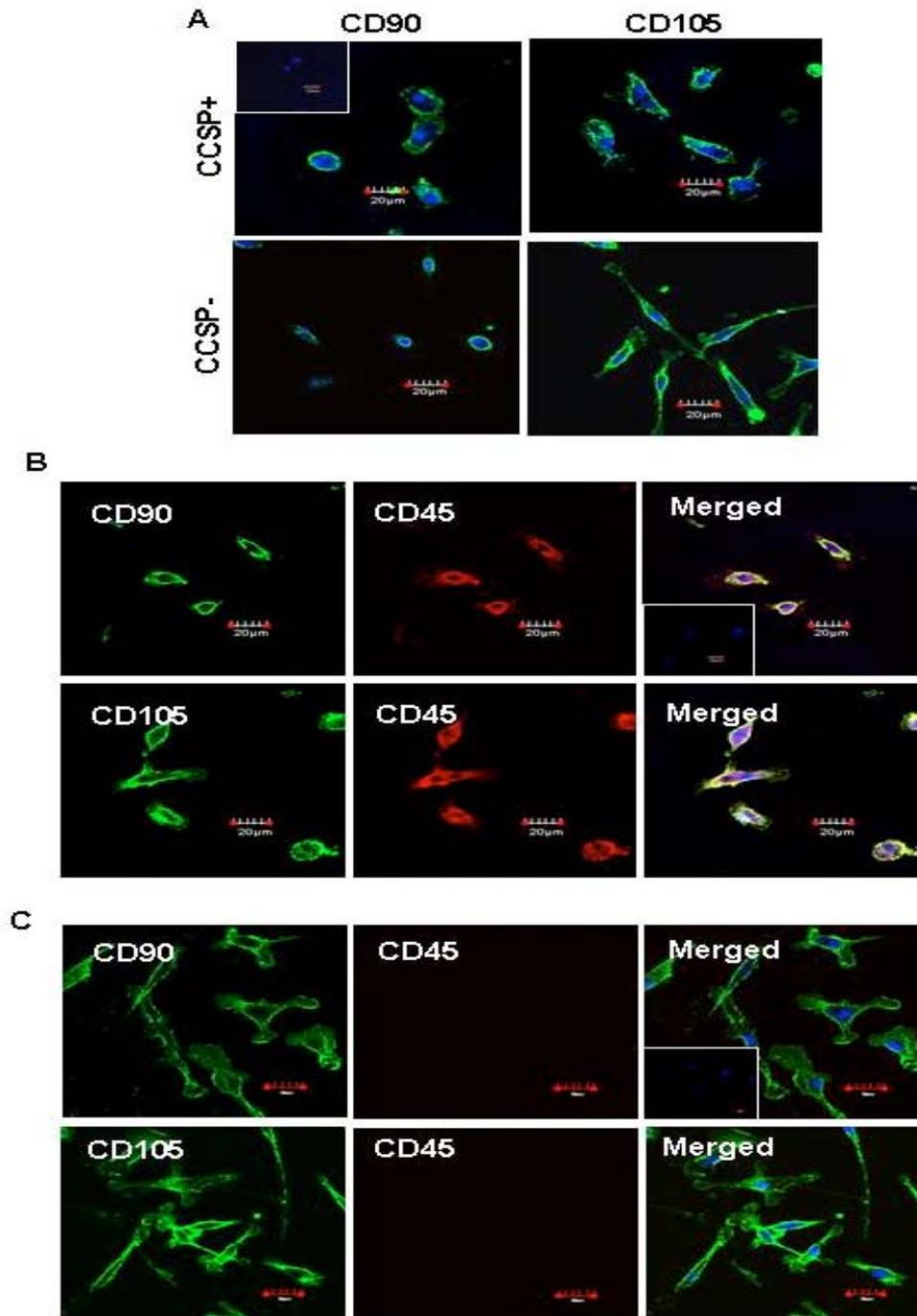


Figure 3-6 Mesenchymal characterization of CCSP⁺ and CCSP⁻ BMC.

(A) Both CCSP⁺ and CCSP⁻ cells express the mesenchymal cell markers CD90 and CD105. (B) Most CCSP⁺ cells co-express both hematopoietic and mesenchymal markers, CD45 and CD90/105 respectively (C) but not CCSP⁻ cells. Insets are representative isotype staining controls. Isotype stains for boxes without inset are the same as the previous image. Hoechst counterstain was used to visualize nuclei (blue). N \geq 4 per group.

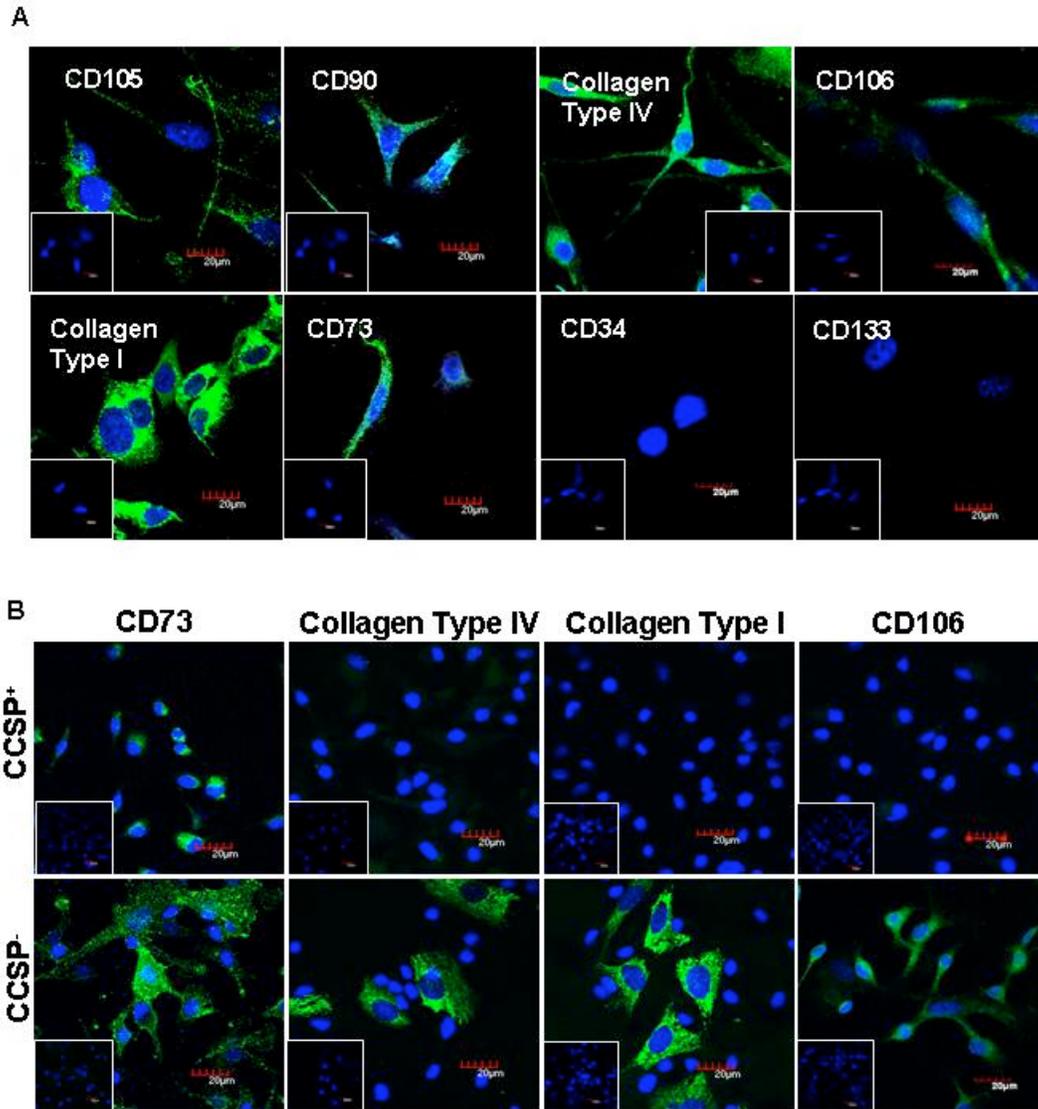
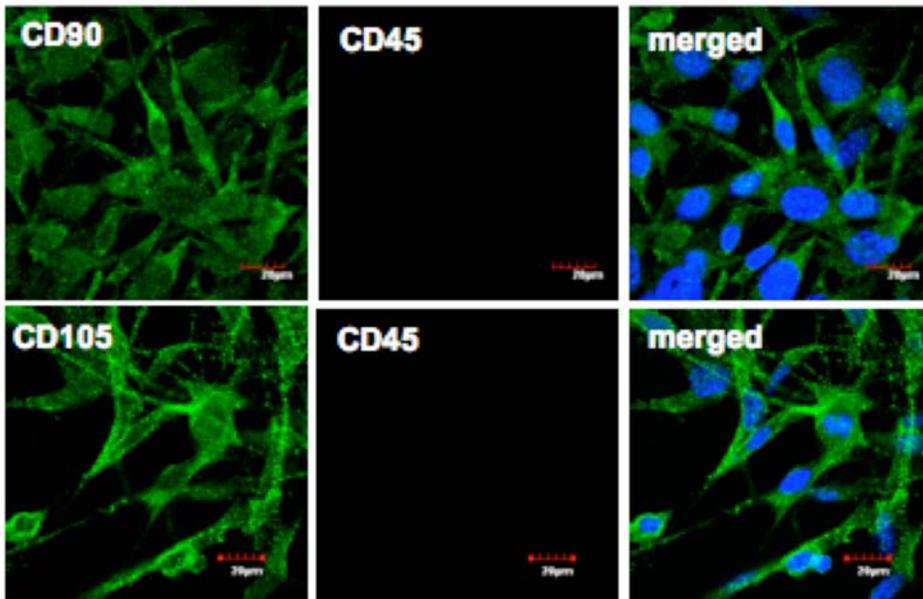


Figure 3-7 CCSP⁺ cells express some but not all mesenchymal stem cell or circulating fibrocyte markers.

(A) Mouse mesenchymal stem cells were used as positive cell controls for the immunostaining. Mesenchymal stem cells showed positive staining for CD73, CD90, CD105, CD106, collagen Type I and IV but not hematopoietic stem cell markers CD34 and CD133. Inset shows respective isotype staining. (B) Representative photomicrographs of CCSP⁺ and CCSP⁻ cells stained for other mesenchymal stem cell and fibrocyte markers. CCSP⁺ cells express CD73 but not CD106, collagen Type I and IV, markers of MSC. Some CCSP⁻ cells express CD73, collagen Type IV and collagen Type I, a marker of circulating fibrocytes. Inset shows respective isotype staining. N \geq 4 per group.

Mesenchymal stem cells



Fibroblasts

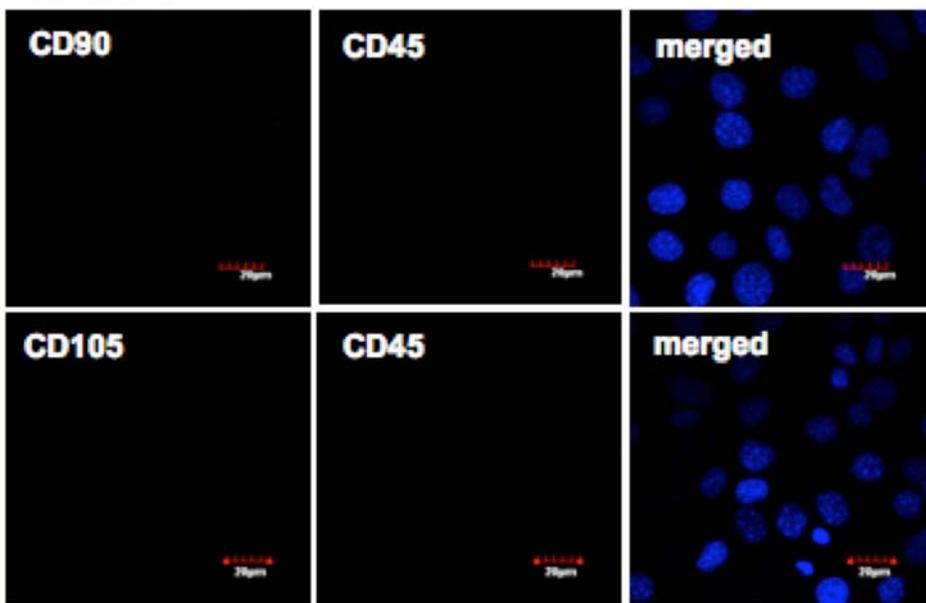


Figure 3-8: Mouse MSC and fibroblasts were used as negative controls for double staining for CD45 and CD90/CD105.

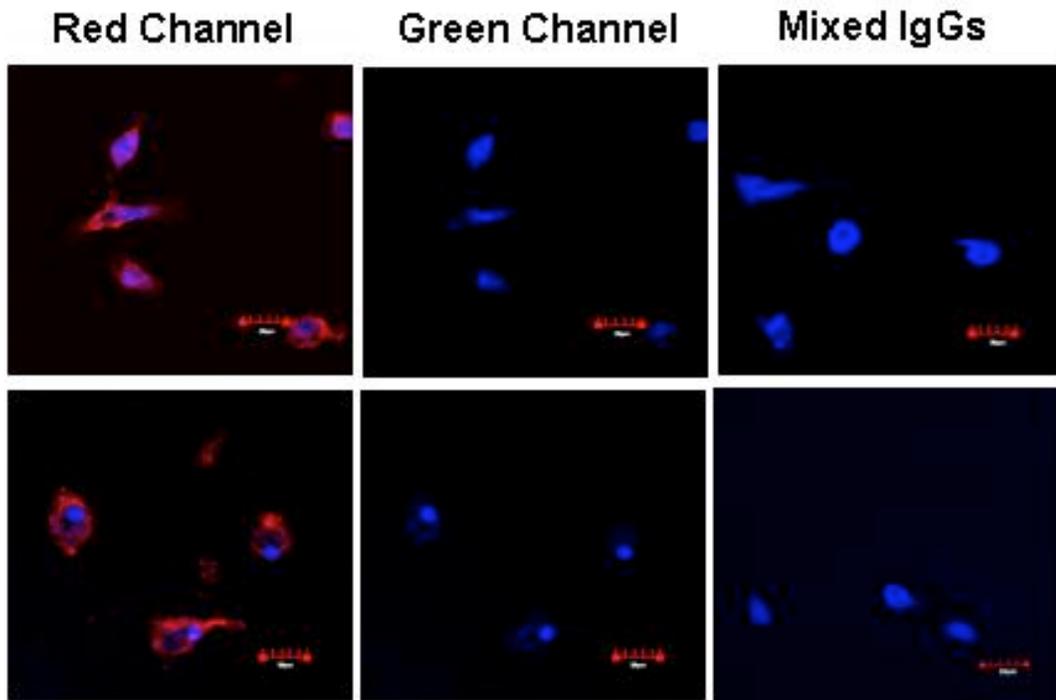


Figure 3-9 No cross reactivity of secondary Alexa Fluor IgGs (green channel) with anti-CD45-PE (red channel) primary antibody. Alexa Fluor 488 IgG stained on CCSP⁺ cells. Mix of isotypes show no cross reactivity either.

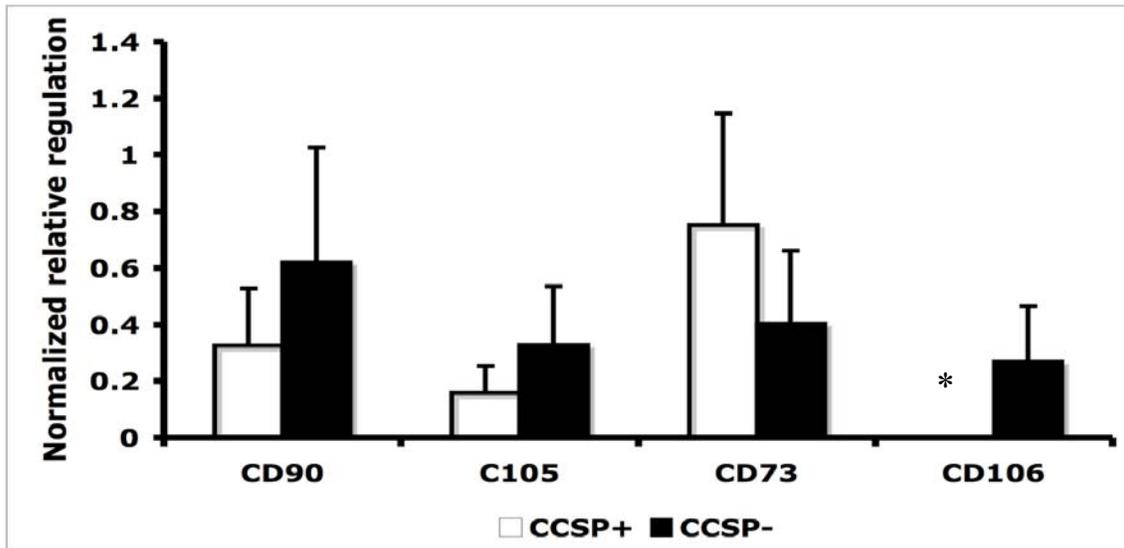
**Table 3-4: Flow cytometric analysis of bone marrow CCSP⁺ cell phenotype (N=3).
Mean±SEM**

	Fresh	7-day cultured
CD90	14.4 ± 3.5%	8.7 ± 2.1%
CD105	22.3 ± 5.5%	58.5 ± 4.4%
CD73	3.2 ± 0.9%	5 ± 2%
Sca-1	12.3 ± 7.5%	13.3 ± 5.4%
CD45	40 ± 4.7%	81.5 ± 5.1%
CD34	1.3 ± 2.3%	6.5 ± 5.8%
CD31	12.3 ± 7.6%	1.8 ± 0.7%
CD14	0 ± 0.6%	0 ± 0.4%
CD90/CD45	2 ± 0.4%	4 ± 1.1%
CD105/CD45	13 ± 3.1%	38.7 ± 6.2%

**Table 3-5: Flow cytometric analysis of bone marrow CCSP⁻ cell phenotype (N=3).
Mean±SEM**

	Fresh	7 day cultured
CD90	2.7 ± 0.8%	13.5 ± 2.8%
CD105	0.4 ± 0.3%	68.3 ± 10.1%
CD73	11.4 ± 2.7%	6.5 ± 1.9%
Sca-1	5.6 ± 0.7%	10.3 ± 6.1%
CD45	0 ± 0.5%	0 ± 0.4%
CD34	0 ± 0.1%	0 ± 0.1%
CD31	0 ± 0.1%	0
CD14	0 ± 0.3%	1.3 ± 0.5%
CD90/CD45	0 ± 0.2%	0 ± 0.3%
CD105/CD45	0 ± 0.1%	0 ± 0.3%

A



B

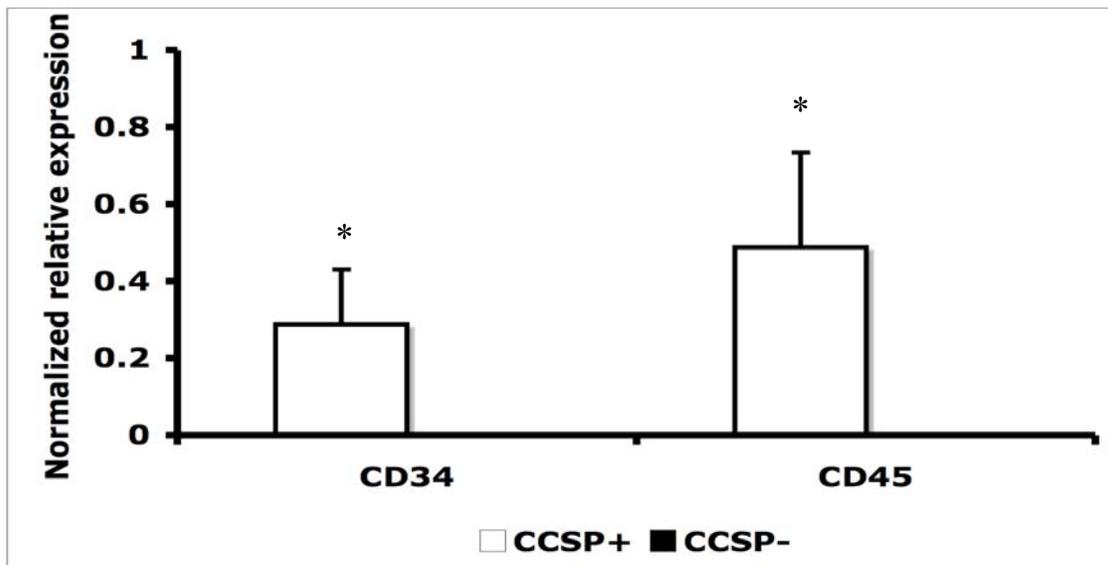


Figure 3-10 Real time PCR confirms mRNA expression of mesenchymal and hematopoietic cell markers in sorted 7-day cultured BMC.

(A) CCSP⁺ cells express certain MSC genes (CD90, CD105, CD73) but not other marker CD106. CCSP⁻ cells express CD90, CD105, CD73, CD106. (B) CCSP⁺ cells also express hematopoietic markers CD34 and CD45 but not CCSP⁻ cells. N=3 per group. *P<0.01 compared to CCSP⁻ cells.

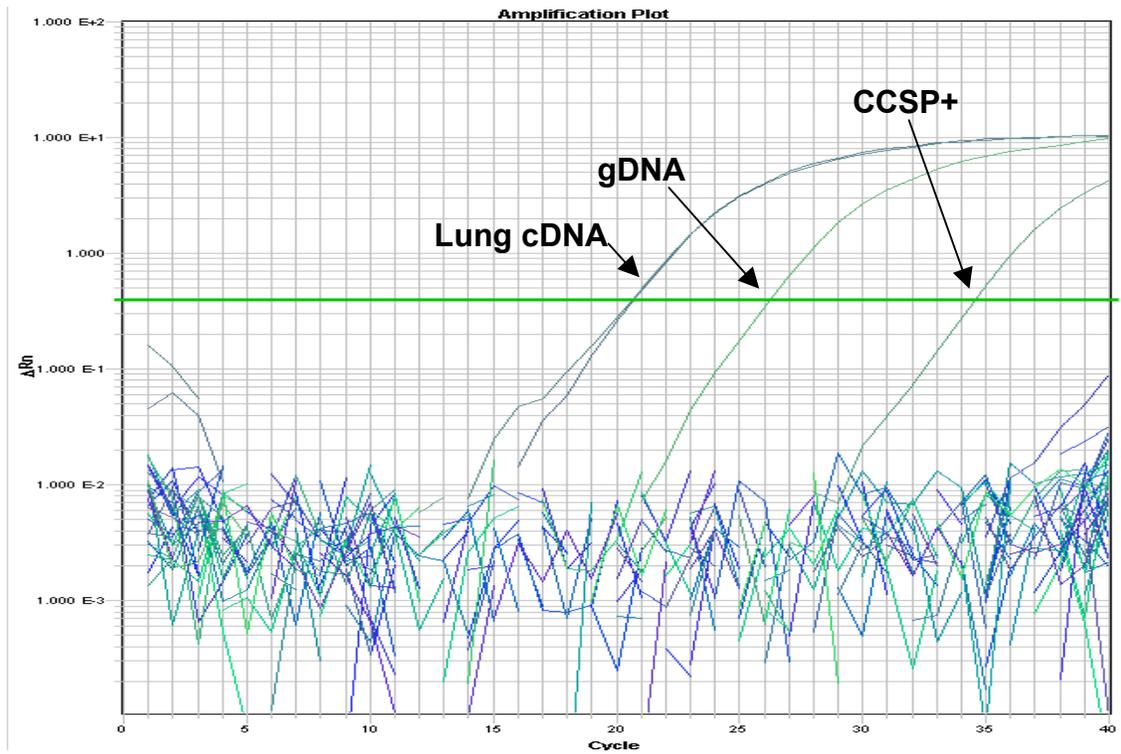


Figure 3-11: Hematopoietic cells do not express CCSP mRNA

Real time PCR amplification data demonstrating lack of CCSP mRNA in all hematopoietic cell lineages except from lung cell extracts, genomic DNA from early hematopoietic stem cell lineage and CCSP⁺ BMC.

Multi-epithelial lineage differentiation of CCSP⁺ BMC

To determine whether CCSP⁺ cells could give rise to other epithelial cell lineages, plastic-adherent BMC were sorted into CCSP⁺ and CCSP⁻ cells after 7 days in culture. After an additional 2 weeks they were then cultured in air-liquid interface conditions for 4 weeks. Airway epithelial cells were used as positive controls for both the air-liquid interface cultures and immunofluorescence staining (Figure 3-13A). To ensure specificity of colocalized fluorescent signals, control tracheal epithelial cells were stained with one specific primary antibody, excited with the appropriate excitation (350, 488, 532, or 633nm) lasers, while monitoring emission wavelength images for the specific fluorophore and the other channels used in the experimental groups. Mixed IgGs were used as control staining to ensure specificity of the primary antibodies (Figure 3-13B). Background fluorescence was low in the other fluorophore channels suggesting observed co-localization was not an artifact of “bleed-through” between the fluorescence channels.. Cell suspensions were prepared by cytopsin for immunofluorescence staining. CCSP⁺ cells can give rise to SP-C⁺ (type II alveolar cell marker), AQP5⁺ (type I alveolar cell marker) and SP-C⁺AQP5⁺ cells (Figure 3-14). CCSP⁻ cells only gave rise to SP-C⁺ cells (Figure 3-15). In addition, CCSP⁺ cells can also give rise to K14⁺ (basal cell marker), acetylated α -tubulin⁺ (ciliated cell marker), and acetylated α -tubulin⁺SP-C⁺ cells (Figure 3-14). Most CCSP⁺ cells cultured under air liquid interface for 4 weeks lose CCSP expression and gain other epithelial characteristics such as protein expression of SP-C, K14 and/or AQP5. Few cells that remain CCSP⁺ do coexpress several other epithelial markers. Furthermore, ALI cultured CCSP⁺ cells lose the hematopoietic marker CD45 and mesenchymal markers CD90/CD105 (Figure 3-16).

Immediately post-sorted CCSP⁺ cells did not express other epithelial genes except very low levels of SP-C whereas CCSP⁻ cells did not express any epithelial genes (Figure 3-17A) After 2 weeks of culture, the level of all epithelial markers increased in CCSP⁺ cells while only SP-C mRNA levels increased in CCSP⁻ cells (Figure 3-17B).

After 4 weeks of air-liquid interface culture, epithelial gene expression (CCSP, SP-C, AQP5, K5, K14, K18) was increased in CCSP⁺ cells while only SP-C expression was seen in CCSP⁻ cells (Figure 3-18).

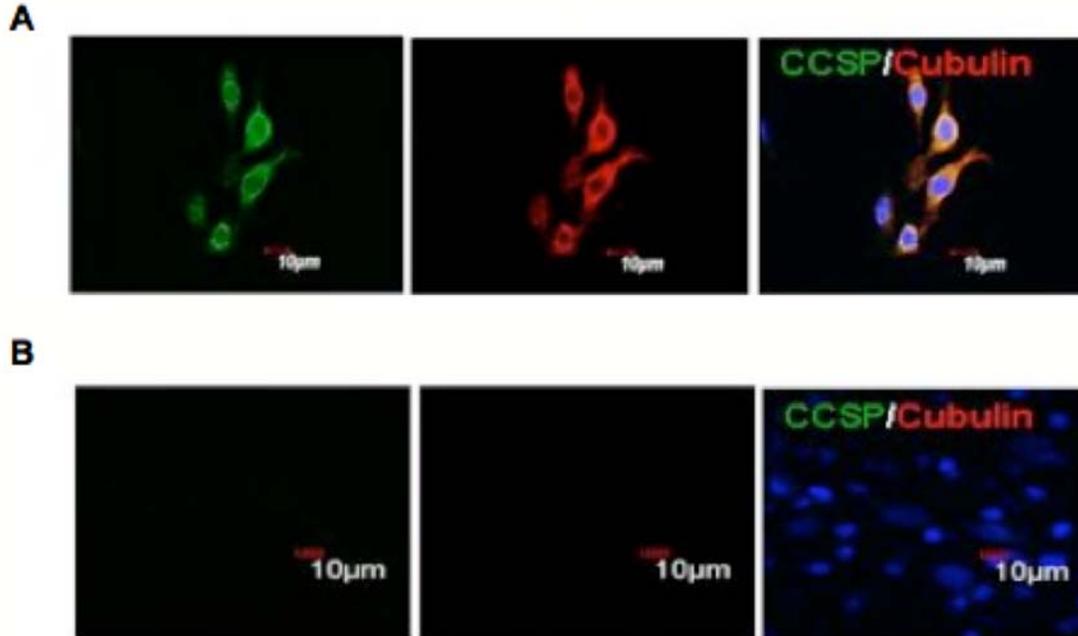


Figure 3-12: CCSP⁺ cells express a receptor known to bind CCSP protein on the cell surface.

CCSP⁺ cells express cubulin on the cell surface (A) but not CCSP⁻ cells (B). Hoechst counterstain was used to show all nuclei (blue).

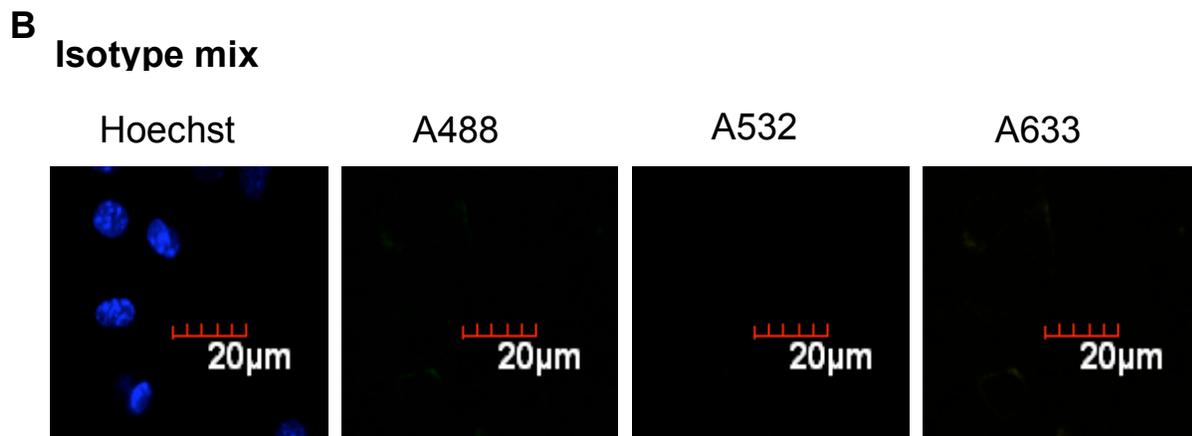
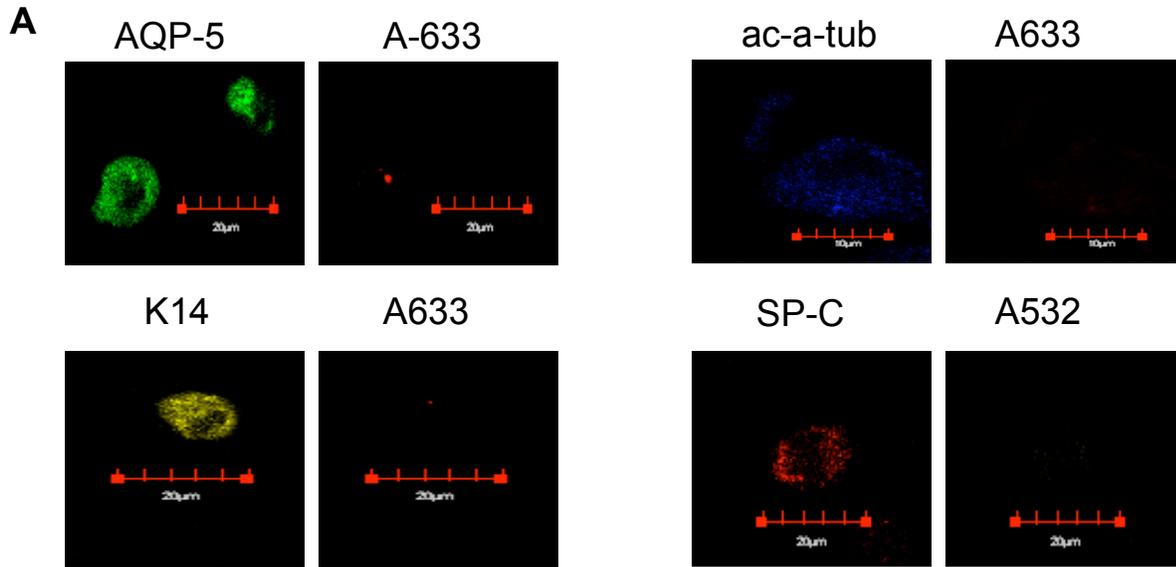


Figure 3-13: Immunofluorescence staining for airway epithelial markers - Airway epithelial cells.

Airway epithelial cells were cultured under air liquid interface for 4 weeks, isolated and stained for various epithelial markers. **(A)** To ensure no “bleed through” of the fluorescence from the signal, the specific primary antibody was excited with the appropriate excitation (350, 488, 532, or 633nm) lasers, while monitoring emission wavelength images for the specific fluorophore. **(B)** Mixed isotypes were stained to ensure specificity of the primary antibodies.

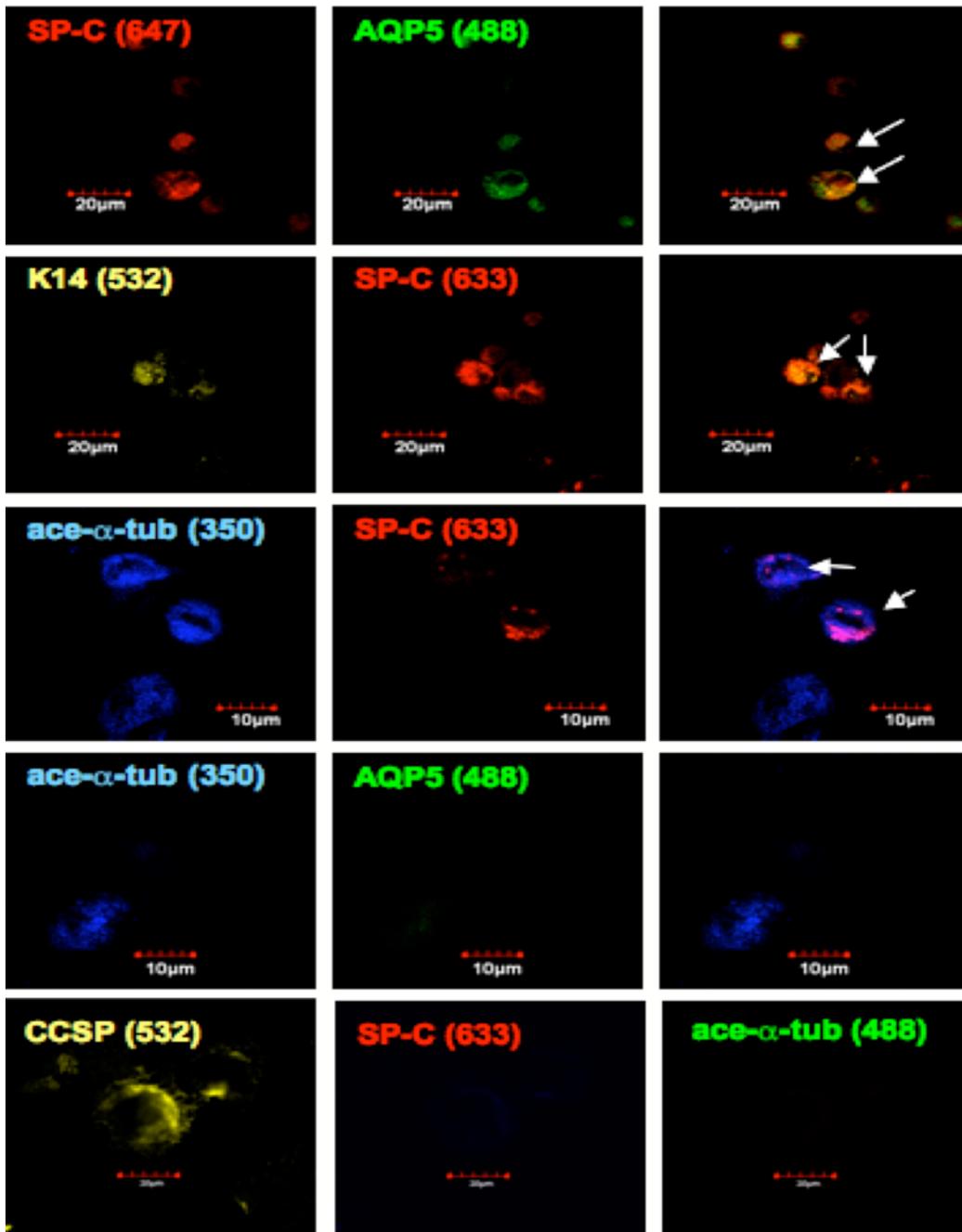


Figure 3-14 Epithelial multilineage differentiation potential of CCSP⁺ BMC

After 4 weeks in air-liquid interface culture, CCSP⁺ cells can give rise to a multitude of epithelial lineage cells including SP-C⁺ only, SP-C⁺AQP5⁺, SP-C⁺K14⁺, K14⁺ only, acetylated α -tubulin⁺ only, acetylated α -tubulin⁺SP-C⁺ cells where as CCSP⁻ cells only gave rise to SP-C⁺ cells. White arrow points to double positive cells. Inset is a representative isotype staining control. Hoechst counterstain was used to visualize nuclei (blue). In parenthesis is the fluorescent emission wavelength for the stains.

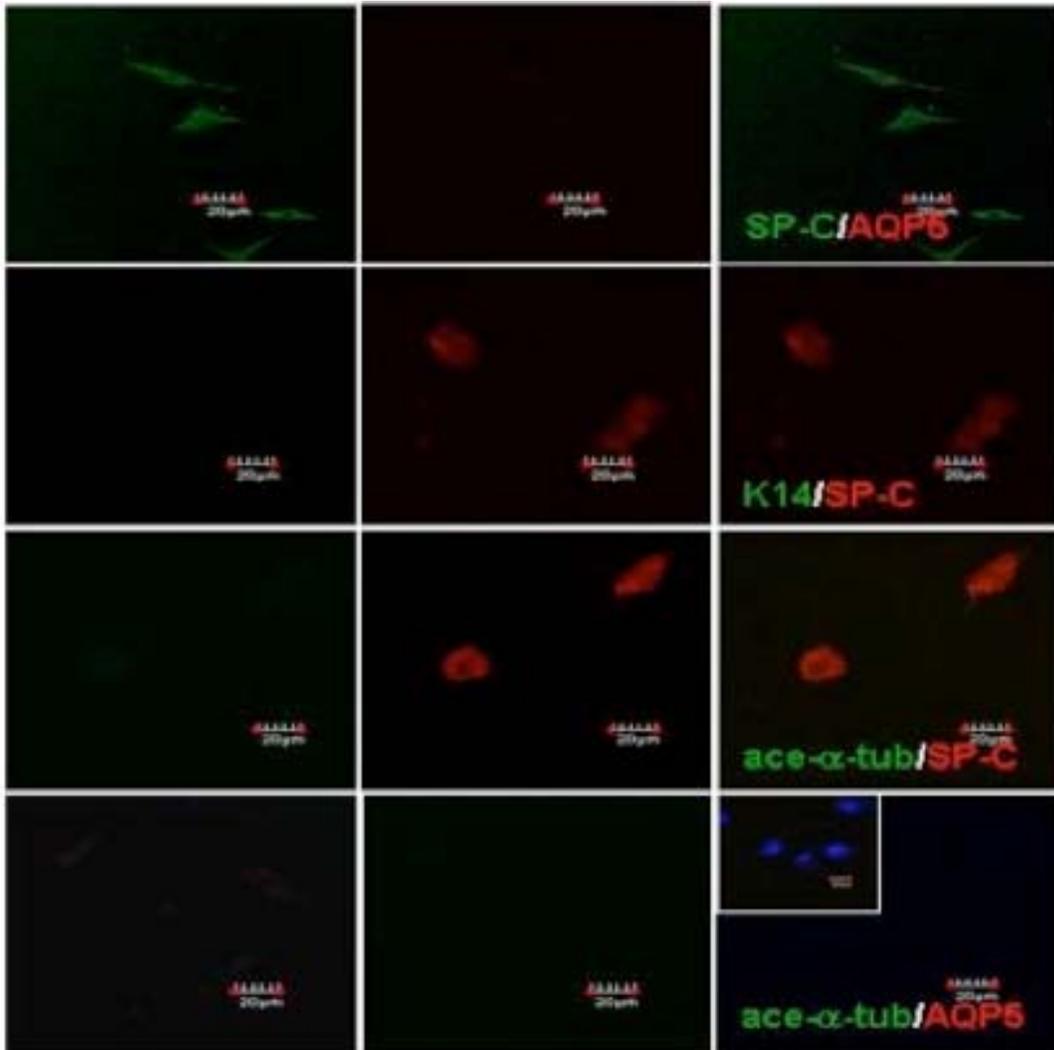
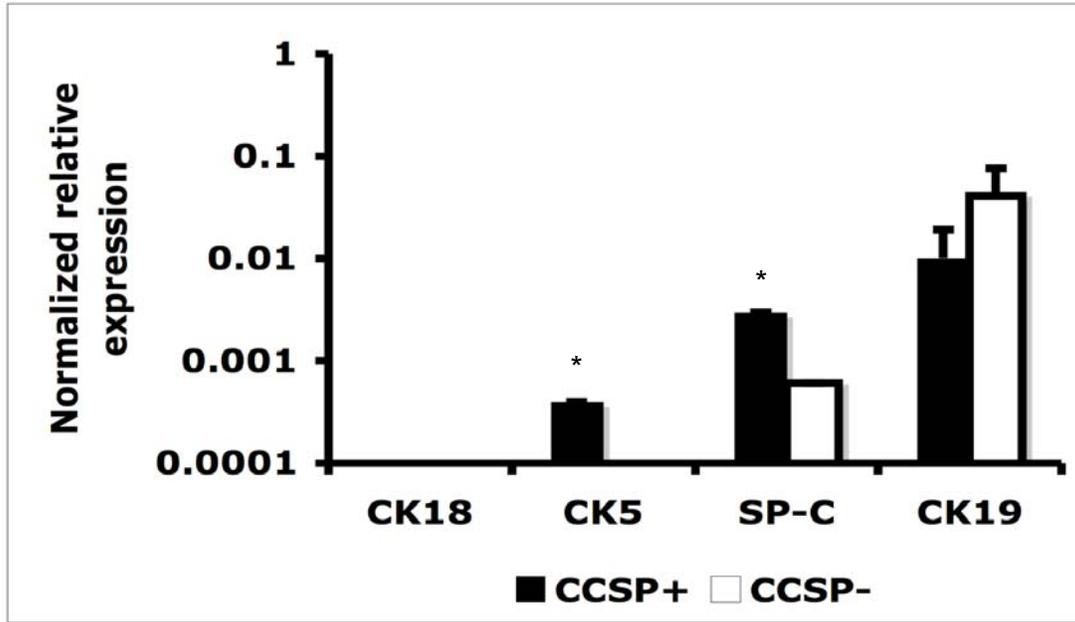


Figure 3-15 CCSP⁻ BMC do not have multilineage differentiation potential.

CCSP⁻ cells only give rise to SP-C-expressing cells after air liquid interface cultures. Insets are representative isotype staining controls. Hoechst counterstain was used to visualize nuclei (blue).

A



B

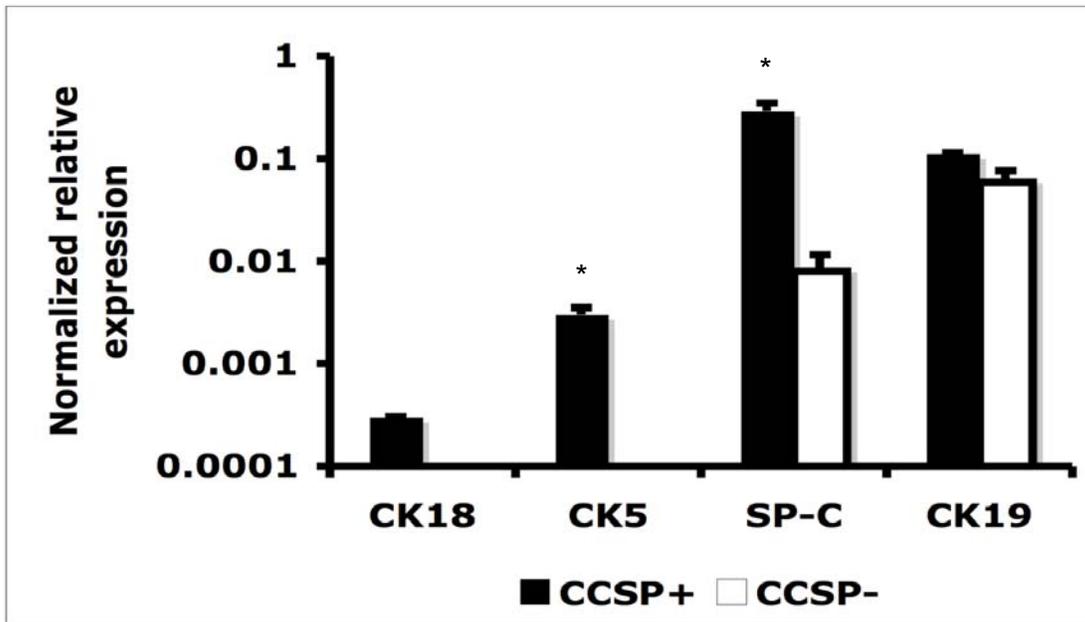


Figure 3-16 Real time PCR comparing immediately post-sorted and cultured post-sorted cells.

(A) BMC immediately after sorting show low but detectable levels of epithelial-specific (CK5, SP-C, CK19) gene expression in CCSP⁺ cells. CCSP⁻ cells have low levels of SP-C and CK19. (B) Sorted cells after 14 days of culture show higher levels of CK5, SP-C and CK19 and the expression of CK18. GAPDH was used as housekeeping gene for normalization of expression levels. Each bar represents normalized relative levels compared to TEC. n=4 sets of cells from four different animals. *P<0.01 compared to CCSP⁻ cells.

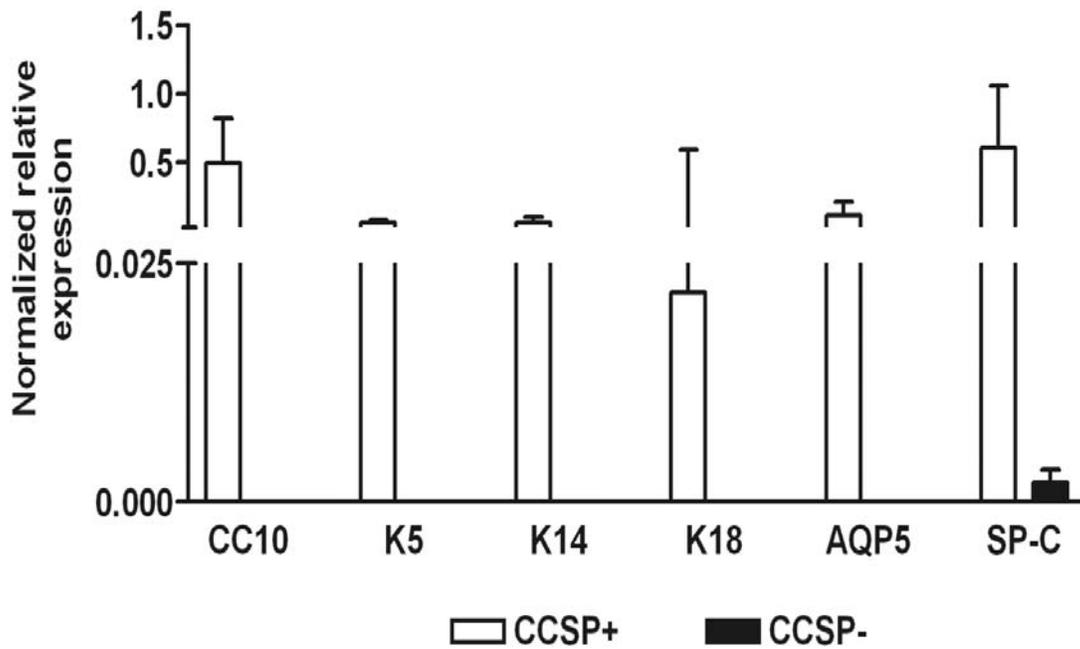


Figure 3-17 Real time PCR confirms mRNA expression of epithelial markers of air-liquid interface cultured cells

CCSP⁺ cells (white bar) cultured under air-liquid interface for 4 weeks express CCSP, K5, K14, AQP5 and SP-C mRNA. CCSP⁻ cells (black bar) only express SP-C mRNA. Normalized to house-keeping gene GAPDH and expressed as relative expression to lung epithelial cells. N=3 sets of cells per group.

Bone marrow CCSP⁺ cells are differentially retained in injured airways

To determine whether the CCSP⁺ BMC more efficiently retained in the lung, 7-day plastic-adherent BMC were sorted into CCSP⁺ and CCSP⁻ populations. Cells were plated, cultured for 2 additional days to recover following the stress of sorting, and labelled with CMTMR prior to injection transtracheally or intravenously into naphthalene-injured recipients as previously described (Wong et al., 2007). Four days following cell injection, the mice were sacrificed and lungs harvested. Donor BMC were detected by fluorescence microscopy for CMTMR (left lung) and retention quantified by real-time PCR for the SRY gene (right lung). When injected into the injured lungs more CCSP⁺ cells were found in or on the airway epithelium than CCSP⁻ cells (Figure 3-18A). Of the 10⁶ BMC injected (to both lungs), about 10% of the CCSP⁺ cells were detected in the right lung after transtracheal delivery compared to about 1% after intravenous delivery. Retention in the lung was far greater for CCSP⁺ than CCSP⁻ cells (*p<0.01, Figure 3-18B).

Bone marrow CCSP⁺ cells express epithelial ion channel proteins and have some epithelial ion transport properties following air liquid interface cultures

Epithelial ion channel (ENaC and CFTR) and tight junction protein (E-cadherin) mRNA was detected in CCSP⁺ BMC but not CCSP⁻ cells by real time PCR (Figure 3-18C). Double immunofluorescence staining for CFTR and ENaC showed that the CCSP⁺ cells express both proteins (Figure 3-19A) while most CCSP⁻ cells express neither. Rare CCSP⁻ cells express small levels of CFTR but not ENaC. Real time PCR confirm mRNA expression of both CFTR and ENaC in CCSP⁺ cells and low and undetectable levels of

CFTR and ENaC respectively in CCSP⁻ cells. Perforated patch clamp recordings were carried out in CCSP⁻ and CCSP⁺ cells to examine whether these cells express functional CFTR and ENaC. The total transmembrane conductance at different levels of membrane potential (V_M) was revealed by a voltage-ramp, and the CFTR- and ENaC-mediated conductance was determined by addition of forskolin (3 μ M), glybenclamide (100 μ M) and amiloride (100 μ M), respectively, to the bath solution (Figure 3-19B). We found that the total conductance in CCSP⁺ cells was larger than that in CCSP⁻ cells (Figure 16B; Measured at -50 mV, CCSP⁺: -205 ± 12 pA, n = 5 cells; CCSP⁻ cell: -54 ± 8 pA, n =5 cells; P < 0.005). Application of forskolin and glybenclamide, respectively, did not alter the total conductance of CCSP⁺, suggesting that CFTR is not functional. Amiloride significantly suppressed the total conductance (Figure 3-19B), suggesting that ENaC is indeed functional in these cells. Consistent with their lack of ion channel expression, treating CCSP⁻ cells with glybenclamide and amiloride did not change their total conductance.

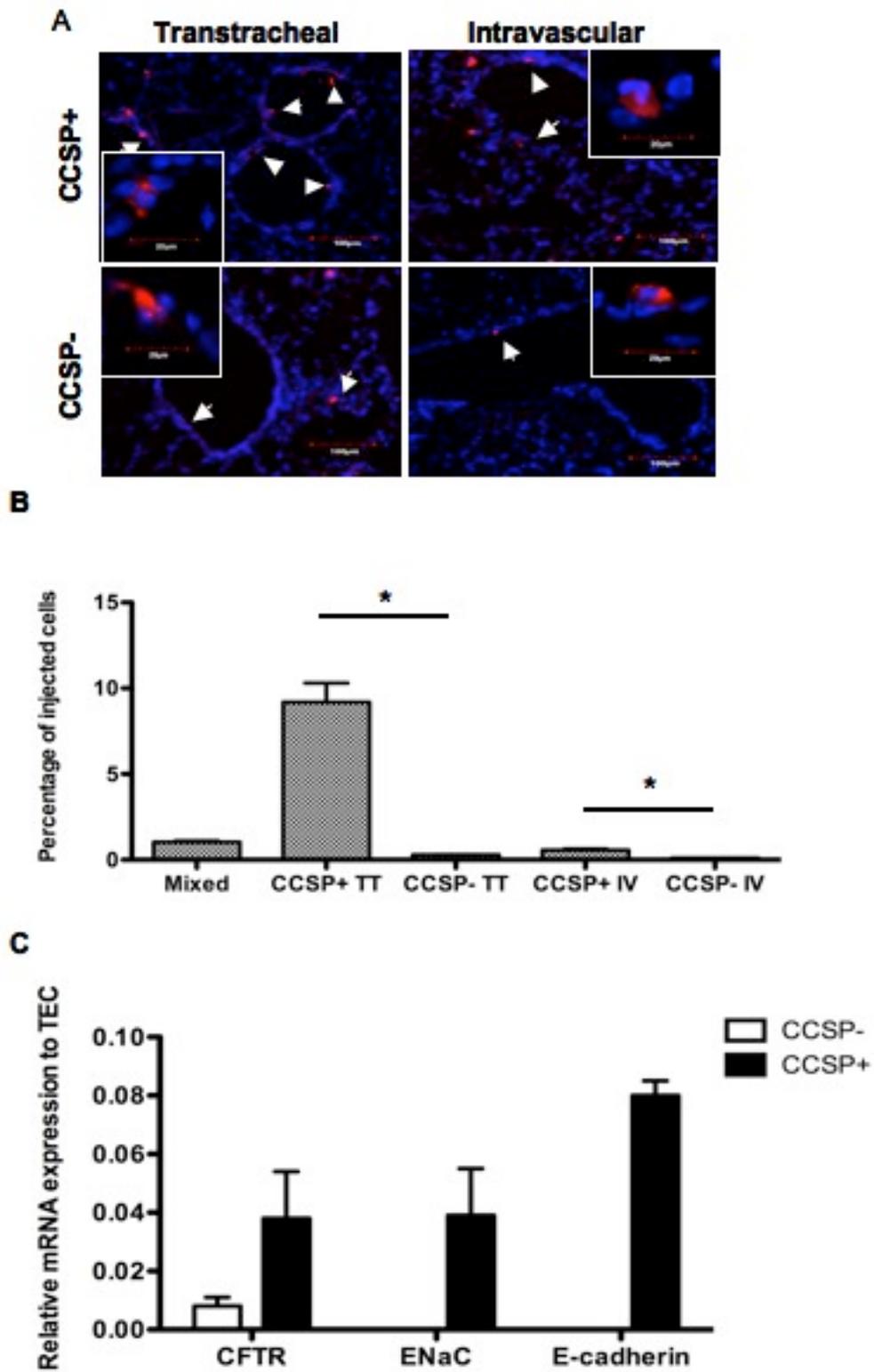


Figure 3-18 Retention potential of CCSP⁺ BMC

(A) Both transtracheal (TT) and intravenous (IV) delivery of CCSP⁺ or CCSP⁻ BMC (red) after naphthalene-induced airway injury show greater airway retention of CCSP⁺ cells than CCSP⁻ cells. **(B)** Of the 10⁶ cells injected, transtracheal delivery of CCSP⁺ cells showed the most cell retention (9%) measured 4 days after cell injection compared to whole bone marrow injection and intravenous cell delivery. N=4 mice per group. *P<0.01 compared to CCSP⁻ cells. **(C)** Detection of gene transcripts for the ion channel genes CFTR and ENaC and the tight junction protein E-cadherin was detected by real time RT-PCR of CCSP⁺ cells. CCSP⁺ BMC levels of CFTR and ENaC corresponded to 4% of the levels detected in airway epithelial cells while E-cadherin levels corresponded to 8%. Low but detectable levels of CFTR (<1%) could also be detected in CCSP⁻ cells.

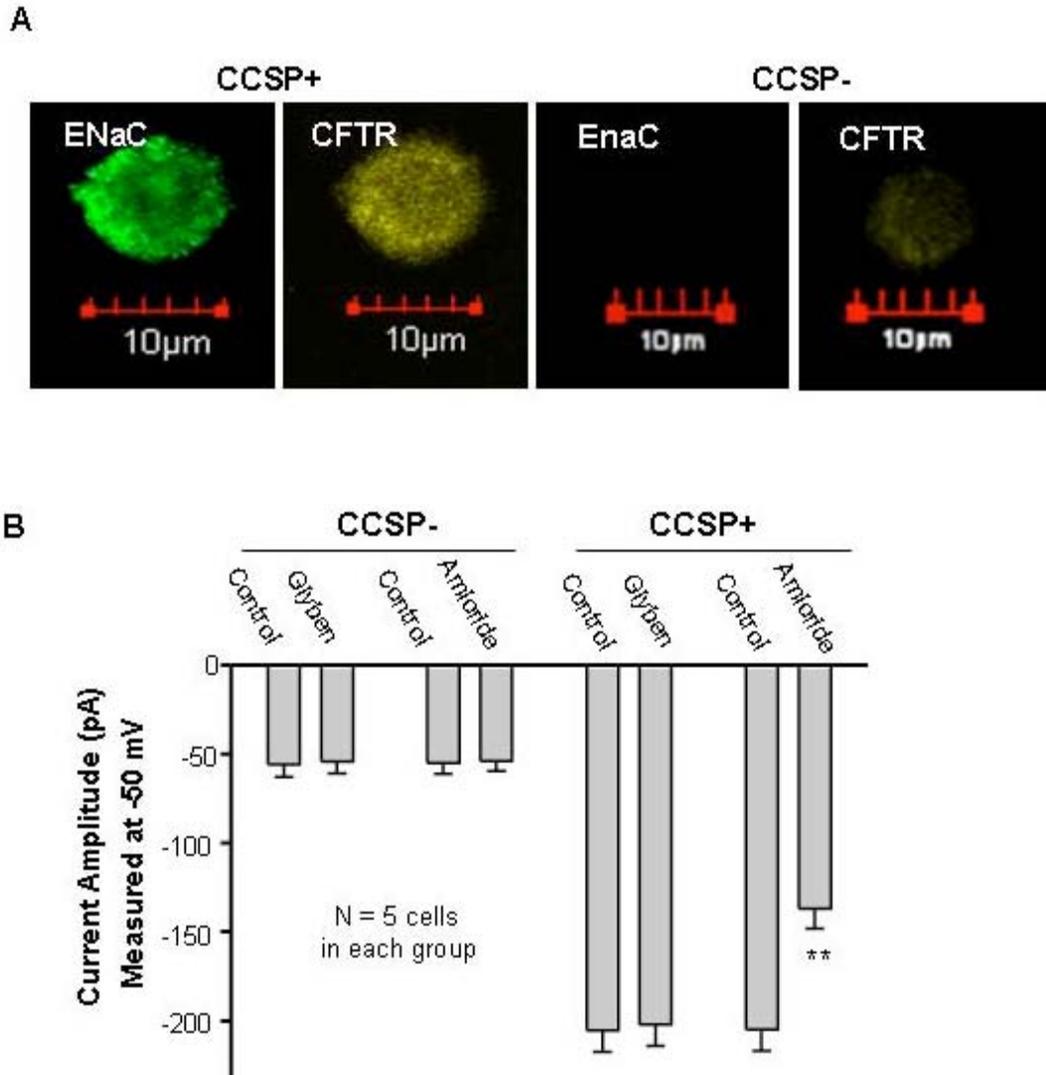


Figure 3-19 Epithelial functional potential of CCSP⁺ BMC.

(A) CCSP⁺ cells co-expressed CFTR (green) and ENaC (red) proteins. Hoechst counterstain was used to visualize nuclei (blue). (B) Perforated patch clamp recordings show the total conductance in CCSP⁺ cells was larger than in CCSP⁻ cells. Treating CCSP⁻ cells with glybenclamide and amiloride did not change their total conductance. Application of glybenclamide did not alter the total conductance of CCSP⁺ cells, but amiloride significantly suppressed the total conductance suggesting that CCSP⁺ cells express functional ENaC properties. N=5 cells per group. **P<0.005 compared to control.

Naphthalene-induced lung injury transiently increases the CCSP⁺ BMC population in the bone marrow and peripheral blood

To explore whether endogenous CCSP⁺ BMC may be involved in lung repair, we assessed CCSP⁺ cells in the bone marrow and peripheral blood after naphthalene-induced lung injury. Freshly isolated BMC from naphthalene-injured and uninjured mice were assessed for CCSP. In contrast to the lung, where naphthalene depletes CCSP⁺ cells (Stripp et al., 1995; Wong et al., 2007), the number of CCSP⁺ cells in the bone marrow and peripheral blood increased and peaked at 2 days of injury (Figure 3-20A). The number of CCSP⁺ cells remained elevated for 4 days and returned to near baseline levels by 20 days. In contrast to lung epithelial cells, freshly isolated and cultured BMC expressed low levels of CYP2E1 but do not express CYP2F2, the P450 isoenzyme involved in naphthalene metabolism and generation of toxic metabolites leading to lung epithelial cell death (Figure 23).

To determine whether endogenous CCSP⁺ BMC can migrate from the bone marrow to the injured lung, bone marrow transplantation of either whole bone marrow or CCSP⁺ cells (3×10^5 cells per mouse) from wild-type mice mixed with whole bone marrow (2×10^7 cells) from CCSP-null mice and injected into female CCSP-null recipients after lethal irradiation. As controls, whole marrow from CCSP-null mice or 3×10^5 CCSP⁻ BMC from wild-type mice were mixed with whole bone marrow from CCSP-null mice. Thirty days after marrow reconstitution, half of the mice from each group received an injection of naphthalene. Naphthalene-injured and uninjured mice were then sacrificed 4 or 30 days following injury. Bone marrow of mice that received bone marrow containing CCSP⁺ cells show detectable levels (1.6%) of CCSP⁺ cells in the bone

marrow after 30 days (Figure 3-20B). This short term re-populating ability suggests that this BMC population can at least be described as progenitors. As in wild type animals, the number of CCSP⁺ cells more than doubled to over 4% in the bone marrow at 4 days after naphthalene injection. On the contrary, mice that received CCSP-null marrow showed no detectable CCSP⁺ cells in the marrow (Figure 3-20B) or in the airways (Figure 3-21) even after naphthalene injury. Anti-CCSP staining of wild-type airways without injury shows distinct brown staining of CCSP-expressing epithelial cells. Staining of serial sections of wild-type airways with isotype immunoglobulins show low background non-specific staining. Isotype staining of the group receiving CCSP⁺ bone marrow transplant show some background brown staining perhaps due to radiation lung injury. Mice that received wild-type whole marrow had some positive cells in the uninjured airways and more CCSP⁺ cells lining the airway epithelium after injury (Figure 3-21). Mice that received CCSP⁺ BMC had greater numbers of CCSP⁺ cells lining almost the entire airway epithelium especially after injury. On the contrary, mice that received CCSP⁻ cells had few positive cells in airways with a few more lining the airways after injury whereas mice that received CCSP-null marrow demonstrated no CCSP⁺ cells at all.

Western blotting for CCSP also confirmed significant levels of CCSP expression in the lungs of recipients of BM transplants with whole wild-type marrow (Figure 3-20D) and knockout marrow supplemented with CCSP⁺ cells (Figure 3-20D). Expression was seen also in kidney but not in the heart or liver (Figure 3-20E). CCSP staining was observed in the kidney epithelium but not seen the heart or liver (Figure 3-22). Isotype staining (inset) shows no brown positive staining in contrast to staining of a kidney from an uninjured animal.

To determine whether donor CCSP⁺ BMC were producing CCSP in the lungs, and if so, where, transplants were repeated using freshly isolated donor CCSP⁺ cells from transgenic mice that constitutively express GFP (*BActGFP*). These donor cells were also Sca-1 positive. Double immunofluorescence confocal microscopy for GFP (green) and CCSP (red) of the lung from a *BActGFP* mouse showed co-localization of red and green fluorescence (Figure 24A) but not in the negative controls. Isotype-matched (negative control) staining of the CCSP knockout bone marrow transplant group (Figure 24B). Wild type recipients of CCSP⁺ BMC showed infrequent donor-derived CCSP⁺ cells with or without naphthalene lung injury. Most of the epithelium was either negative for donor cells (Figure 24C) or when they could be found; they did not express CCSP (Figure 24D). An rare example of a CCSP⁺ donor-derived cell (Figure 24E). In CCSP knockout recipients however, donor-derived epithelial cells could easily be found, even without naphthalene injury (Figure 24F, G, white arrows). Thirty days after naphthalene-induced airway injury in CCSP knockout recipients of CCSP⁺ bone marrow transplants, donor-derived CCSP⁺ cells were found to line a much larger proportion of the airway epithelium (Figure 24I, K). In addition, recipient-derived epithelial cells (non-GFP⁺) also stained weakly positive for CCSP protein (Figure 24H,J, red arrows), suggesting possible absorption of the CCSP protein produced by the donor cells. Not all donor cells expressed CCSP in the airway epithelium (marked by asterisk), even though all of the starting donor BMC were CCSP⁺ at the time of marrow transplantation. Quantification of the extent to which the donor (GFP⁺CCSP⁺) cells contribute to the repopulation of the airway (Figure 24L). Normal GFP mouse airway contains 86.01±8.18% GFP⁺CCSP⁺ cells. No airway-specific injury following bone marrow transplantation of

GFP⁺CCSP⁺Sca-1⁺ cells into CCSP knockout mice show $56.25 \pm 21.6\%$ GFP⁺CCSP⁺ cells after 32 days and $25.52 \pm 13.62\%$ after 62 days. Radiation has been shown to induce significant lung injury that can promote marrow cell engraftment in the lungs (Theise et al., 2002) therefore observation of donor cells in the airways is not surprising. Naphthalene-induced airway injury showed an increase in $56.3 \pm 11.01\%$ after 2 days of naphthalene injury (Day 32) and $39.47 \pm 17.94\%$ after 30 days following naphthalene injury (Day 62). Rare (<0.5%) donor cells were found in the airways of wild-type recipients with and without naphthalene-induced airway injury.

To determine whether airway retained GFP⁺CCSP⁺ donor cells differentiated into mature epithelium, triple immunofluorescence staining for GFP, CCSP and cytokeratin-18 was performed (Figure 25). We found co-expression of GFP⁺CCSP⁺CK18⁺ cells suggesting that the donor BMC expressing CCSP have differentiated and matured into an epithelial phenotype. Since real time PCR on sorted CCSP⁺ and CCSP⁻ cells show no other epithelial gene expression such as CK18, CK5 or AQP5, the in vivo detection of CCSP⁺CK18⁺ donor cells must be a result of phenotypic conversion possibly induced by the lung niche.

The presence of the CCSP protein in native CCSP knock out lung epithelial cells following marrow reconstitution with CCSP⁺ cells suggests that the protein may be transferred. We believe that donor CCSP⁺ cells may secrete the protein into the airway fluid which then gets absorbed by neighbouring native lung cells. In collaboration with Dr. Barry Stripp (Duke University), a sensitive ELISA assay developed in his lab was performed on bronchioalveolar lavage (BAL) fluid from the lungs of CCSP knockout

mice that received bone marrow transplant with CCSP⁺ cells (Figure 3-26). We were able to detect CCSP protein in the BAL fluid of these recipient lungs.

SDF-1 induces chemotaxis of cultured bone marrow CCSP⁺ cells

We speculated that CCSP⁺ cells might be involved in repair of lung injury and migration might involve the SDF-1-CXCR4 axis. Low levels of SDF-1 could be detected in uninjured lungs. SDF-1 increased after naphthalene injury and returned toward basal levels after day 10 (Figure 3-27B). A fraction of the CCSP⁺ cells express CXCR4 (26%, Figure 3-27B). SDF-1 induced migration of both CCSP⁺ (Figure 3-27C). Migration was partially reduced with blocking antibody towards CXCR4 in CCSP⁺ cells but not CCSP⁻ cells.

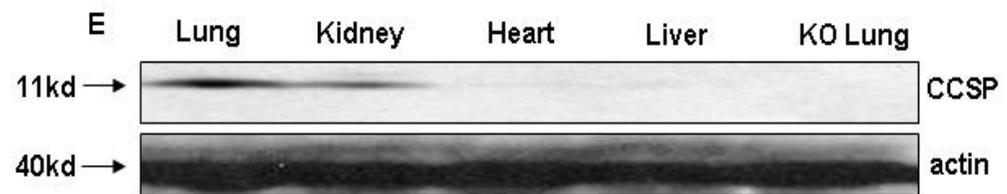
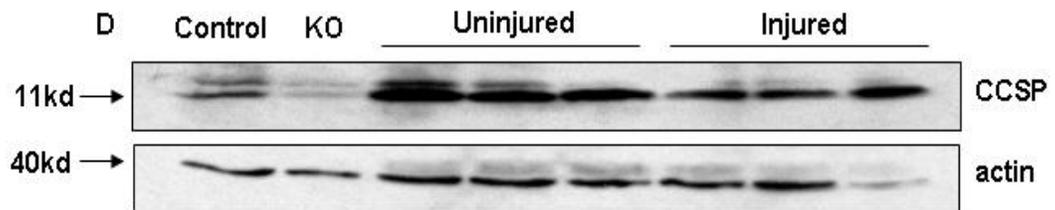
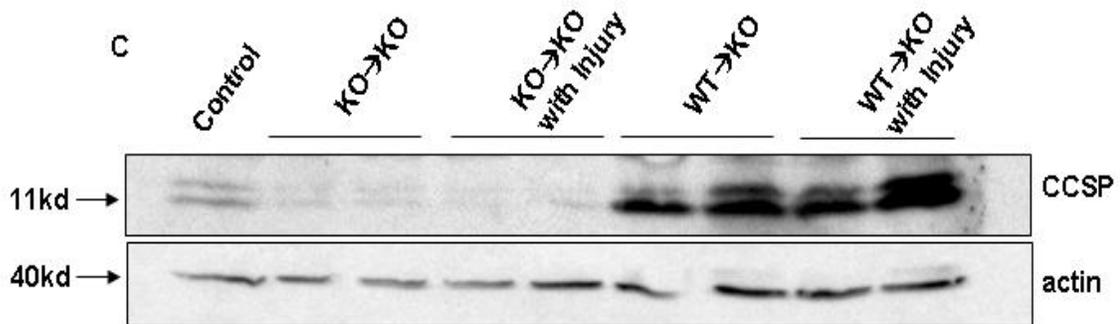
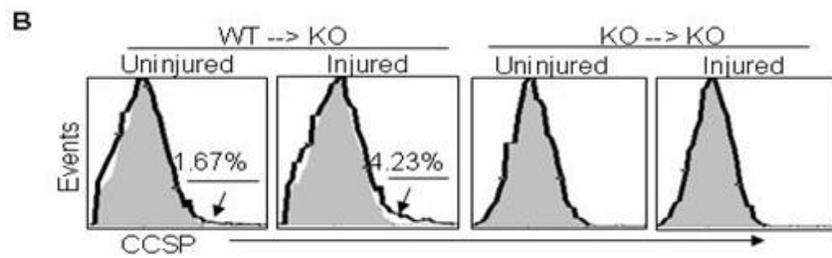
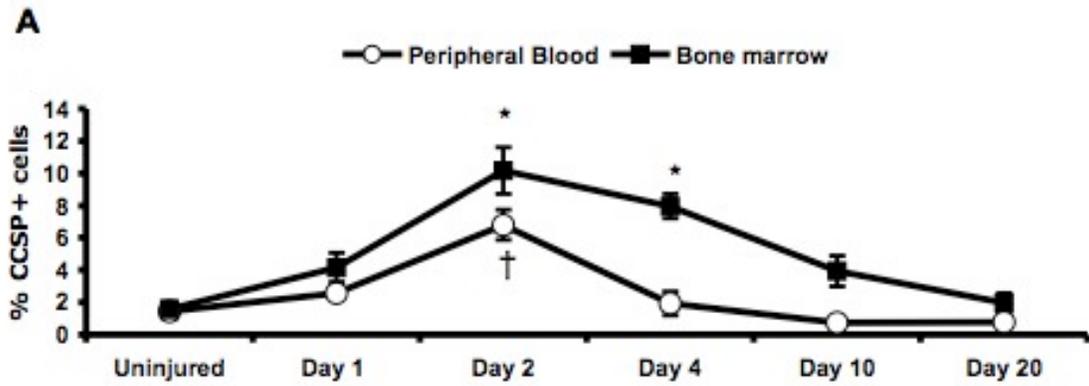


Figure 3-20 Endogenous bone marrow CCSP⁺ cells can respond to airway injury and home to airway epithelium

(A) Bone marrow and peripheral blood of mice after naphthalene-induced airway injury show a transient increase in CCSP⁺ cells peaking at 2 days of airway injury and declining to baseline levels by 20 days. **(B)** Flow cytometry of bone marrow after transplantation of wild-type whole marrow into CCSP-null recipients show existence of CCSP⁺ BMC fraction in the marrow 32 days after reconstitution without airway injury. Airway injury (4 days after naphthalene injection) increases the CCSP⁺ BMC fraction in the marrow of these mice. On the contrary, mice that received CCSP-null marrow have no detectable CCSP⁺ BMC with or without airway injury. **(C)** Western blot confirms the presence of the 11kD band corresponding the CCSP protein in the lungs of mice that received bone marrow transplant from wild-type whole marrow but not CCSP^{-/-} knockout (KO) marrow. **(D)** Mice that received KO marrow supplemented with wild-type CCSP⁺ cells and no airway injury showed greater CCSP protein levels in the lungs than mice that received a dose of naphthalene-induced airway injury. **(E)** CCSP protein was also detected in the kidneys but not the heart and liver of mice receiving wild-type marrow. Probing for beta-actin was done as internal protein loading control. N=3 per group. *P<0.01 compared to uninjured peripheral blood. †P<0.05 compared to uninjured bone marrow.

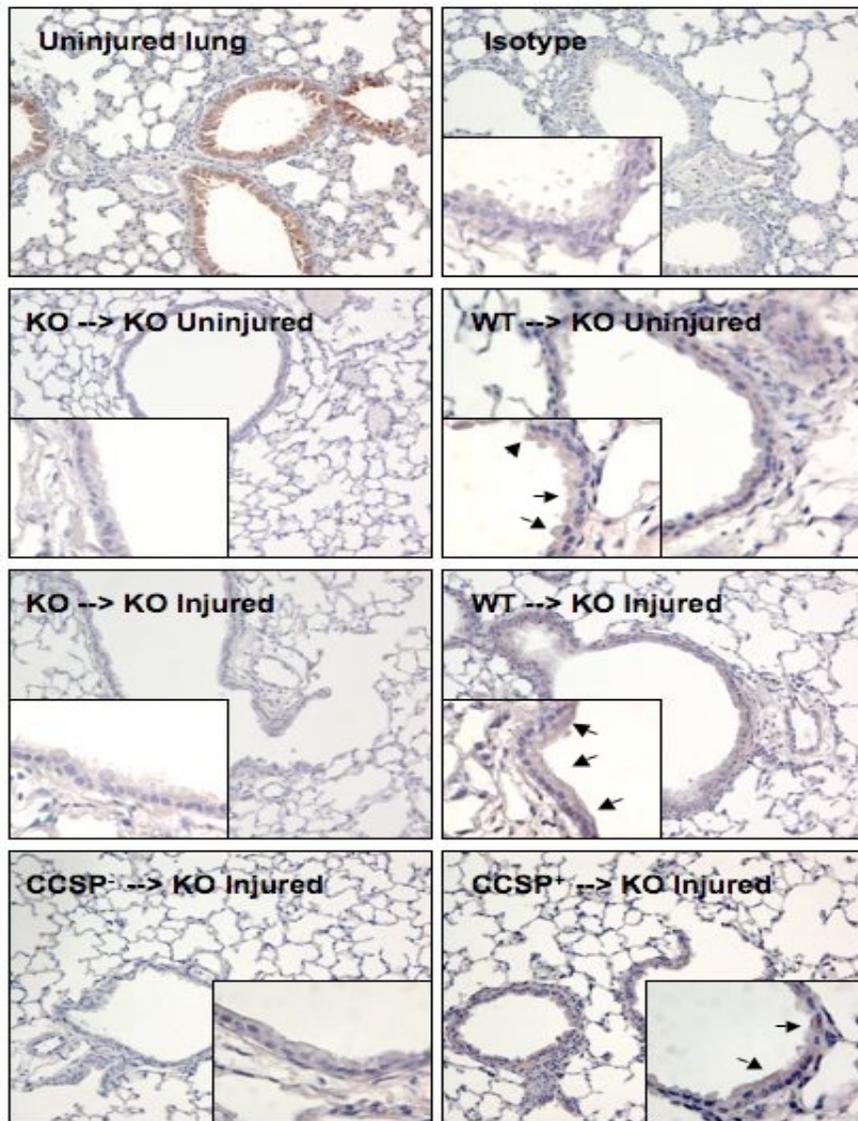


Figure 3-21 Endogenous bone marrow CCSP⁺ cells can reconstitute the airway epithelium.

Representative images of airways from CCSP-null mice receiving bone marrow transplantation of wild-type, CCSP-null or CCSP-null enriched with CCSP⁺ or CCSP⁻ cells from wild-type marrow. CCSP staining (brown) show brown staining in the airways (arrows) of mice receiving CCSP⁺ BMC transplantation but not in others. Inset show enlarged image of 2 CCSP⁺ cells in the airways. Arrows point to positive cells.

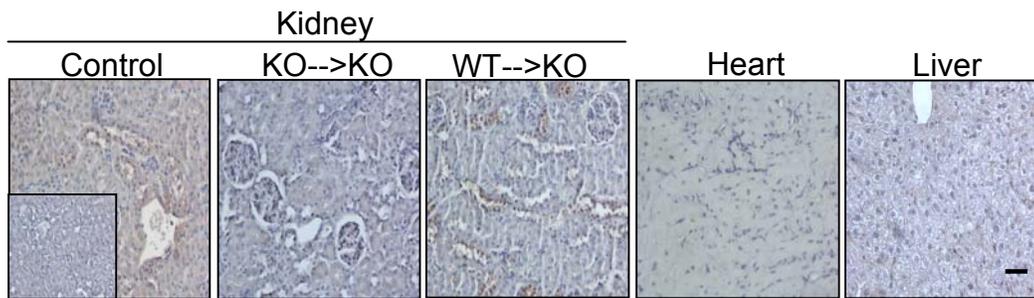


Figure 3-22: CCSP⁺ cells are found in the kidneys but not heart or liver.

CCSP⁺ cells (brown staining) was also found in the kidney epithelium but not the heart myocardium or liver epithelium. Control kidney is from uninjured mouse. Inset shows isotype staining. $n \geq 3$ mice per group for each experiment. Scale bar = 50 microns

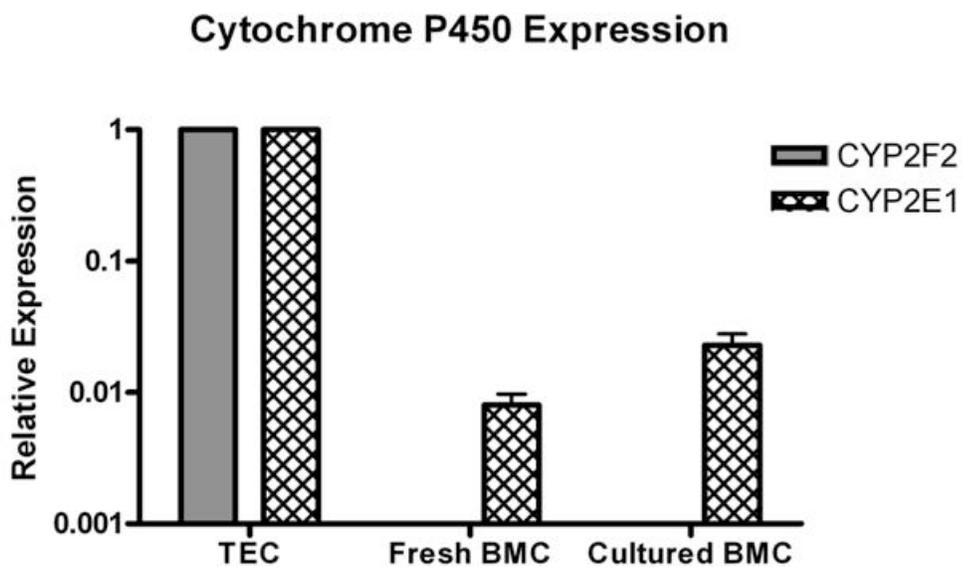


Figure 3-23 Cytochrome P450 enzyme expression

Real time PCR of freshly isolated and cultured BMC do not show detectable levels of CYP2F2 mRNA. Normalized to tracheal epithelial cell and normalized to GAPDH house-keeping gene. N=4 sets of cells per group.

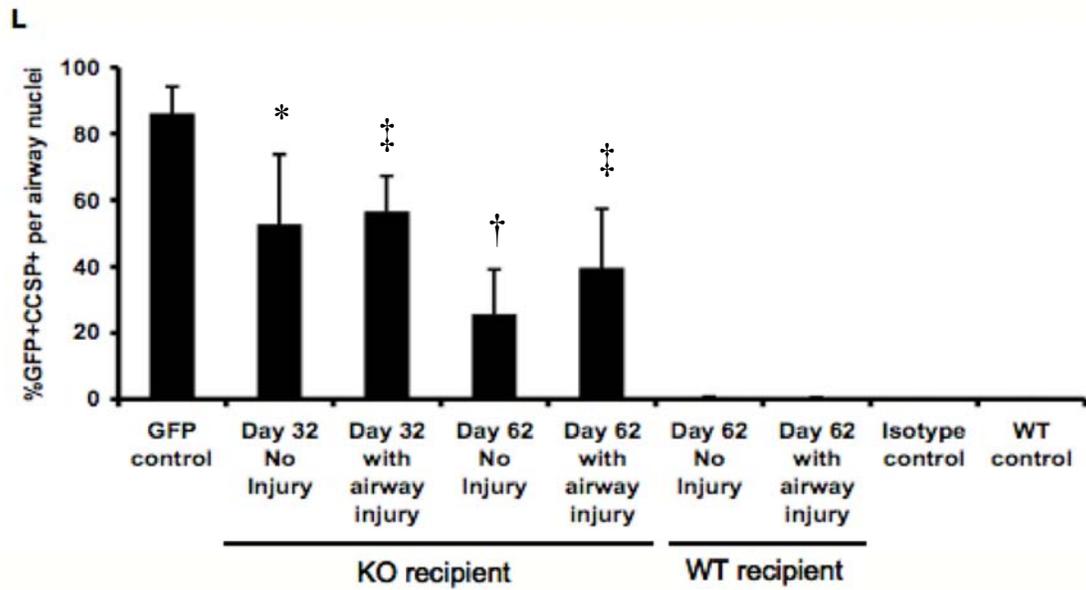
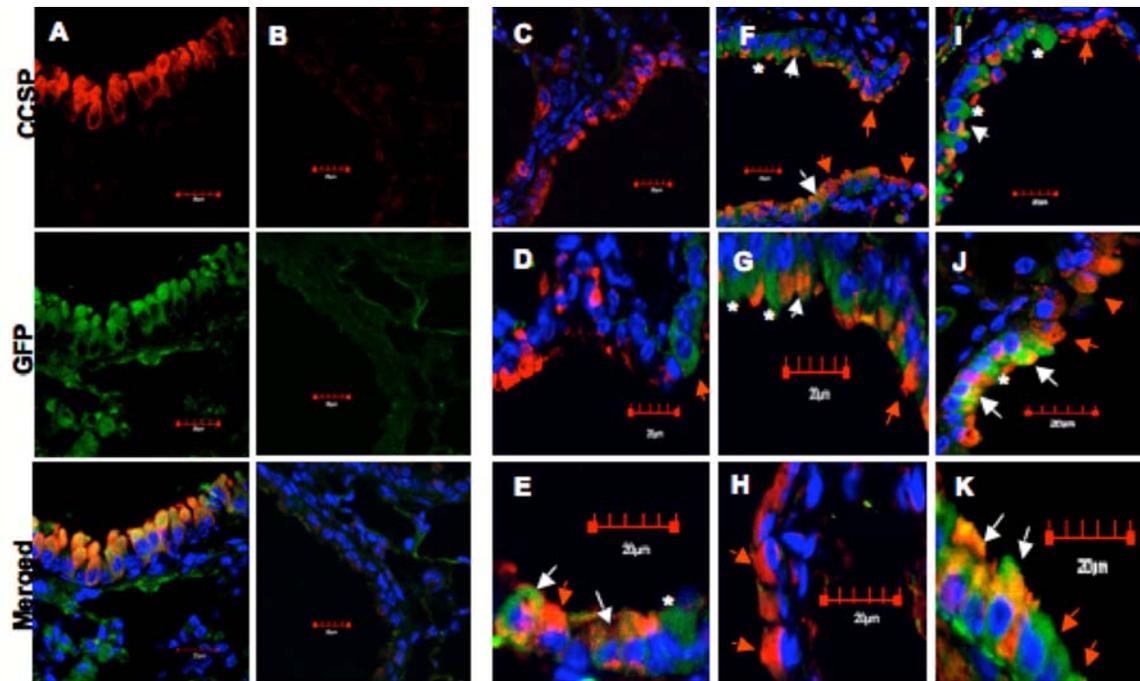


Figure 24: Airway localized donor BMC express CCSP

(A-K) Representative double immunofluorescence staining for GFP (green) and CCSP (red) of lung sections from BMT recipient mice that received CCSP⁺Sca-1⁺ cells from GFP⁺ donors. Asterisk marks GFP⁺ cell that is negative for CCSP. White arrows points to donor-derived cells positive for CCSP. Red arrows point to CCSP⁺ cells that are not donor-derived. The white trace demarcates a large section of donor-derived epithelium. A: GFP⁺ (positive control) lung, B: Isotype staining of lung from BMT recipient, C-E: BMT recipients were wild-type background, C: low, D,E: high power images. F-H: BMT recipients were CCSP knockout. 60 days following BMT without naphthalene (F: low, G,H: high power) or with naphthalene injury at 30 days post-BMT (I:low, J,K: high power) injury. **(L)** Quantification of donor CCSP⁺ cells in the airways. Each bar represents mean \pm SEM (n=3 per group).. *P<0.01 compared to WT Uninjured group; †P<0.05 compared to WT Uninjured group; ‡P<0.001 compared to WT Injured group.

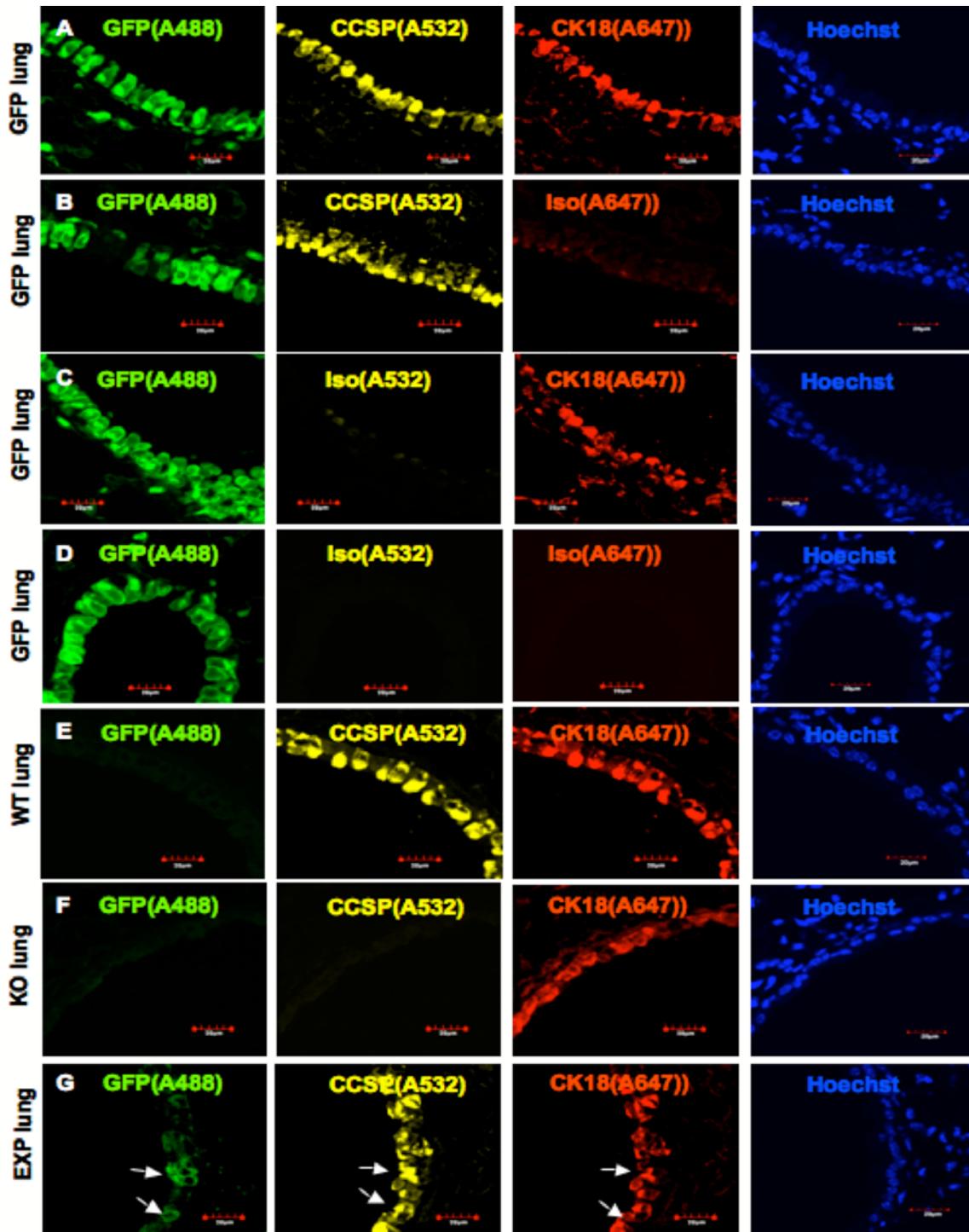


Figure 3-25 Airway retained GFP⁺CCSP⁺ donor cells express epithelial-specific cytokeratin-18.

(A-F) Staining controls for GFP, CCSP and CK18. (G) Representative experimental group (EXP lung) show coexpression of GFP, CCSP and CK18. N=4 per group

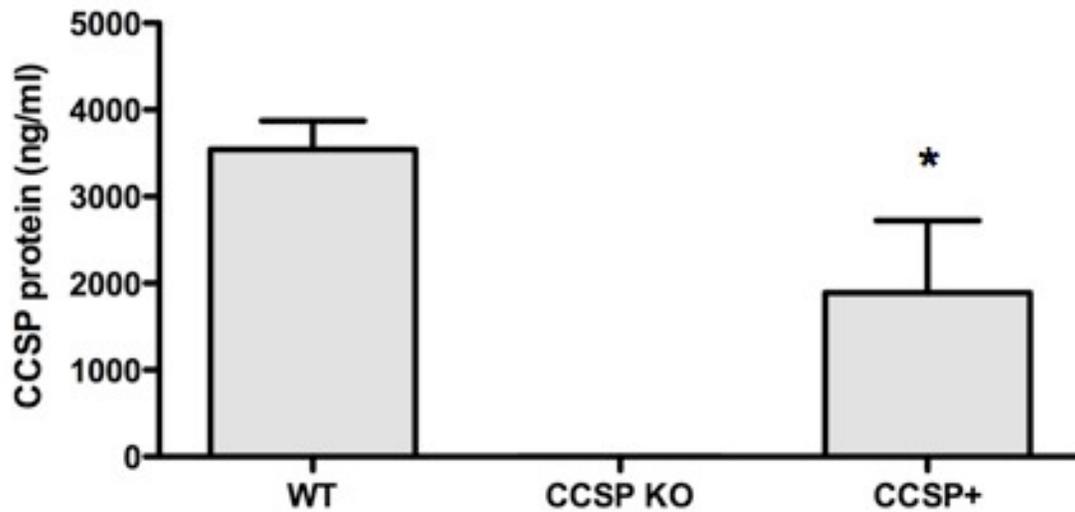


Figure 3-26 CCSP protein can be detected in the bronchioalveolar lavage fluid of bone marrow transplant CCSP knockout mice.

Sensitive ELISA detection of CCSP protein showed significant restoration of CCSP protein in the BAL fluid of CCSP knockout (KO) that received wild-type (WT) CCSP⁺ supplemented marrow. *P<0.0001 compared to CCSP knockout (KO) group.

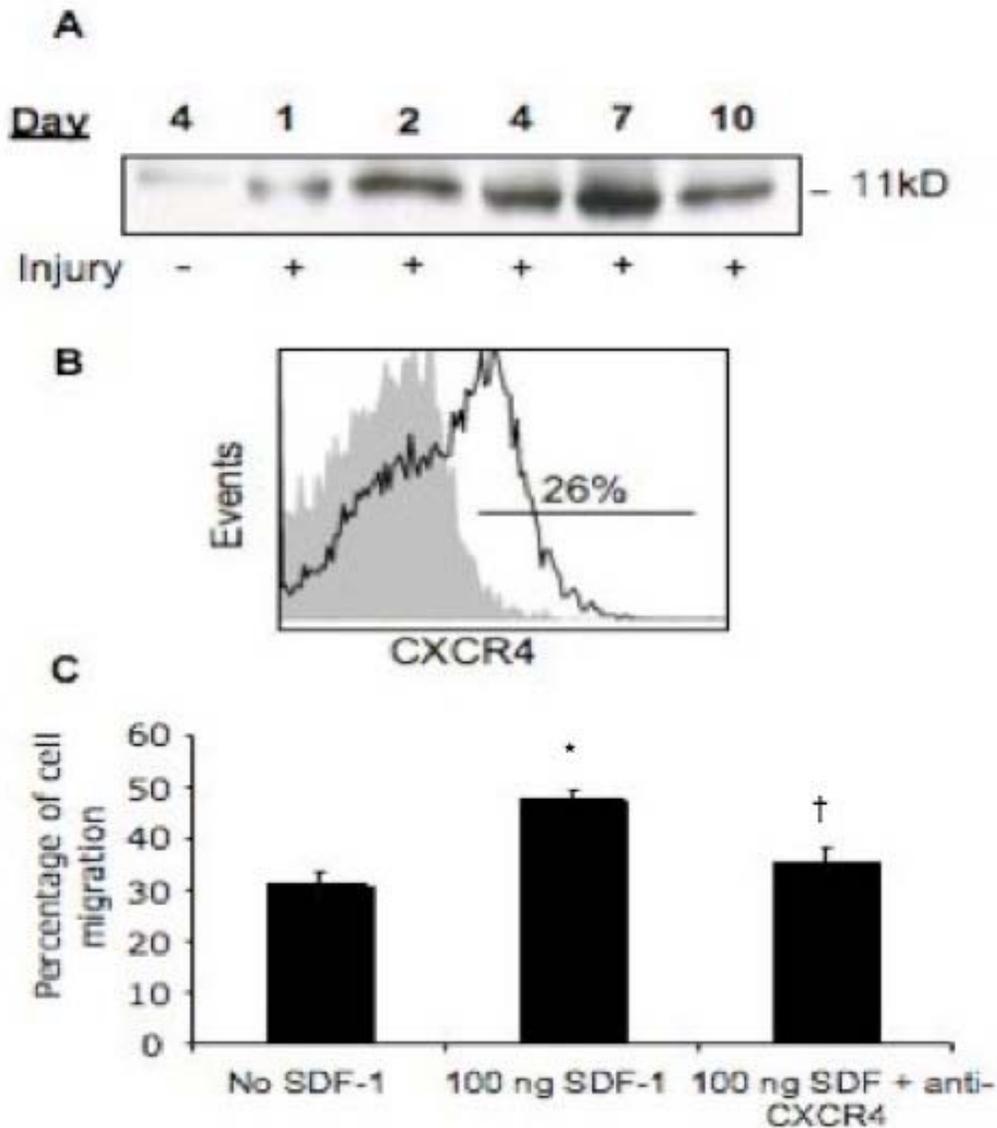


Figure 3-27 SDF-1 induces in vitro chemotaxis of bone marrow CCSP⁺ cells.

(A) Naphthalene-injured lungs show up-regulated protein levels of SDF-1 as early as 1 day after naphthalene injection up to 7 days and declining at 10 days. Uninjured lungs show baseline levels of SDF-1 protein. (B) A fraction of CCSP⁺ cells express CXCR4 (26%). (C) CCSP⁺ cells can migrate towards SDF-1, which can be partially inhibited with anti-CXCR4 antibody. *P<0.001 compared to no SDF-1, †P<0.01 compared to SDF-1-treated cells. N=4 per group.

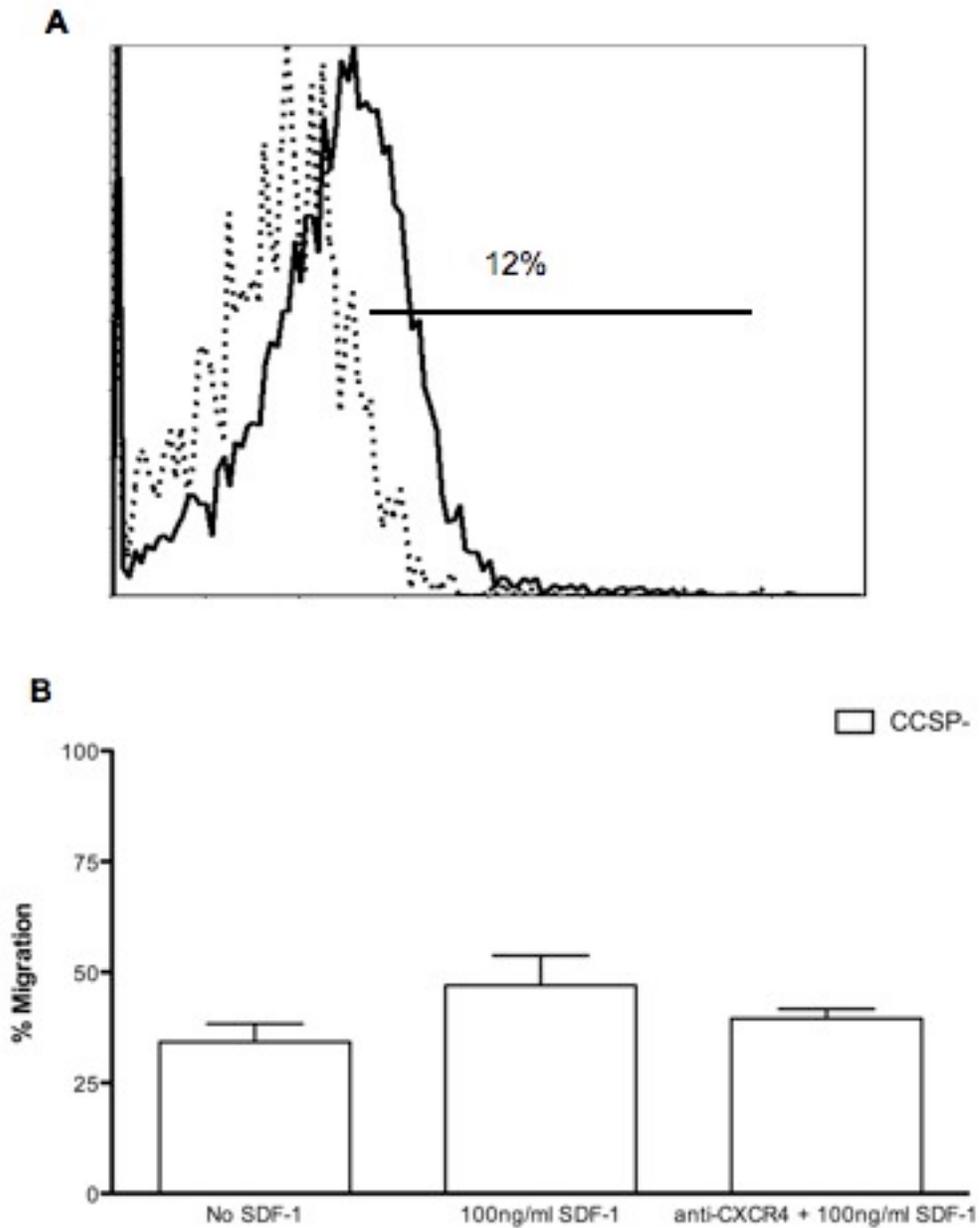


Figure 3-28 SDF-1 does not significantly affect in vitro chemotaxis of bone marrow CCSP⁻ cells.

(A) A small fraction of CCSP⁻ cells express CXCR4 (12%). (C) CCSP⁻ cells can migrate towards SDF-1 but the migration is not inhibited with anti-CXCR4 antibody. N=4 per group.

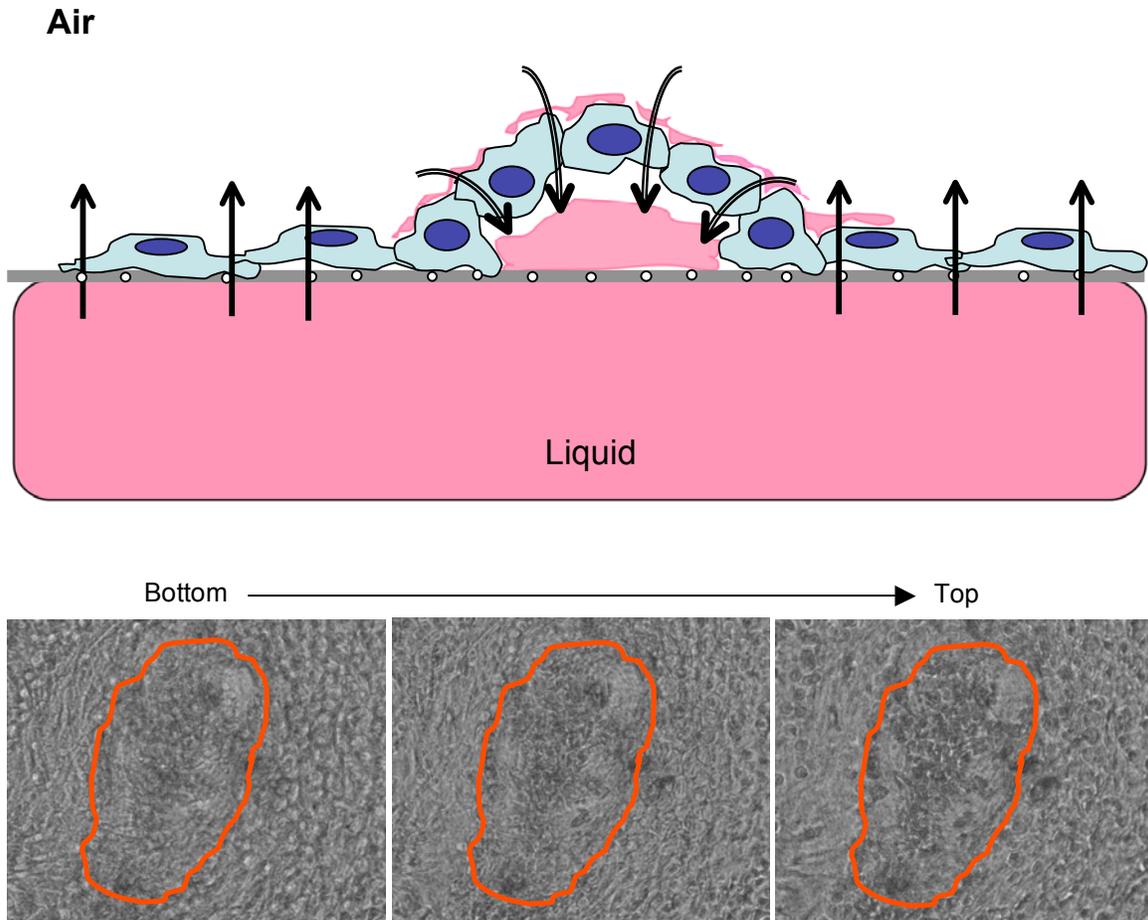


Figure 3-29 Dome formation from CCSP⁺ cells.

Formation of “dome” is a common feature of an epithelial monolayer of cells (Goodman and Crandall, 1982). A dome is a fluid-filled mound commonly found in epithelial cell monolayers actively transporting solutes from above the monolayer of cells to the substratum with fluid following passively. As the fluid fills under the cell monolayer, some cells mound up due to the hydrostatic pressure resulting in a dome structure surrounded by cells that remain attached. Domes can be visualized by adjusting the focus so that attached cells can eventually appear out-of-focus and cells that form the domes appear in-focus. CCSP⁺ cells formed dome-like structures (orange outline of a dome in CCSP⁺ cells cultured in air liquid interface). N=3 sets of cultures per group.

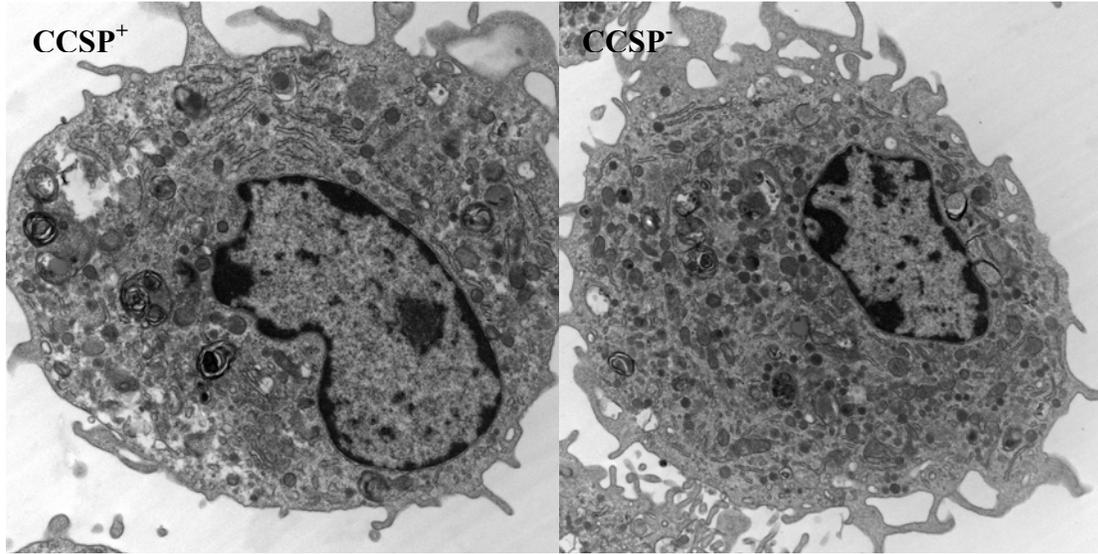


Figure 3-30 Transmission electron micrographs of sorted air liquid interface-cultured CCSP⁺ and CCSP⁻ cells

Representative transmission electron photomicrographs of CCSP-sorted cells do not convincingly show lamellar-like bodies in CCSP⁺ or CCSP⁻ cells after air-liquid interface culture for 4 weeks. This was confirmed by Dr. Ernie Cutz, a staff pathologist at Sick Children's Hospital, Toronto. N=6 sets of cells per group.

3.5 Discussion

Our studies demonstrate the existence of a newly discovered bone marrow cell population in both human and mouse marrow that express an epithelial secretory protein, CCSP. These cells can be distinguished and isolated by surface expression of CCSP. In a murine model of airway injury, both marrow and blood CCSP⁺ cells increased. Under air-liquid interface conditions, CCSP⁺ could be induced to differentiate into various cell lineages expressing markers of ciliated cells, Type I, Type II, and/or basal cells. Differentiation into specific lineage-committed epithelial cell such as Type II (SP-C⁺ only) or Type I (AQP5⁺ only) cells was limited. These cells can form dome-like structures, a hallmark of ion and fluid transport of lung epithelial cells (Figure 3-29). Furthermore, CCSP⁺ BMC expressed proteins involved in important epithelial functions including SP-C, CFTR and ENaC and exhibited sodium transport properties consistent with ENaC function. However, using transmission electron microscopy we did not find definitive ultrastructural features classically seen in epithelial cells such as lamellar bodies and cilia (Figure 3-30) suggesting these cells at least in vitro have limited epithelial differentiation potential. Alternatively, perhaps examination of cells after ALI culture and then sorted into only SP-C positive cells would yield more convincing results. Transtracheal or intravenous delivery of CCSP⁺ cells as a mode of cell therapy was found to have great potential to reconstitute the airway epithelium, more so than a mixed population of bone marrow cells or CCSP⁻ cells. Bone marrow transplantation of CCSP⁺ BMC into CCSP knockout mice demonstrated airway CCSP expression, probably due to both localized engraftment and absorption of the CCSP protein secreted by CCSP-expressing cells. CCSP expression was restored at the protein and mRNA levels although substantial airway repopulation was

only observed in CCSP knockout mice receiving CCSP⁺ BMC, compared to mice with normal pulmonary expression of CCSP, suggesting that absence of the CCSP protein itself may impair endogenous regenerative potential.

The presence of the CCSP protein in native CCSP knock out lung epithelial cells following marrow reconstitution with CCSP⁺ cells suggests that the protein may be transferred. In collaboration with Dr. Barry Stripp, a sensitive ELISA generated in his lab was used to detect small levels of CCSP protein. We were able to detect CCSP protein in the BAL fluid of the CCSP knockout transplant recipient lungs that received CCSP⁺ cell supplemented marrow. To support this possibility of absorption/intake of secreted material, it has been shown that genetic information may be transferred from radiation-injured lung epithelial cells through microvessels that get absorbed by marrow cells (Aliotta et al., 2007). Absorption of these RNA-filled microvessels by marrow cells results in phenotypic alteration of the cells that express lung-specific genes and proteins and can influence their engraftment potential. It is likely that the converse is also true whereby genetic information and/or protein are leaked and absorbed by CCSP⁺ BMC that can be absorbed by lung epithelial cells affecting their differentiation potential such as expression of CCSP.

Many reports have suggested a possible role of bone marrow stem/progenitor cells in tissue regeneration (Camargo et al., 2003; Horwitz et al., 1999; Kerjaschki et al., 2006; Kocher et al., 2001; Okamoto et al., 2002b; Otani et al., 2002) however the specific subpopulations of BMC involved remains elusive in the lung. Reports of bone marrow cell-derived chimerism in the pulmonary epithelium remain controversial as some studies suggest that hematopoietic precursors contribute to the lung epithelium (Krause et al.,

2001; Mattsson et al., 2004) while others suggest that these cells are exclusively mesenchymal-derived (Ortiz et al., 2003; Rojas et al., 2005). Our studies may help to reconcile these findings. The population described here expresses CCSP, a marker notably shown to identify endogenous pulmonary progenitor (Giangreco et al., 2002; Hong et al., 2001; Reynolds et al., 2004) and stem cells (Kim et al., 2005), while also expressing hematopoietic markers, and are found in the plastic adherent fraction and express some markers used to characterize mesenchymal cells. According to the International Society for Cellular Therapy, CD73, CD90 and CD105 are markers that define a mesenchymal stromal cell population (Horwitz et al., 2005). However, CD90 and CD105 are also known to be expressed in hematopoietic precursors (Pierelli et al., 2001; Sumikuma et al., 2002). Studies are currently being done to determine whether bulk CCSP⁺ cells and sub-fractions can give rise to mesenchymal, hematopoietic and other tissue-specific cell lineages. Several reports have suggested that there is a developmental relationship between hematopoietic stem cells and their mesenchymal progenitors (Dominici et al., 2004; Olmsted-Davis et al., 2003). Non-adherent hematopoietic cells that can reconstitute the blood have also been shown to give rise to stromal cells suggesting the existence of an early precursor for both cell lineages. Indeed, Sca-1-deficient mice have impaired hematopoietic stem cell and progenitor cell function (Ito et al., 2003a) and appear to have mesenchymal progenitor cell self-renewal deficiency leading to age-dependent osteoporosis (Bonyadi et al., 2003). Future work will need to be done to determine the relationship CCSP⁺ cells have with these early hematopoietic/mesenchymal precursors.

Bone marrow-derived cells have been shown to give rise to lung epithelial-like cells *in vitro* (Spees et al., 2003; Wang et al., 2005) and *in vivo* (Krause et al., 2001; Mattsson et al., 2004; Okamoto et al., 2002a; Rojas et al., 2005) however, the specific population of BMC and the molecular mechanisms involved remains unknown. Culturing CCSP⁺ BMC under air-liquid interface for 4 weeks to induce epithelial differentiation resulted in multi-epithelial lineage differentiation. CCSP⁺ BMC differentiated into CCSP⁺ cells (suggestive of Clara cells), SP-C⁺ (Type II alveolar cells), AQP5⁺ (Type I alveolar cells), K14⁺ (basal cells), acetylated α -tubulin⁺ (ciliated cells), and intermediary cells co-expressing multiple lineage markers such as acetylated α -tubulin⁺SP-C⁺ double positive cells. Coexpression of CCSP and SP-C in the CCSP⁺ population after 4 weeks air liquid interface cultures suggests a resemblance to the recently described bronchioalveolar stem cell (BASC) (Kim et al., 2005). However, in addition to being CD34⁺, our CCSP⁺ BMC are CD45⁺ and CD31⁺ which makes them distinct from the BASC population. It remains unclear whether differentiation could lead to down-regulation of CD45 and CD31 and thereby become BASC cells in the lungs. On the contrary CCSP⁻ cells only gave rise to SP-C⁺ cells suggesting a much more limited differentiation potential of these cells. Although CCSP⁺ cells express phenotypic markers of various lung epithelial cell lineages, transmission electron microscopy of CCSP⁺ BMC did not definitively confirm epithelial ultrastructural features (Figure 3-30) suggesting these cells, under the current culture conditions, do not have full differentiation potential. The full extent of differentiation possible using culture conditions such as specific media (Ali et al., 2002), coculture with epithelial cells (Wang et al., 2005), or differentiation in

vivo were not tested but these conditions might yield more distinct and committed epithelial cell populations.

Other studies have suggested that bone marrow contains cells that already express epithelial-specific proteins. Gompert identified a K5⁺ cell in the freshly isolated bone marrow and peripheral blood that is recruited to the airway epithelium following tracheal transplantation (Gomperts et al., 2006). CCSP⁺ cells described here expressed K5 mRNA but not protein. Cytokeratin-5 protein was expressed after further growth in culture suggesting these CCSP⁺ BMC may be a precursor cell for circulating K5⁺ cells. Tissue-committed neural, cardiac, liver and muscle stem/progenitor cells have also been suggested to reside in the bone marrow (Kucia et al., 2004b; Kucia et al., 2006a; Kucia et al., 2005b; Kucia et al., 2006b) however identification was based on gene expression of a whole marrow population. It is unclear whether a single stem/progenitor cell can express gene transcripts of all tissues or specific cell populations expressed different tissue-specific gene transcripts. The latter concept would suggest each tissue contain a stem/progenitor cell that exists in the bone marrow and is mobilized to its specific tissue upon injury (Kucia et al., 2004a; Kucia et al., 2006b). Thus our study provides the foundation for further investigation into the identification of tissue-specific progenitor or stem cell populations in the bone marrow and the role of these cells in tissue regeneration.

Bone marrow CCSP⁺ cells may have substantial value as a source material for cell replacement therapies for epithelial diseases. Intrapulmonary stem cells that reside in distinct niches in the lung (Giangreco et al., 2002; Hong et al., 2004b; Kim et al., 2005; Reynolds et al., 2000b; Reynolds et al., 2007; Summer et al., 2003) are considered the

primary source of epithelial regeneration, however isolation and expansion of autologous cells from such sources is difficult and limits their practical utility. Bone marrow CCSP⁺ cells could be easily isolated and expanded. Unlike terminally differentiated lung CCSP⁺ cells, CCSP⁺ BMC did not express the specific cytochrome P450 isoenzyme, CYP2F2 (Stripp et al., 1995), which degrades naphthalene into toxic metabolites and were not destroyed following naphthalene treatment. On the contrary, naphthalene increased the number of CCSP⁺ cells in the bone marrow and blood. This relative resistance to naphthalene injury is also seen in endogenous lung CCSP⁺ progenitor cells. Far more marrow-derived cells were identified in the lungs, when the recipient did not express CCSP. It may be possible that the CCSP protein may play a role in promoting a conducive environment for BMC to engraft.

While we can not rule out the possibility that cell fusion may occur in our model, this explanation seems unlikely because the number of CCSP⁺ donor cells that repopulated the airway epithelium of CCSP knockout mice was quite high and studies including our own have suggested rare cell fusion events by BMC (Loi et al., 2006; Wong et al., 2007; Zander et al., 2005). Harris used a robust Cre-lox strategy to demonstrate that BMC (containing hematopoietic and mesenchymal stem/progenitors) do not contribute to lung, liver and skin epithelia by cell fusion with local epithelial cells (Harris et al., 2004). Since CCSP⁺ cells may also exist in the BMC fraction studied by Harris, it suggests cell fusion is unlikely an explanation for the higher level of donor cell repopulation we have observed.

SDF-1 has been shown to be a common chemoattractant for bone marrow-derived progenitors (Abbott et al., 2004; Kucia et al., 2004a; Kucia et al., 2004b) expressing the

cognate receptor CXCR4 (Kucia et al., 2005a; Ratajczak et al., 2004). SDF-1 levels increased in response to naphthalene-induced airway injury. A fraction of bone marrow CCSP⁺ cells do express CXCR4 and can migrate in response to SDF-1 chemoattraction in vitro. Gomperts (Gomperts et al., 2006) showed that K5⁺CXCR4⁺ cells from the marrow and peripheral blood can regenerate the large airways in response to SDF-1-induced migration to the tracheal epithelium. Impairing the SDF-1/CXCR4 axis involved in the recruitment of these cells to the injured airway resulted in a squamous metaplasia phenotype.

In summary, we report a newly discovered epithelial-specific progenitor cell in the bone marrow that can repopulate the airway and kidney epithelium but may also participate in regenerating epithelia of other tissues such as skin and GI tract. The discovery of these cells in human bone marrow suggest a potential application of these cells in cell replacement strategies for treatment of various epithelial diseases. Due to the pluripotent and multipotent nature of stem cells such as embryonic stem cells, hematopoietic stem cells and mesenchymal stem cells, ensuring stem cell differentiation along the appropriate cell lineage for tissue regeneration remains a challenge. Studies have attempted to differentiate stem cells in vitro (Choi et al., 2005; Samadikuchaksaraei et al., 2006; Wang et al., 2005) in hopes to deliver these cells in vivo for tissue regeneration. Since the CCSP⁺ cells have a greater propensity to engraft in the lung and have multi-epithelial differentiation potential, therapeutic delivery of CCSP⁺ cells as opposed to hematopoietic and mesenchymal stem cells may more successfully promote lung epithelial regeneration.

Chapter 4

Characterization of a multipotent epithelial-like bone marrow stem cell population with airway reconstitution potential

4.1 Rationale

The adult bone marrow has been shown to contain tissue-specific progenitor cells. In Chapter 3 we identified a novel cell population in the bone marrow that express the airway progenitor cell protein, CCSP, and has greater propensity to home to the lung, and can differentiate in vitro into multiple epithelial lineages. We believe that the true stem cell behaviour of this population resides within a subset also expressing Sca-1.

Objective of study: To characterize the multi-differentiation and self-renewal potential of CCSP⁺Sca-1⁺ subpopulation of CCSP⁺ BMC.

Results: The bone marrow-derived epithelial progenitor cell population contains rare cells that display characteristic features of multipotent stem cells. These CCSP-expressing cells express the stem cell antigen, Sca-1. A smaller subpopulation coexpress markers of pluripotency including OCT-4, Nanog, and SSEA1. CCSP⁺Sca-1⁺ BMC have a clonogenic expansion potential in vitro with an efficiency of clonogenic expansion from a single CCSP⁺Sca-1⁺ cell of ~40%. CCSP⁺Sca-1⁺ cells from secondary and tertiary clones also exhibit robust clonogenic potential suggesting self-renewal. Importantly, these rare cells have pulmonary reconstitution potential following airway engraftment. Furthermore, these stem cells can give rise to a limited variety of “epithelial” cell types after air-liquid interface culturing conditions although full differentiation of the epithelium is not yet demonstrated.

Conclusions: Collectively, we demonstrate that the adult mouse marrow harbors epithelial-like stem cells that can be isolated, expanded in culture and subsequently induced to differentiate into “quasi” pulmonary epithelial cells. These CCSP⁺Sca-1⁺ cells exhibit significant pulmonary reconstitution potential. On-going work assesses the self-renewal and multilineage differentiation into mesoderm, endoderm and neuroectoderm potential of these cell in vivo.

4.2 Introduction

Tissue-specific stem cells have been identified in the marrow compartment of adult bone (Kucia et al., 2004a; Kucia et al., 2004b). Extrapulmonary stem/progenitor cells are conventionally said to “transdifferentiate” into lung cells following pulmonary engraftment however recent studies have suggested that the bone marrow may contain rare subsets of progenitor cells that already express proteins classically thought to be exclusive to the epithelium (see Chapter 3). Gomperts identified cytokeratin-5-expressing (CD45⁺CXCR4⁺) cells that exists in the bone marrow and peripheral blood and are increased after injury (Gomperts et al., 2006). Blockade of the CXCL12/CXCR4 axis involved in the recruitment of these cells to the injured airway subsequently resulted in a squamous metaplasia phenotype suggesting the extrapulmonary epithelial cells play an important role in epithelial regeneration. We have previously identified a novel bone marrow cell population that expressed the airway progenitor cell marker Clara cell secretory protein (CCSP). These cells can respond to airway injury, differentiate into

multiple distinct epithelial-like cells, have some epithelial functional properties in vitro, and finally have greater propensity to be retained in the lung.

Here we hypothesized that this CCSP⁺ bone marrow cell population may contain a stem cell subset that has self-renewal and multipotent differentiation potential. To test this, we identified and isolated a CCSP⁺Sca-1⁺ subset in the CCSP⁺ cell fraction and determined its self-renewal ability, multi-lineage differentiation capabilities, and pulmonary engraftment and reconstitution potential. In a single cell clonogenic assay, about 40% of the CCSP⁺Sca-1⁺ cells gave rise to primary clones which further gave rise to secondary and tertiary colonies in vitro. CCSP⁺Sca-1⁺ cells that were retained in the lung proliferated and regenerated the epithelium after airway injury suggesting in vivo differentiation potential. Characterization of the CCSP⁺Sca-1⁺ subset showed a small population that expressed markers of embryonic stem cell self-renewal and pluripotency, Oct-4, Nanog and SSEA-1. All of the CCSP⁺Sca-1⁺ cells also expressed SP-C resembling a bronchioalveolar stem cell (Kim et al., 2005) (BASC) phenotype although they were also CD45⁺. In air-liquid interface cultures, CCSP⁺Sca-1⁺ cells give rise to various airway and alveolar epithelial cell types. In summary, the adult mouse bone marrow harbors epithelial-like stem cells that can be isolated, expanded in culture and subsequently differentiated into pulmonary epithelial cells which may be a source for cell replacement therapies.

4.3 Materials and Methods

Animals

Adult male (4-6 weeks) C57Bl6, CD1 (Charles River Laboratories, Wilmington, MA), Bactin-GFP (C57Bl/6 mice constitutively expressing GFP under the control of the beta-actin promoter, Jackson Laboratories) and transgenic K18GFP (GFP is driven by the cytokeratin-18 promoter; CD1 background) mice were used as BMC donors (Wong et al., 2007). Age and strain-matched C57Bl/6 and CD1, females were used as recipients respectively. All animal procedures were approved by the University Health Network Animal Care Committee. All mice received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research, and the Guide for the Care and Use of Experimental Animals formulated by the Canadian Council on Animal Care.

Naphthalene-induced airway injury and transtracheal cell delivery

Mice were given intraperitoneal injections of naphthalene (>99% pure; Sigma Chemical, St. Louis, MO), 200 mg per kg body weight as previously described (Wong et al., 2007). Cell injections were performed 2 days following naphthalene-injury. Recipient mice were anaesthetized and injected transtracheally with 10^6 unfractionated cultured K18GFP bone marrow cells or 1.8×10^5 CCSP⁺Sca-1⁺ from cultured K18GFP cells resuspended in 0.05 ml PBS.

Bone marrow cell harvest and marrow transplantation

Bone marrow cells were harvested and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco-Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin (Gibco, Grand Island, NY) as previously described (Wong et al., 2007).

Cell Sorting

Plastic-adherent BMC cultured for 7 days were sorted for CCSP⁻ or CCSP⁺ Sca-1⁺ cells using the MoFlo Cell Sorter (Becton Dickinson, Franklin Lakes, NJ USA) with low flow pressure of 20 PSI. Briefly, non-specific binding on BMC (10^7) was blocked with blocking buffer containing 5% normal goat serum and 2% BSA for 20 minutes on ice. Cells were then stained with anti-CCSP antibody (1:500, Upstate Labs), anti-Sca-1 (Ly6A)-PE (1:200, Chemicon) or a mixed cocktail of anti-CCSP and anti-Ly6A-PE followed by AlexaFluor488 secondary IgG detection for 30 minutes on ice. The cells were washed at least 3 times between primary and secondary staining steps and following secondary antibody staining.

Assays of self-renewal

In vitro clonogenic assay

To quantitatively examine the clonogenic capacity and self-renewal of CCSP⁺Sca-1⁺ cells, a single CCSP⁺Sca-1⁺ cell per well was seeded into a 96-well plate pre-coated with irradiated DR4 mouse embryonic fibroblast (MEF, ATCC). To distinguish donor BMC from fibroblasts, mice expressing GFP driven by the β -actin

promoter were used as donor cells. Only wells that contained a single GFP⁺ cell per well 16 hours after plating was further analyzed for clonal expansion. Cells were subsequently counted for GFP⁺ cells after 1, 4, 7 and 10 days in culture. After 2 weeks, 1 colony was re-sorted for CCSP⁺Sca-1⁺ cells and re-plated single cell/well into MEF-coated 96-well plate to assess for secondary and tertiary generation of colonies.

In vivo CCSP⁺Sca-1⁺ BMC reconstitution potential

Airways of female CD1 mice were injured with naphthalene as previously described (Wong et al., 2007). Two days following injury, 10⁶ unfractionated 7-day cultured cells or 1.8 x10⁵ CCSP⁺Sca-1⁺ cells from K18EGFP mice were delivered transtracheally into female recipients. A second dose of naphthalene was administered 30 days after the first to assess the proliferative and reconstitutive potential of the “engrafted” donor CCSP⁺Sca-1⁺ cells. To test the self-renewal potential, CCSP⁺Sca-1⁺GFP⁺ cells were sorted from the lungs of primary recipients (R1) and re-transplanted into naphthalene-injured secondary recipients (R2). An increase in donor male-specific genomic DNA was used as a measure of increased donor cell number. An increase in K18-GFP mRNA was reflective of donor cell differentiation into epithelial-like cells. This was confirmed and localized with anti-GFP staining of the airway epithelium.

Immunostaining

Assessment of stem cell and epithelial cell marker expression

Immunofluorescence staining for CCSP (07-623, Upstate), proSP-C (AB3428, Chemicon, Temecula, CA), cytokeratin 18 (K18: clone Ks 18.04, RDI, Flanders, NJ), cytokeratin 5/8 (K5/8: MAB3228, Chemicon), cytokeratin 14 (K14, Vector Labs), Sca-1

(Ly-6A/E-PE, clone D7, BD Pharmingen, San Diego, CA), CD45 (clone 30-F11, BD Pharmingen), CD34 (553732, BD Pharmingen), CD31 (MCA1364, Serotec), collagen Type IV, vimentin, CD90, CD105, CD106, CD73 (all from BD Biosciences), collagen Type I (Rockland), Oct-4 (ab18976, Abcam, Cambridge, MA), SSEA-1 (ab16287, Abcam), Nanog (ab21603, Abcam), CFTR (MAB1660, R&D Systems), ENaC (AB3532P, Chemicon), acetylated alpha-tubulin IV (ab24610, Abcam), and AQP-5 (178615, Calbiochem) was performed as previously described (See Chapter 3). Briefly, adherent cells were fixed with 4% paraformaldehyde (PFA), washed twice, and incubated with the above primary antibodies overnight at 4°C. AlexaFluor-488-conjugated or phycoerythrin (PE)-labelled secondary antibodies were applied after 3 successive washes with PBS and incubated at room temperature for 1 hour. Nuclei were stained with Hoechst dye (1:5000, Molecular Probes) for 5 minutes. Slides were mounted with immunofluorescent mounting medium. Negative controls were treated with appropriate non-specific IgG matched for isotype. Images of representative fields were acquired using an Olympus FluoView 1000 Confocal Imaging System (Olympus, Melville, NY). Embryonic stem cells, mesenchymal stem cells, hematopoietic stem cells and fibroblasts were used as positive and negative cell controls for the staining.

Assessment of donor cells in recipient lungs

Recipient lungs were fixed at constant pressure (20 cm H₂O) with 1:1 v/v of OCT and PBS. Frozen sections (5 µm thick) were prepared and stained for GFP (catalog # AB6556, Abcam). Briefly, sections were fixed with ice-cold acetone for 2 minutes, air-dried and washed with PBS containing 0.1% Triton X-100. Non-specific binding was

blocked with 5% normal goat serum for 2 hours followed by overnight incubation with anti-GFP primary antibody (1:1000) at 4°C. After extensive washes, the slides were then treated with AlexaFluor-488-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:500; Molecular Probes-Invitrogen), counterstained with Hoechst (nuclear) dye (1:5000, Sigma) and mounted with immunofluorescence mounting medium (Vector Laboratories, Burlingame, CA). Isotype controls were used for non-specific binding. Stainings were visualized with a Nikon microscope.

Real-time PCR analysis

Total RNA was prepared from sorted BMC ($n \geq 3$ sets of BMC from different animals) using the RNeasy Kit (Qiagen, Valencia, CA). For RNA, reverse transcription for first-strand cDNA was generated using Superscript II (Sigma) according to manufacturer's protocol. Primers specifically detecting expression of pluripotent stem cell markers Oct-4 and Nanog are shown in Table 4-1.

The real time PCR conditions and instrumentation was as follows: 50°C for 2 min; 95°C for 10 min; 35 cycles at 94°C for 15 sec; 60°C for 1 min followed by dissociation using the ABI7900HT robot and analyzed with SDS 2.0 software. GAPDH was determined to be the most stable house-keeping gene by geNorm program therefore it was used as the housekeeping gene to normalize gene expression levels using REST-384 (relative expression software tool, available at www.Gene-Quantification.com) program.

Table 4-1: Primers

Gene	Forward primer 5'→3'	Reverse primer 5'→3'	Notes
K18Intron	CACTCACCCCACCCAT CC	CTCCTCGCCCTTGCT CAC	Detects transgenic DNA
OCT4	AGTTGGCGTGGAGACT TTGC	CAGGGCTTTCATGTC CTGG	ESC marker
Nanog	TCTTCCTGGTCCCCACA GTTT	GCAAGAATAGTTCT CGGGATGAA	ESC marker
Sca-1	AGGAGGCAGCAGTTAT TGTGG	CGTTGACCTTAGTAC CCAGGA	Stem cell marker

Flow Cytometry

CCSP⁺Sca-1⁺ sorted cells from freshly isolated bone marrow from C57Bl/6 mice were stained with antibodies against CD90, CD105, CD45, CD34, CD31, OCT4, Nanog, SSEA1 in FACS buffer (2% BSA, 0.05% sodium azide in PBS) for 30 minutes on ice after blocking with buffer containing 5% normal goat serum and 2% BSA to block non-specific binding. For intracellular proteins, cells were pre-treated and stained with FACS buffer containing 0.05% saponin. After primary antibody treatment, the cells were washed and incubated with AlexaFluor488 secondary IgG (1:500, Molecular Probes) on ice for 30 minutes. Relative expression was compared to isotype controls for all staining. A FACSCalibur flow cytometer and Expo32 Software (Becton-Dickinson, San Jose, CA) were used to assess expression of these markers. Red blood cell lysis buffer (Sigma) was used to lyse contaminating red blood cells prior to staining.

Differentiation assays*Air-liquid interface for epithelial cultures*

TEC and CCSP⁺Sca-1⁺ or CCSP⁺Sca-1⁻ were cultured at ALI for one month as

previously described (Chapter 3). After 2 weeks in submerged culture, only the bottom of the transwell membrane was exposed to media. CCSP⁺Sca-1⁺ or CCSP⁺Sca-1⁻ BMC were cultured in media containing 75% MTEC and 25% DMEM. All cells were cultured under ALI for 4 weeks.

Statistical Analysis

Data are presented as mean \pm SEM. For comparison of freshly isolated bone marrow cell and cultured plastic-adherent bone marrow cell phenotype one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests (Prism 4.0) were performed. Statistical significance was defined as $p < 0.05$.

4.4 Results

A subpopulation of the CCSP⁺ cells in the bone marrow also express Sca-1⁺

We have previously shown that a small population of freshly isolated BMC in both murine and human marrow expressed CCSP and can expand in vitro (See Chapter 3). To determine whether the CCSP⁺ BMC fraction contained a stem cell subpopulation, 7-day cultured BMC were stained for CCSP and the stem cell antigen (Sca)-1. Within the CCSP⁺ fraction, 10% also expressed Sca-1 which corresponds to 2% of the total cultured cells (Figure 4-1A). To rule out the possibility of artifactual expression of these markers due to ex vivo culturing, freshly isolated cells from the bone marrow were also assayed for CCSP and Sca-1 expression. A small (~0.1%) fraction of the freshly isolated BMC coexpress CCSP and Sca-1 express. To confirm expression of these markers, CCSP⁺Sca-

1⁺ cells were sorted directly from freshly isolated marrow and assayed for mRNA levels by real time PCR. Indeed the sorted CCSP⁺Sca-1⁺ cells expressed CCSP mRNA (Figure 4-1B). To determine whether this population of cells can expand in culture, male CCSP⁺Sca-1⁺ cells were plated directly on plastic culture dishes or cocultured with female CCSP⁻ cells (Figure 4-2A). CCSP⁺Sca-1⁺ cells cultured alone did not survive beyond 7 days of culturing suggesting a co-dependence on the CCSP⁻ cell population. CCSP⁺Sca-1⁺ cells cocultured with CCSP⁻ cells show an increase in cell number (Figure 4-4-2B) and SRY genomic DNA (Figure 4-2C) after 14 days in culture.

Naphthalene-induced lung injury transiently increases the CCSP⁺Sca-1⁺ BMC population in the bone marrow and peripheral blood.

We reasoned that CCSP⁺Sca-1⁺ BMC may be involved in endogenous lung repair mechanisms. To determine whether the CCSP⁺Sca-1⁺ cells in the bone marrow can respond to naphthalene-induced lung injury, freshly isolated BMC from naphthalene-injured and uninjured mice were assessed for CCSP⁺Sca-1⁺ levels. In contrast to the lung (Stripp et al., 1995; Wong et al., 2007), the number of CCSP⁺Sca-1⁺ cells in the bone marrow increased as early as 2 hours of naphthalene injury, peaked levels between 2-10 days of injury (~0.3%) (Figure 4-3G). By 20 days after naphthalene-induced injury, CCSP⁺Sca-1⁺ BMC levels returned to near baseline levels (compared to uninjured ~0.1%).

The peripheral blood was also assessed for CCSP⁺Sca-1⁺ cells. A small level of CCSP⁺Sca-1⁺ cells was detected in the blood of uninjured mice (~0.25%, Figure 4-3G). The number of circulating CCSP⁺Sca-1⁺ cells increased significantly up to ~0.45% 1 day

of lung injury corresponding to the early phase of maximal airway injury. The level of circulating CCSP⁺Sca-1⁺ cells declined at 2 days and returned back to near baseline levels by 20 days

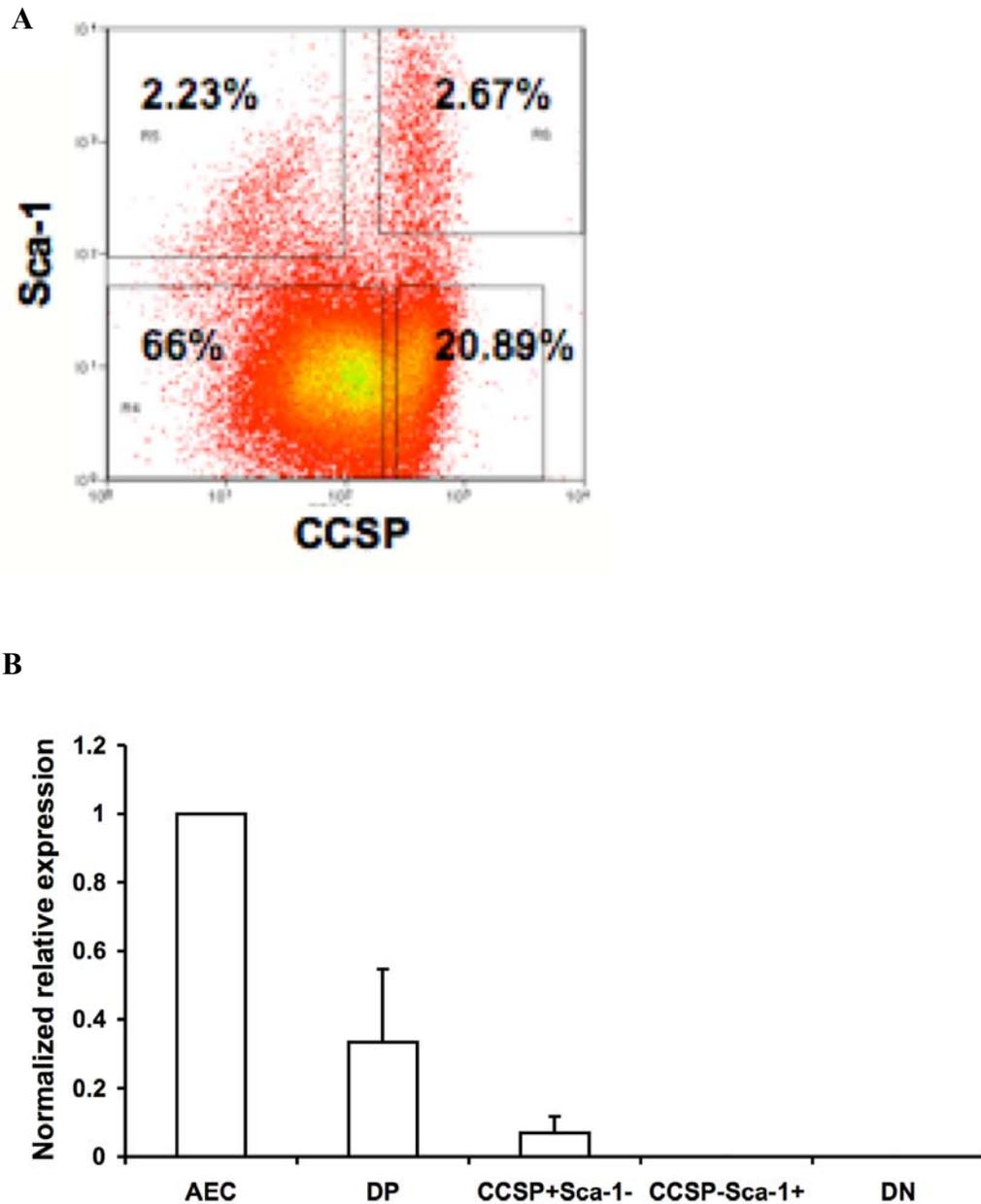
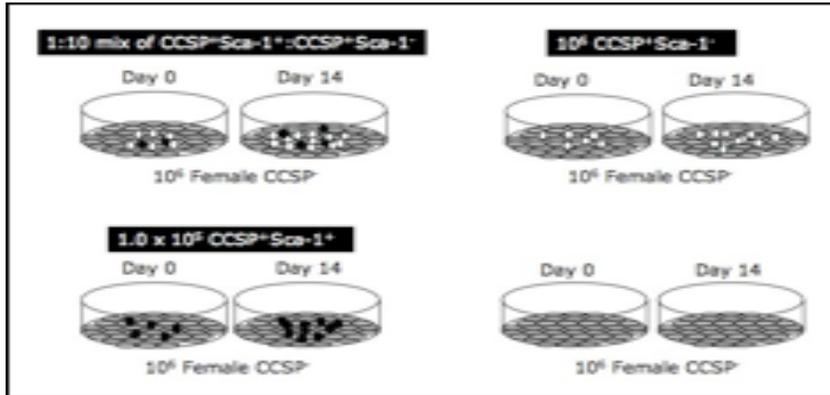


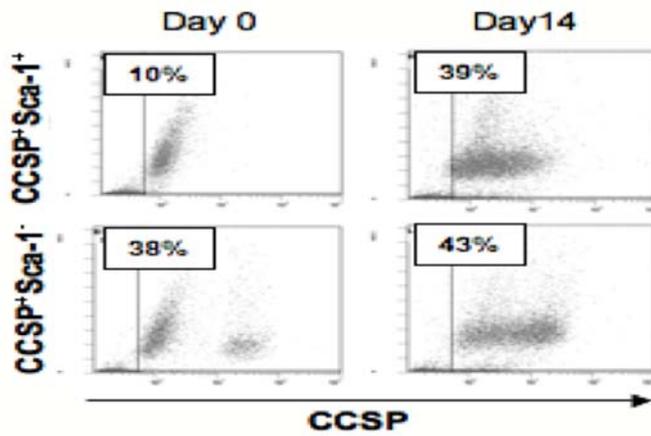
Figure 4-1: Identification of CCSP⁺Sca-1⁺ cells

(A) Flow cytometric analysis of 7-day cultured plastic-adherent bone marrow cells show a small subpopulation of CCSP⁺ cells (>2.5%) coexpress the stem cell antigen (Sca)-1. (B) Real time PCR confirm mRNA expression of CCSP in CCSP⁺Sca-1⁺ (double positive, DP) and CCSP⁺Sca-1⁻ but not in CCSP⁻Sca-1⁺ or CCSP⁻Sca-1⁻ (double negative, DN) cells. AEC; airway epithelial cells. Samples were normalized to GAPDH and expressed as relative expression to AEC. N=4 per group.

A



B



C

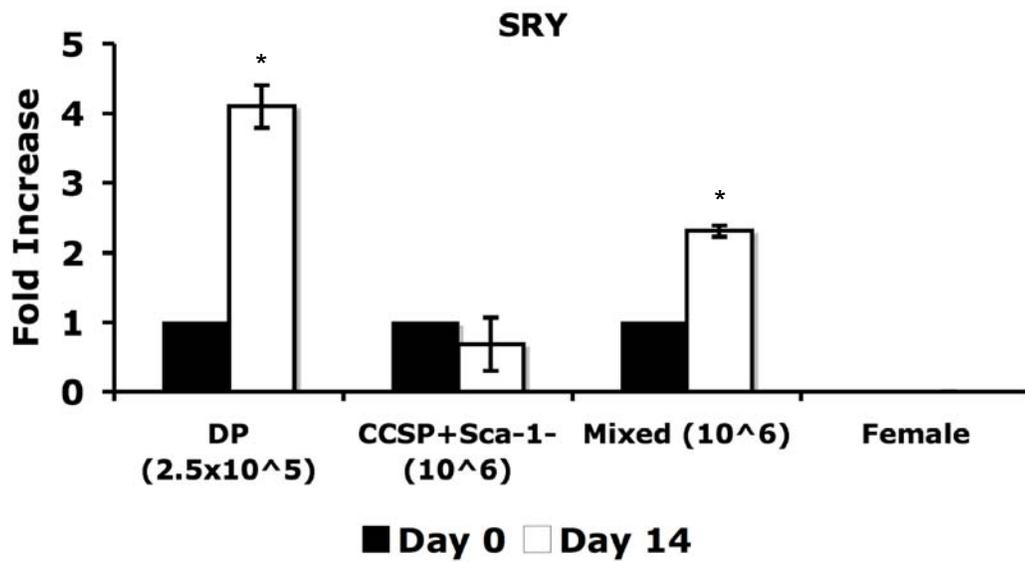


Figure 4-2: CCSP⁺Sca-1⁺ are highly proliferative and expand 4-fold after 14 days in culture.

(A) Coculture experiment to assay for the growth characteristics of CCSP⁺Sca-1⁺ cells. Male CCSP⁺Sca-1⁺ cells were mixed with CCSP⁺Sca-1⁻ in a 1:10 mix (10⁶ total donor cells) and cultured with female CCSP⁻ cells. In another group, 10⁶ CCSP⁺Sca-1⁺ or CCSP⁺Sca-1⁻ were cultured with CCSP⁻. Growth was measured as the difference in number of donor cells by flow cytometry (B) or real time PCR (C) at day 0 (cocultured for 1 hour) and day 14. While CCSP⁺Sca-1⁻ show no significant increase in cell number after 14 days in culture, CCSP⁺Sca-1⁺ show a ~4-fold increase. PCR for male sex chromosome SRY confirm the increase in donor SRY DNA in DP group. Cultured with mixed male cells showed a 2.5-fold increase in SRY DNA. N=4 per group. *P<0.01 compared to Day 0.

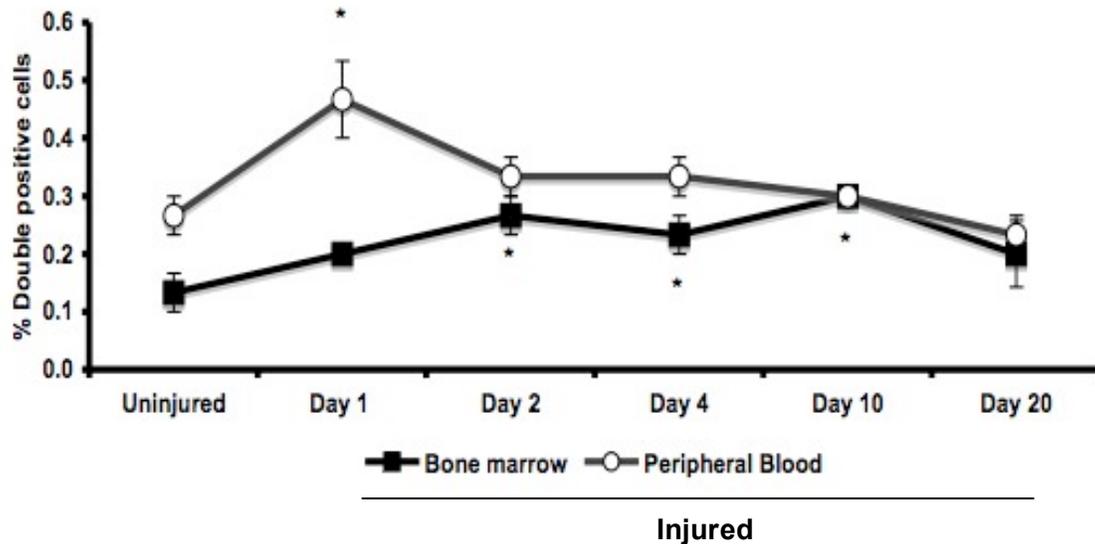


Figure 4-3: Naphthalene-induced lung injury transiently increases the CCSP⁺Sca-1⁺ BMC population in the bone marrow and peripheral blood

The average baseline levels of CCSP⁺Sca-1⁺ (double positive) cells in the bone marrow and peripheral blood are 0.1% and 0.25% respectively. The percentage of double positive cells increase in the bone marrow during the early stage of airway injury (Day 1-2) and remain elevated during the course of maximal airway injury (Day 4-10) and returns back to near baseline levels by Day 20. The percentage of double positive cells in the peripheral blood increase early on Day 1 but quickly returns to near baseline levels by Day 2. Data represents Mean ± SEM; N=4 per group. *P<0.05 compared to uninjured controls.

CCSP⁺Sca-1⁺ cells express both genes associated with mesenchymal and hematopoietic cells.

Expression profiling of the CCSP⁺Sca-1⁺ population revealed that these cells expressed genes that are classically associated with mesenchymal (CD90, CD105 and CD44) cells although levels of CD73 and CD106 gene expression were low (Figure 4-4A). In addition, these cells express CD45, a hematopoietic cell marker in adult bone marrow (Figure 4-4B). Interestingly, the CCSP⁺Sca-1⁻ subset, which could be the progeny of the double positive cells, express much less CD45, at least at the mRNA level. This data suggests a heterogenous population of cells within the CCSP⁺Sca-1⁺ fraction.

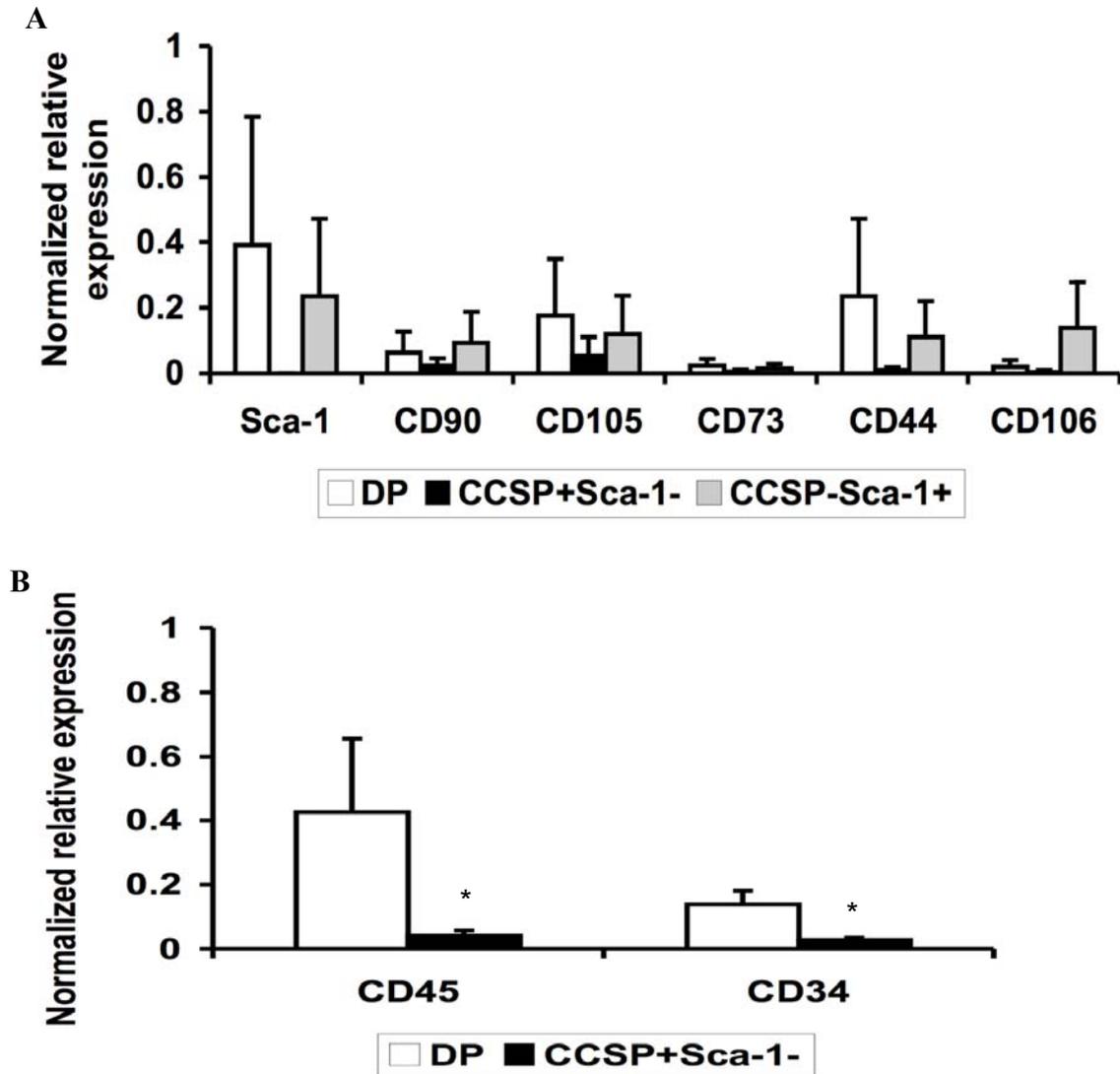


Figure 4-4: CCSP⁺Sca-1⁺ cells express both genes associated with mesenchymal and hematopoietic cells

Cells were sorted from 7-day cultured plastic-adherent bone marrow cells and analyzed for genes associated with bone marrow mesenchymal (A) and hematopoietic (B) cells. Each gene expression was normalized to the house-keeping gene GAPDH and expressed relative to mesenchymal stem cell (MSC) or whole bone marrow. Mean ± SEM; N=3 per group. *P<0.005 compared to DP cells.

On the contrary, CCSP⁺Sca-1⁻ cells express very low levels of all mesenchymal and hematopoietic genes. CCSP⁻Sca-1⁺ cells express similar levels of mesenchymal genes as CCSP⁺Sca-1⁺ cells but do not express the hematopoietic genes CD45 and CD34. Real time PCR of hematopoietic cells of various developmental stages found lack of CCSP mRNA expression in the known hematopoietic cell lineages (See Chapter 3, Figure 3-10).

A small population of bone marrow CCSP⁺Sca-1⁺ cells express pluripotent stem cell markers OCT4, Nanog and SSEA1

To examine the possibility that a stem cell population were enriched in CCSP⁺Sca-1⁺ fraction, CCSP⁺Sca-1⁺ cells were assessed for expression of pluripotent stem cell antigens including the transcription factors associated with pluripotency, OCT4 and Nanog and the cytoplasmic stage-specific embryonic antigen-1 (SSEA-1). Sorting for the CCSP⁺Sca-1⁺ population shows that these cells also expressed OCT-4, Nanog and SSEA1 (Figure 4-5). PCR analysis of these cells for OCT4 and Nanog confirm the gene expression of these stem cell markers (Figure 4-6).

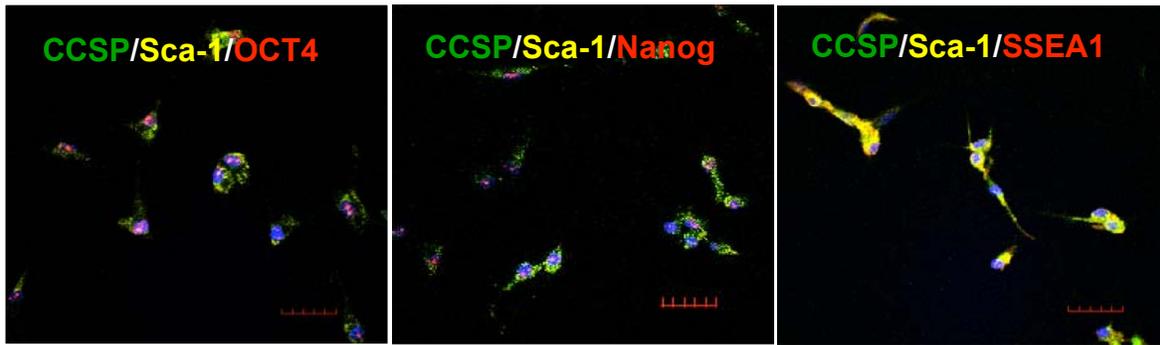


Figure 4-5: Bone marrow CCSP⁺Sca-1⁺ cells express pluripotent stem cell markers

A small subpopulation of CCSP⁺Sca-1⁺ cells sorted after 7 days in culture coexpress markers of pluripotency, the transcription factor octomer-4 (OCT4), Nanog and the stage-specific mouse embryonic protein (SSEA)-1. CCSP⁺ (green) cells were sorted and double stained for Sca-1 (yellow) and stained with OCT4, Nanog or SSEA1 (red). N=4 per group.

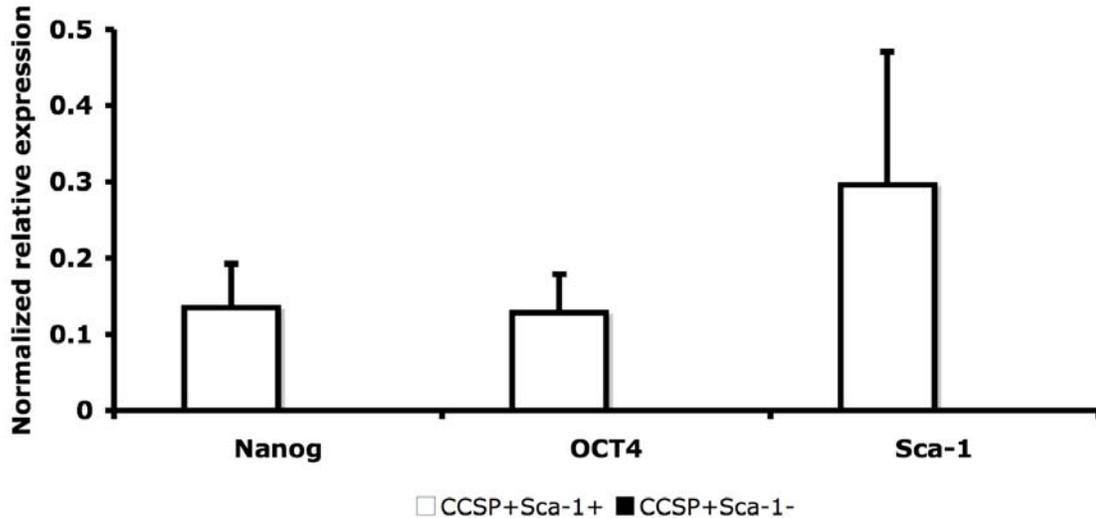


Figure 4-6: CCSP⁺Sca-1⁺ cells express Oct4 and Nanog mRNA

Real time PCR confirms mRNA expression of Oct 4 and Nanog in CCSP⁺Sca-1⁺ cells but not in CCSP⁺Sca-1⁻ cells or CCSP⁻Sca-1⁺ cells. Each gene expression was normalized to the house-keeping gene GAPDH and expressed relative to mouse ESC. Mean ± SEM. N=4 per group.

Bone marrow CCSP⁺Sca-1⁺ cells have self-renewal potential *in vitro* and *in vivo*

To provide evidence that the CCSP⁺Sca-1⁺ cells not only express markers of stem cell self-renewal, we attempted clonal analysis of cells arising from the CCSP⁺Sca-1⁺ cells. A single bone marrow CCSP⁺Sca-1⁺ cell from mice constitutively expressing GFP under the beta-actin promoter (BACT-GFP/Bl/6) were sorted by FACS into each well of a 96-well plate pre-coated with irradiated MEF (Figure 4-7A). To ascertain that 1 cell was sorted in each well, a GFP⁺ cell was identified under fluorescence microscopy within 16 hours after sorting (Figure 4-7B). Only wells that contained 1 cell in each well were further assessed for clonal expansion up to 14 days in culture. Single cell cultures of CCSP⁺Sca-1⁺ cells plated on feeders formed colonies (Figure 4-7B) with a clonal frequency of 40% (Figure 4-7C). To test self-renewal, single cells from one colony from the first clone (P0). The progenies of these clones also gave rise to secondary (P1) and tertiary (P2) colonies at a frequency of 40%. Plating of CCSP⁺Sca-1⁻ cells produced colonies at a frequency of about 20%. The size of the colonies derived from CCSP⁺Sca-1⁺ cells appeared to be larger than the CCSP⁺Sca-1⁻ cells (Figure 4-7D). The number of cell division however was no different between the two cell types suggesting that the CCSP⁺Sca-1⁻ cells were dieing at a higher rate than CCSP⁺Sca-1⁺ cells (Figure 4-7E).

We have previously shown that 7-day plastic adherent BMC could remain in the airways up to 120 days following cell delivery (Wong et al., 2007). To determine the proliferative potential of CCSP⁺Sca-1⁺ BMC in airway epithelial regeneration, CCSP⁺Sca-1⁺ BMC (2×10^5 cells/mouse) or mixed unfractionated BMC (10^6 cells/mouse) from male K18EGFP mice were injected transtracheally into naphthalene-injured airways of female recipients two days following injury. After 30 days, a group of

mice were sacrificed while another group of mice were injected with a second dose of naphthalene and assessed for donor BMC repopulation 2 or 20 days following the second injury (Figure 4-8A). The right lung was harvested for real-time PCR assessment of donor DNA level using specific primers designed to recognize K18 intron sequence and K18EGFP transgene expression using intron-spanning primers (Wong et al., 2007). The left lung was harvested for anti-GFP immunofluorescent staining. Airway injured and uninjured recipient mice that did not receive K18EGFP BMC were used as controls. As expected, no transgenic DNA was detected in mice that did not receive donor K18EGFP BMC (Figure 4-8B). Transgenic DNA was detected in recipient lungs at all timepoints. The level of transgenic DNA in mice that received CCSP⁺Sca-1⁺ cells was more than 10-fold less than the mice that received the mixed population of cells after 30 days although these mice were injected with only 5-fold fewer cells to begin with. However, after the second dose of airway injury (day 32), the level of transgenic DNA did not change in mice receiving CCSP⁺Sca-1⁺ cells compared to day 30 while transgenic DNA decreased dramatically in the group that received mixed cells. By day 50, corresponding to 20 days following the second dose of naphthalene injury and a time when airway epithelium has completely regenerated (Wong et al., 2007), there was an increase in transgenic DNA in both groups suggesting CCSP⁺Sca-1⁺ cells are not only resistant to naphthalene injury but can proliferate during epithelial repair. Assessment of K18-GFP transgene expression showed a substantial increase in transgene expression in the lungs of mice that received CCSP⁺Sca-1⁺ cells (Figure 4-8C, *P<0.05 compared to day 30).

To confirm the PCR data, anti-GFP staining was done on contralateral lung sections. After the second injury, an increase in GFP⁺ cells was found in the airway

epithelium of mice that received CCSP⁺Sca-1⁺ cells (Figure 4-8D). On the contrary, a decrease in GFP⁺ cells was found in mice 2 days after the second injury that received a mixed population of cells with a gradual reappearance of more GFP⁺ cells by 20 days.

To determine the in vivo self-renewal potential of the engrafted CCSP⁺Sca-1⁺ cells after 30 days in the lungs, donor CCSP⁺Sca-1⁺ cells (also GFP⁺, 9.7x10⁴ cells per mouse) were re-isolated from the lungs of the first recipient (R1) and injected transtracheally into naphthalene-injured secondary recipients. After 30 days, some mice were sacrificed while others received a second dose of naphthalene and sacrificed at day 50 (Figure 4-9). Detectable levels of transgenic DNA were found in mice that received CCSP⁺Sca-1⁺ cells. Similar to the R1 group, an increase in transgenic DNA was observed at day 50 after the second dose of naphthalene-injury (Figure 4-9B). Transgene expression also increased at day 50 (Figure 4-9C, *P<0.05 compared to day 30).

Bone marrow CCSP⁺Sca-1⁺ cells differentiate into various epithelial cell lineages

To determine whether CCSP⁺Sca-1⁺ cells could give rise to other epithelial cell lineages, sorted CCSP⁺Sca-1⁺ cells were cultured in air-liquid interface for 4 weeks. After 4 weeks, cells were trypsinized from the membrane and resuspended for cytospin preparations for immunofluorescence staining. CCSP⁺Sca-1⁺ cells can give rise to distinct populations of cells that co-expressed epithelial markers of different lineages. SP-C⁺ (Type II alveolar cell marker) that co-expressed AQP5⁺ (Type I alveolar cell marker) cells were found (Figure 4-10). Cells that coexpressed K14 (basal cell marker) and SP-C, K14 and acetylated α -tubulin (ciliated cell marker), K14⁺ only and acetylated α -tubulin⁺

only were also found. Lung tracheal epithelial cells were used as positive controls for both the air liquid interface cultures and immunofluorescence staining.

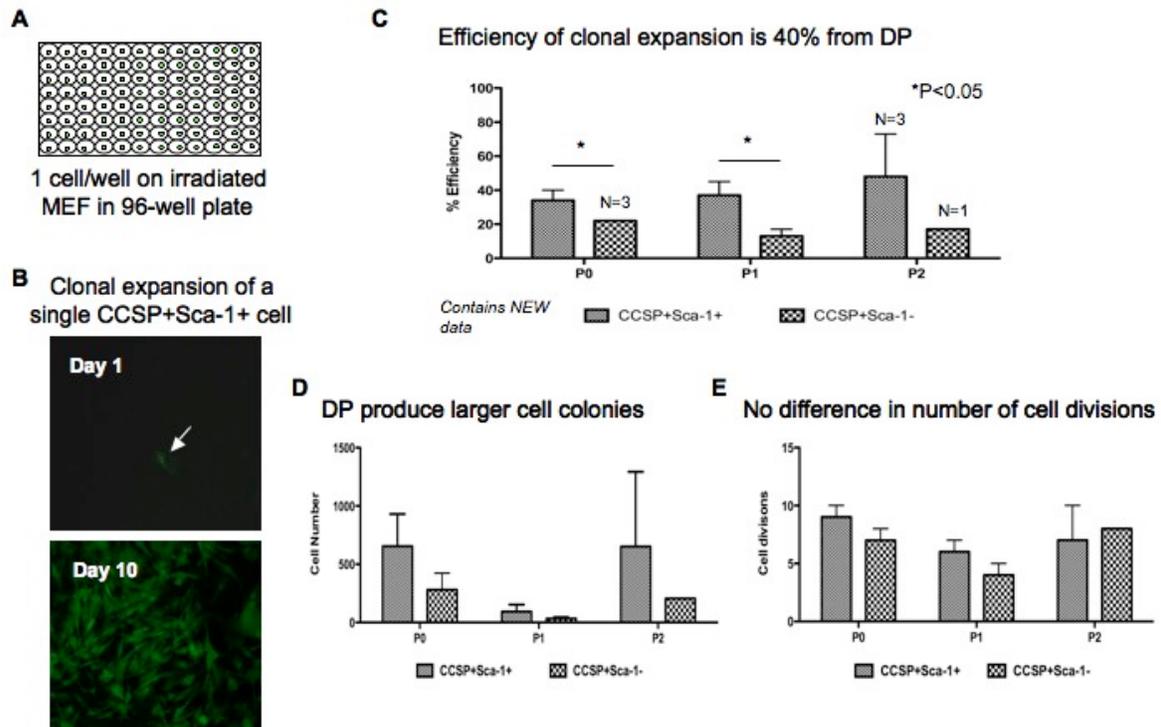


Figure 4-7: Bone marrow CCSP⁺Sca-1⁺ cells have self-renewal potential *in vitro*

(A) Strategy for clonal assessment. (B) Representative photomicrograph of 1 single GFP⁺CCSP⁺Sca-1⁺ cell on a layer of mouse embryonic fibroblasts 1 day after sorting which clonally expanded by 10 days in culture. (C) Efficiency of clonal expansion from a single GFP⁺CCSP⁺Sca-1⁺ cell from parent (P0), second generation (P1) and third generation (P2) clone. (D) Mean cell number at various days in culture derived from a single GFP⁺CCSP⁺Sca-1⁺ cell or GFP⁺CCSP⁺Sca-1⁻ cell. (E) No difference in the number of cell division between the two cell type. Mean ± SEM. N≥1 per group.

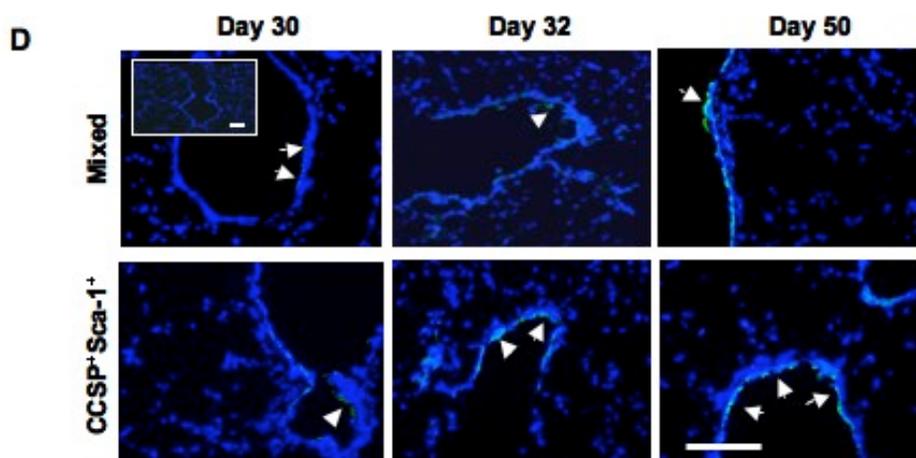
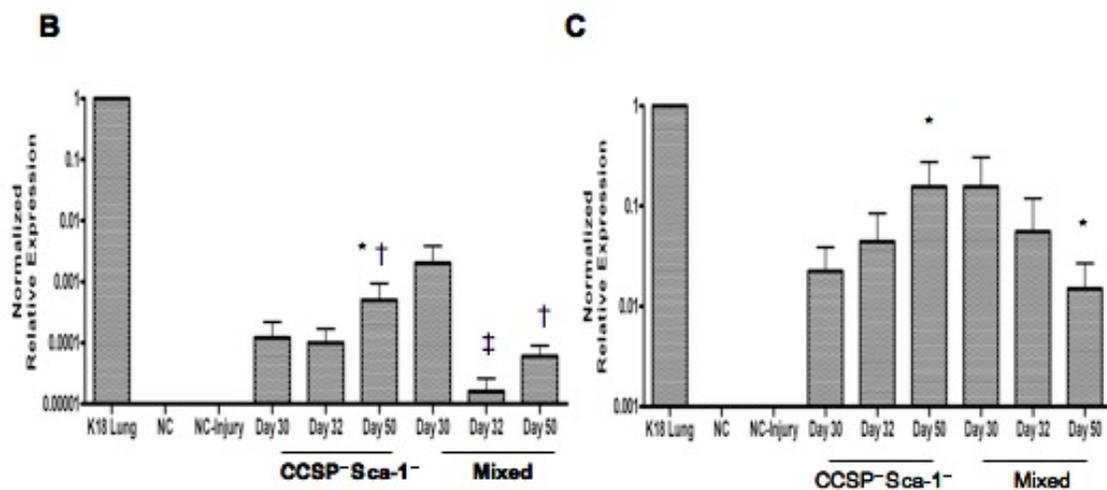
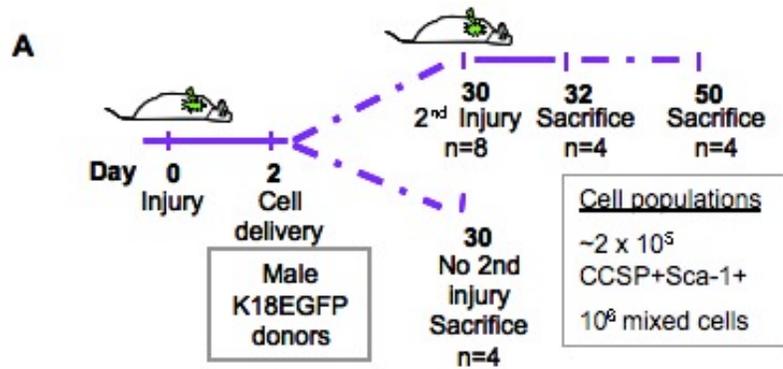
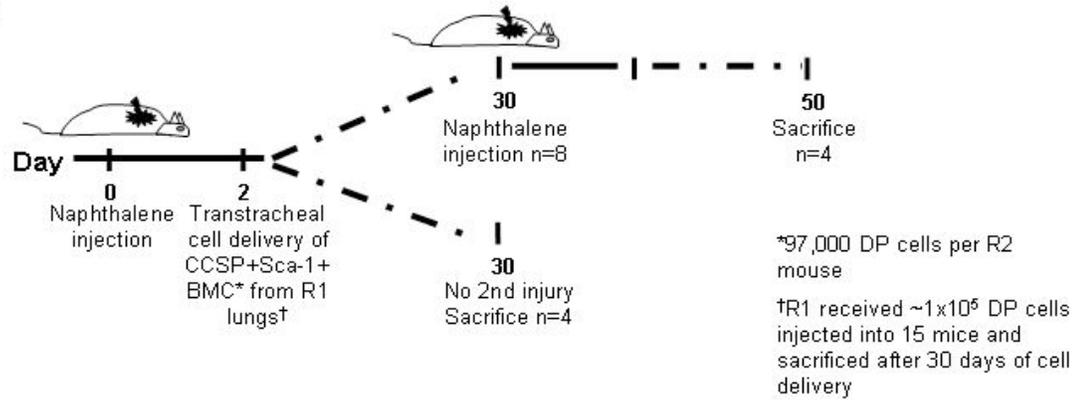


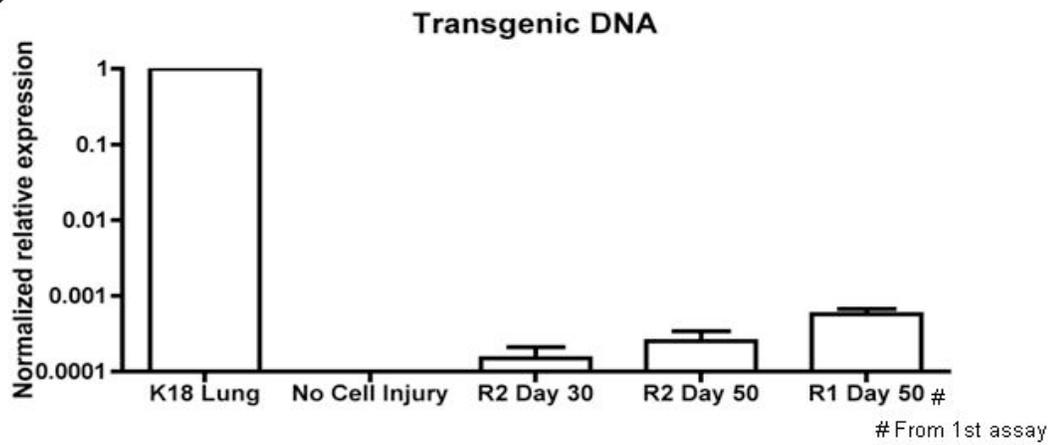
Figure 4-8: Bone marrow CCSP⁺Sca-1⁺ cells have reconstitution potential *in vivo*

(A) Schematic diagram of the *in vivo* reconstitution assay. **(B)** The level of transgenic DNA in mice that received CCSP⁺Sca-1⁺ cells compared to more than 10-fold less than the mice that received the mixed population of cells after 30 days even though these mice were delivered with 5-fold less cells to begin with. However, after the second dose of airway injury (day 32), the level of transgenic DNA did not change in mice receiving CCSP⁺Sca-1⁺ cells while transgenic DNA decreased dramatically in the mixed group. By day 50, there was an increase in transgenic DNA in both groups. * P<0.05 compared to Day 30 group. †P<0.05 compared to day 32. ‡P<0.001 compared to day 30. **(C)** Transgene expression increased overtime in mice that received CCSP⁺Sca-1⁺ cells. Mice that received the mixed population of cells showed a reduction in transgene expression. Expression was normalized to the house-keeping gene GAPDH and expressed relative to lung sample from K18EGFP mouse. Mean ± SEM * P<0.05 compared to Day 30 group. **(D)** Anti-GFP staining of the left lung show isolated GFP⁺ cells in the airway epithelium at day 30 and 32 but later reconstitutes the airway epithelium at day 50. N=4 per group.

A



B



C

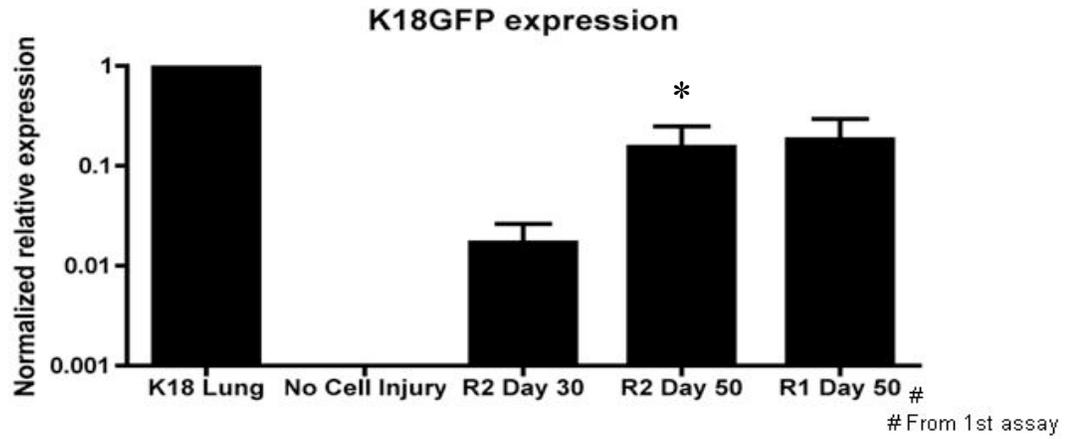


Figure 4-9: Airway engrafted donor CCSP⁺Sca-1⁺ BMC have secondary reconstitution potential *in vivo*

(A) Schematic diagram of the *in vivo* reconstitution assay. (B) The level of transgenic DNA in mice that received CCSP⁺Sca-1⁺ cells from the first recipient (R1) was 5000-fold less than control K18GFP transgenic lung. After the second dose of airway injury, the level of transgenic DNA increased. (C) A corresponding increase in transgene expression was observed in R2 mice. Level of expression reached similar levels to R1 mice at day 50. Each gene expression was normalized to the house-keeping gene GAPDH and expressed relative to lung sample from K18EGFP mouse. * P<0.05 compared to Day 30 group.

Mean ± SEM. N=4 per group.

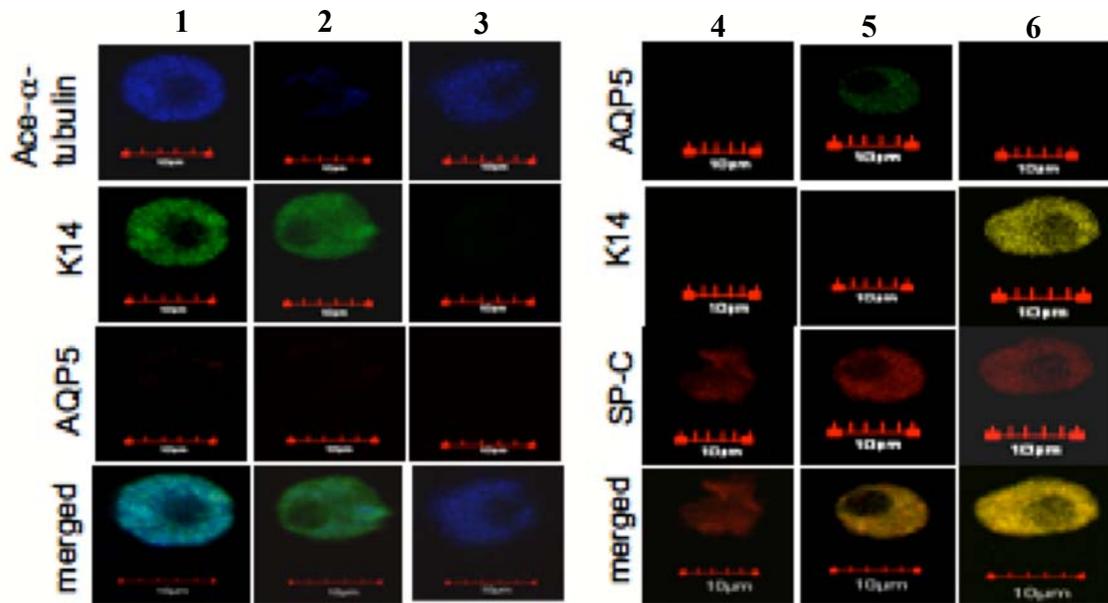


Figure 4-10: Multi-lineage epithelial differentiation of CCSP⁺Sca-1⁺ cells

Representative photomicrograph of CCSP⁺Sca-1⁺ cells after 4 weeks in air liquid interface cultures. Each column represents the same cells triple stained for epithelial markers of different lineages (airway, alveolar). N=4 per group.

4.5 Discussion

We have identified a cell population in the adult mouse marrow that has properties of stem cells and has pulmonary reconstitution potential. These bone marrow-derived epithelial-like stem/progenitor cells expressing CCSP and Sca-1 were also found in the peripheral blood. Both bone marrow and blood CCSP⁺Sca-1⁺ cells proliferated during airway epithelial injury. Preliminary in vitro studies suggest a hierarchy where these double positive cells do not arise from CCSP⁺Sca-1⁻ or CCSP⁻ cells. These cells expressed genes associated with mesenchymal and hematopoietic cell lineages and may explain conflicting data published in recent years that both hematopoietic and mesenchymal stromal cell populations may have pulmonary reconstitution potential. These cells are detectable in fresh unmanipulated bone marrow but adhere readily to plastic. We might speculate that these cells (or their progeny with reduced CD45 expression) could have been co-isolated in protocols designed to enrich for MSC (Kotton et al., 2001; Krause et al., 2001). A small subpopulation of expressed markers associated with pluripotency suggesting these might indeed be stem cells with self-renewal and multidifferentiation potential. Indeed, a single CCSP⁺Sca-1⁺ cell could give rise to a colony of cells at an average ~40% efficiency in vitro which was serially repeatable to at least the third generation. Engrafted K18GFP CCSP⁺Sca-1⁺ cells in the airway epithelium could proliferate in situ. Histologically, these cells expressed a K18-driven GFP reporter suggesting acquisition of an epithelial phenotype and appeared to be on the airway epithelium after airway injury. CCSP⁺Sca-1⁺ cells could be induced to differentiate into various epithelial cell types including cells expressing phenotypic characteristics of

ciliated cells, Type I, Type II and airway basal cells however full scale differentiation into a mature epithelium remains to be demonstrated.

Expression of pluripotent markers in adult stem cells

We identified a small population of CCSP⁺Sca-1⁺ BMC that express pluripotent markers Oct4 and Nanog. Although expression of Oct-4 and Nanog are classically observed in embryonic stem cells, there is growing evidence to suggest that adult stem cells also express these transcription factors. Oct-4 and Nanog expressing cells have recently been identified in multipotent adult stem cells in human hair follicles (Yu et al., 2006), in a subset of endothelial progenitor cells that are CD14⁺CD34^{low} (Romagnani et al., 2005) and in amniotic epithelial cells (Miki et al., 2005). OCT-4 and Nanog expression was recently identified in cancer stem/progenitor cells (Gu et al., 2007) and seminoma and breast carcinoma cells (Ezeh et al., 2005). OCT-4-expressing stem cells could also be found in isolated mouse lung cells (Ling et al., 2006). In the adult bone marrow of mice, a rare OCT-4-expressing embryonic-like pluripotent stem cell has been identified that can give rise to cells of all three germ layers in vitro (Kucia et al., 2006a). These cells adhere to fibroblasts and are coisolated with bone marrow plastic-adherent cells. This seems reminiscent of the inability of the CCSP⁺Sca-1⁺ BMC to survive and propagate in cultures devoid of the larger CCSP⁻ cells and MEFs. It is likely that the CCSP⁺Sca-1⁺ population we isolated contain a mixture of stem and progenitor cells. Future studies will need to delineate the stem/progenitor cell hierarchy within the CCSP⁺ fraction as well as determine whether the self-renewing population in vitro are the same as those those regenerate the lung epithelium using genetic tracing studies. The ultimate

test of stem cell activity is a single-cell clonogenic transplantation assay, as has been done in the hematopoietic system (Krause et al., 2001).

Tissue committed neural, cardiac, liver and muscle stem/progenitor cells have been found in the bone marrow (Kucia et al., 2004b; Kucia et al., 2006a; Kucia et al., 2005b; Kucia et al., 2006b). These cells expressed tissue committed markers and are mobilized to the peripheral blood and recruited to the injured tissue. In Chapter 3, we demonstrate that CCSP⁺ BMC can respond to airway injury and migrate to the injured lungs. Although the CCSP⁺Sca-1⁺ population could respond and expand in the bone marrow and blood during airway injury, it remains unclear whether these cells could also migrate to the injured epithelium and contribute to lung cell lineages. It is possible that the CCSP⁺Sca-1⁺ cells reside in the bone marrow and contribute to lung regeneration by generating daughter cells that are CCSP⁺Sca-1⁻ that can mobilize and home to the epithelium. On-going work will assess the contribution of CCSP⁺Sca-1⁺ population in lung regeneration.

Comparison of bone marrow-derived CCSP⁺Sca-1⁺ cells to lung stem cells

Intrapulmonary stem cells that reside in distinct niches (Borthwick et al., 2001; Engelhardt, 2001) in the pulmonary compartments are classically considered the source for epithelial regeneration. For example, in the distal airways, naphthalene-induced toxicity to the Clara cells of the airway epithelium revealed a unique resistant cell population (expressing CCSP) that reside in close proximity to rare neuroendocrine bodies (Hong et al., 2001) of the proximal airways. These CCSP-expressing progenitor cells also reside in the bronchioalveolar duct junctions in the terminal bronchioles

(Giangreco et al., 2002) and could give rise to Clara and Type II cells (Reynolds et al., 2004). Kim showed that a rare subset of CCSP-expressing cells in the bronchioalveolar duct junctions were bronchioalveolar stem cells (Kim et al., 2005). A comparison of the reported adult bronchioalveolar stem cells and the CCSP⁺Sca-1⁺ BMC in our study reveals that they not only share common markers such as Sca-1, CD34, CCSP and SP-C but they are also resistant to airway damage and can proliferate in response to airway injury. Similarly, our CCSP⁺Sca-1⁺ BMC have self-renewal capacity in vitro and are multipotent in epithelial differentiation. However, the bone marrow CCSP⁺Sca-1⁺ cells described here are also phenotypically different from lung bronchioalveolar stem cells in that the former express other hematopoietic markers including CD45, CD117, CD31 but the latter does not. A lung stem cell population that expresses CCSP, Sca-1, OCT-4 and SSEA-1 (all markers common in our bone marrow CCSP⁺Sca-1⁺ cells) has also been identified in neonatal lungs (Ling et al., 2006). Whether these stem cells are a subpopulation of bronchioalveolar stem cells or if there is a common lineage association between these two type of cells remains unknown. The mRNA for CD45 decreased substantially as cells progressed from the double positive to CCSP⁺Sca-1⁻ phenotype. While potentially iconoclastic, we wonder whether bone marrow-derived CCSP⁺Sca-1⁺CD34⁺SP-C⁺ cells may be a source of the previously described lung CCSP⁺Sca-1⁺OCT-4⁺SSEA1⁺ stem cells or CCSP⁺Sca-1⁺SP-C⁺CD34⁺ bronchioalveolar stem cells that have lost surface expression of CD45. This loss of CD45 expression may be triggered upon engrafting into the lung stem niche or differentiation into lung stem cells.

Origin of CCSP⁺Sca-1⁺ cells

This study identified a rare CCSP⁺Sca-1⁺ cell population that phenotypically resembles cells derived of hematopoietic and mesenchymal lineages. While it is understood that the phenotype of a MSC is CD90⁺CD105⁺CD73⁺ (Horwitz et al., 2005), CD90, CD105 and CD73 are also found on blood cells including early hematopoietic stem/progenitor cells (Pierelli et al., 2001; Saalbach et al., 2000), uncommitted CD4 T-cells (Yang et al., 2005), polymorphonuclear leukocytes and monocytes (Saalbach et al., 2000). However, unlike hematopoietic cells, mesenchymal cells lack CD45 expression (Barry et al., 2001; Bieback et al., 2004). Recently it has been demonstrated that multipotent cells with dual hematopoietic and mesenchymal nature exist in human umbilical cord blood (Rogers et al., 2007). These cells phenotypically characterized as CD45⁺lin⁻ umbilical cord blood (UCB) cells could transiently acquire mesenchymal markers vimentin and CD73 in vitro and were capable of mesenchymal but not hematopoietic engraftment in NOD/SCID mice. When cultured in specific media containing FGF4, SCF, and Flt3 ligand (FSFl media), these cells expressed Oct4 and Nanog and were highly expansive. Under specific differentiating culture conditions these cells were able to differentiate into endothelial, neuronal, osteoblasts, and muscle cells however these cells must first have expanded in FSFl media to acquire such multipotent characteristics. As such, these cells were concluded to be neither hematopoietic nor mesenchymal. In comparison. the CCSP⁺Sca-1⁺ cells described here do not require in vitro culturing to acquire mesenchymal cell surface markers. These cells could be directly isolated from the marrow and expanded in culture. We are currently testing the hematopoietic reconstitution potential of these cells. It remains unclear whether the

CCSP-expressing cells are derived from hematopoietic, mesenchymal cell lineages or are unique cell populations in the bone marrow. Furthermore, the mesenchymal reconstitution potential was not determined and therefore whether the CCSP⁺Sca-1⁺ cells expressing CD90, CD105 and CD73 are mesenchymally-derived cells or can give rise to mesenchymal cells remains unknown. In addition to expressing surface markers of both hematopoietic and mesenchymal cells, the CCSP⁺Sca-1⁺ cells express CCSP, a marker known to be expressed in lung epithelial progenitor cells (Giangreco et al., 2002; Hong et al., 2001; Kim et al., 2005; Ling et al., 2006). Therefore, the relationship between accepted lung progenitor cells and those that reside in the bone marrow is unknown. A provocative possibility is that they serve as a reservoir for lung stem cells and may transiently circulate from bone marrow to the lungs. Nonetheless, these CCSP⁺Sca-1⁺ cells exhibit properties of stem/progenitor cells in that they are clonal in single-cell clonal assays, have self-renewal potential *in vivo* and can at least differentiate into multiple epithelial cell types.

In conclusion, we identified a rare bone marrow cell that can be isolated by surface CCSP and Sca-1. These double positive cells have self-renewal and multi-lineage epithelial differentiation potential making them ideal cells for cell-based epithelial regeneration.

Chapter 5

Summary and Future Directions

5.1 Summary

While there is skepticism about the significance and contribution of bone marrow-derived stem/progenitor cells in tissue regeneration, evidence exists both in animal models and clinical lung specimens that these cells can contribute to lung repair and remodeling. As reviewed in Chapter 1, reports have suggested that bone marrow stem cells can phenotypically convert or “transdifferentiate” into lung epithelial cells upon engraftment or by in vitro model systems such as air liquid interface cultures (Kleeberger et al., 2003; Kotton et al., 2001; Krause et al., 2001; Rojas et al., 2005; Suratt et al., 2003; Wang et al., 2005). Recent reports have suggested a role for bone marrow stromal cells in anti-inflammatory mechanisms in the injured lungs (Gupta et al., 2007; Mei et al., 2007; Ortiz et al., 2007; Xu et al., 2007b). However, the contribution of bone marrow cells to lung cell lineages, the significance of the low levels of engraftment seen, and the functional role of such cells in the lung remain controversial (Chang et al., 2005; Kotton et al., 2005b; Loi et al., 2006). Needless to say, future work will be needed to address the relevance of bone marrow stem/progenitor cells in lung biology. Questions to be considered are: Are there separate progenitors capable of mediating epithelial restoration as oppose to lung pathologies such as pulmonary fibrosis? If so, what are the signals involved in recruiting the “good” versus the “bad” bone marrow stem/progenitors to the lung to tip the potential towards lung regeneration as opposed to fibrosis? If a single progenitor can mediate both phenomena, what signal(s) govern the fate choice? What are the cells specifically involved in lung repair and what are the mechanisms by which they do so? Finally, what are the functional roles, whether it is direct or indirect, these cells play in lung remodeling?

The studies presented in this thesis focus on the contribution of bone marrow progenitor cells in airway regeneration and the specific subpopulation of cells that have greater propensity for pulmonary regeneration. In Chapter 2, we used 7-day cultured plastic-adherent BMC populations and compared intravenous (via the jugular vein) and transtracheal cell delivery methods to assess the level of BMC retention in the lungs following naphthalene-induced airway injury. We demonstrated that transtracheal cell delivery yielded greater cell retention in the airways with increasing donor BMC cells expressing the airway CCSP protein 14 days after cell delivery. We concluded that the bone marrow plastic-adherent cell population contained cells that had the potential for airway cell replacement. We are currently working on methods to improve cell engraftment in the lungs by optimizing cell number, timing of cell delivery following injury and determine the subpopulation of BMC for optimal lung regeneration. The goal is to determine whether gene-corrected BMC can restore epithelial function in vivo in a clinically relevant model such as CF.

Like many other previously published reports, a problem with our first study was that we were unclear of the specific subpopulation of BMC that contributed to airway regeneration. However, in Chapter 2 we noticed that a few CCSP-expressing BMC in the airway epithelium were found as early as 1 day following cell delivery. This led us to investigate the possibility of a pre-existing CCSP-expressing BMC population in the plastic-adherent cultures and the bone marrow. In Chapter 3, we identified a small ~2% in mice and ~0.6 to 4% in 5 human volunteer bone marrow to be positive for the CCSP protein. To confirm that these cells indeed expressed the mRNA transcript for CCSP, real time PCR was employed using primers specific for the CCSP gene. The validity of these

assays was confirmed using knockout animals kindly provided by Dr. Barry Stripp (Duke University) where no CCSP-expressing BMC were found. To our knowledge this is the first report identifying newly discovered pulmonary epithelial-specific stem cell in the bone marrow, capable of self-renewal and multi-lineage differentiation capabilities. The discovery of these cells in human bone marrow suggest potential applications of these cells in cell replacement strategies for treatment of various epithelial diseases. Previously, Gomperts has shown that a cytokeratin-5-expressing BMC population existed in the blood and bone marrow that contributed to tracheal epithelial regeneration, although they did not evaluate either self-renewal or differentiation potential (Gomperts et al., 2006). In our hands, the CCSP⁺ cells were not cytokeratin-5⁺. In fact, we could not isolate the same cytokeratin-5⁺ population suggesting a possible difference in the bone marrow isolation techniques. Indeed technical variations in BMC preparations have resulted in significant differences in CCSP⁺ cell yield within our own lab. For example, bone marrow flushing had a significant impact on the ability to isolate the CCSP⁺ cells in mice. It appears that these cells are localized near the epiphysis and wall of the endosteum. Flushing the marrow solely at the body of the femur dramatically reduces the number of CCSP⁺ cells isolated. Future work will need to address the specific location of these cells in the bone marrow by in situ immunohistochemistry of a decalcified longitudinally-sectioned bone.

A significant finding of our work is the potential contribution of these cells in epithelial reconstitution. The ability to expand these progenitor cells ex vivo augments their utility for cell-based therapies. Using bone marrow transplantation strategies with male wild-type whole unfractionated marrow or CCSP⁺ enriched marrow transplanted

into lethally irradiated female CCSP^{-/-} recipients, we observed CCSP protein in the lungs and kidneys of reconstituted mice whereas recipients that received CCSP⁻ cells or whole unfractionated marrow from CCSP^{-/-} animals did not show CCSP expression. Immunohistochemistry for CCSP showed that CCSP seemed to be present but the staining was substantially less intense than in normal control animals and seemed to be diffusely localized to the entire epithelium under low power magnification. Under higher power magnification, rare isolated airway cells with more intense staining for CCSP were found suggesting that these cells may be secreting CCSP protein that is being absorbed by neighbouring cells. Indeed with additional bone marrow transplants using GFP⁺ donors to co-localize GFP and CCSP and cytokeratin-18 in the airway epithelium showed significant numbers of GFP⁺CCSP⁺cytokeratin-18⁺ cells found in the airway epithelium. In addition, neighbouring native epithelium also expressed the CCSP protein supporting the notion of absorption of the protein. We also demonstrated that the CCSP⁺ cells have some epithelial functional capacity such expression of SP-C and ENaC-sensitive transport properties. This is promising since this would support a regenerative purpose of these cells in the lungs.

Complete differentiation into an epithelial cell was not yet achievable with the CCSP⁺ cells using the conditions described. Specifically, culturing these cells under air liquid interface resulted in cells that seemed to coexpress markers of different epithelial cell lineages. Furthermore, electron microscopy of these cells after 4 weeks in air liquid interface did not show ultrastructural features resembling lung epithelium. It is possible that complete epithelial differentiation could be achieved by coculturing with native lung epithelium as shown by Dr. Prockop's group who were able to differentiate human MSC

into epithelial-like cells expressing CFTR under air liquid interface coculture with injured lung epithelium (Spees et al., 2003; Wang et al., 2005). In addition, coculturing CCSP⁺ BMC with distal lung mesenchyme may be a strategy to induce terminal differentiation as has been previously shown with embryonic epithelial cells (Deimling et al., 2007). Mechanical stretch can also be used to induce respiratory epithelial cell differentiation (Sanchez-Esteban et al., 2004). Although CFTR protein was found in CCSP⁺ cells, CFTR-activity was not observed suggesting further differentiation or differentiation along the proper epithelial cell lineage may be required to induce CFTR protein function. It is possible that injured lung epithelium may provide the extracellular matrix support and the soluble factor support for proper epithelial differentiation which is not mimicked in the ex vivo culturing model that we used. In Chapter 4 we show that CCSP⁺Sca-1⁺ cells could also be induced to differentiate into various epithelial cell types including cells expressing markers of ciliated cells, Type I, Type II and airway basal cells however complete differentiation into a mature epithelium was again unachievable. Future work will need to delineate mechanisms to induce adequate or proper differentiation of these cells to specific epithelial phenotypes.

Another key finding in this work is the identification of CCSP⁺ BMC with dual hematopoietic and mesenchymal characteristics. These cells may explain previously conflicting data regarding the contribution of bone marrow to epithelial regeneration as some studies suggested HSC contribute to epithelial cell lineages while others reported that MSC play a role (Krause et al., 2001; Ortiz et al., 2003; Rojas et al., 2005; Suratt et al., 2003). Cell surface markers such as CD45 are commonly associated with hematopoietic cell lineages while CD90, CD105 and CD73 are common antigens used to

identify mesenchymal stem cells (Horwitz et al., 2005). However, studies have shown that CD90, CD105 and CD73 are also found on hematopoietic stem/progenitor cells (Pierelli et al., 2001; Sumikuma et al., 2002; Yang et al., 2005). Since CCSP⁺ cells also express these markers associated with stem/progenitor cells, it may be that CCSP⁺ cells are a subset of stem/progenitors. Indeed, Chapter 4 explores the possibility of a stem cell population within the CCSP⁺ fraction. It remains unknown whether the CCSP⁺ cells have mesenchymal and hematopoietic reconstitution capabilities and whether these cells can give rise to both lineages of cells. One significance of the CCSP⁺CD45⁺ BMC population is their potential ability to reduce proinflammatory responses in the airways following infections. Pam Davis group showed that bone marrow transplantation of wild type marrow into CFTR knockout mice infected with *Pseudomonas aeruginosa* had significantly improved survival rate 50% (from 100% mortality) (Weiss et al., 2006). Analysis of the lung sections found improved lung histology. Furthermore, 97% of the chimeric cells were confined to the CD45⁺ phenotype. It was hypothesized that the CD45⁺ cells could express functional CFTR that can improve lung function. Therefore, the CCSP⁺CD45⁺ cells may play an important role in regulating lung inflammation.

We are currently performing experiments to functionally test the hematopoietic and mesenchymal contribution of the CCSP⁺ cells in vitro and in vivo. Classical in vitro differentiation assays for mesenchymal cell lineages such as chondrogenic, osteogenic and adipogenic cell differentiation are being performed. Bone marrow transplantation of CCSP⁺ and Sca-1⁺ cells from beta-actin GFP⁺ marrow are underway and we will assess the hematopoietic and mesenchymal contribution of these cells in vivo. Recently, a report from Casper's group identified a CD45⁺lin⁻ cell population in the hematopoietic fraction

of human umbilical cord blood that exhibited MSC differentiation characteristics and stromal cell engraftment but not hematopoietic engraftment potential when cultured in media enhanced with specific growth factors (FGF4, SCF, flt3 ligand) (Rogers et al., 2007). This is in contrast to our finding since the bone marrow CCSP⁺ cells express both HSC and MSC markers were not a product of specific growth factor stimulation.

In Chapter 4, we further investigate these cells and describe important characteristics of the subset which are CCSP⁺Sca-1⁺ (also known as “double positive”). A small subpopulation of CCSP⁺Sca-1⁺ cells expressed markers associated with pluripotency (Oct4 and Nanog) suggesting these cells may have broad differentiation capabilities. We are currently undertaking multilineage (epithelial, cardiomyocyte, and neuronal) differentiation assays using cells derived from a single CCSP⁺Sca-1⁺ cell to determine the plasticity of these cells. To determine the clonal expansion potential of these cells, we employed an *in vitro* single cell clonal expansion assay and found that a single CCSP⁺Sca-1⁺ cell could give rise to a colony of cells at an average ~40% efficiency to at least the third generation of replating. To test the airway reconstitution potential of these cells *in vivo*, we delivered CCSP⁺Sca-1⁺ from K18EGFP mice transtracheally into the airway epithelium of wild-type recipients after airway injury. After 30 days, these mice received a second dose of naphthalene-injury to determine whether the donor CCSP⁺Sca-1⁺ cells could proliferate and regenerate the airway epithelium. We found that these cells did not die in response to naphthalene toxicity but proliferated in response to the second dose of naphthalene which suggests these cells have proliferative capabilities *in vivo*. Furthermore, to assess self-renewal *in vivo*, we re-isolated the donor CCSP⁺Sca-1⁺ cells from the lungs of the first recipient, re-injected

them into the airways of a secondary recipient and found similar levels of donor DNA after 30 days of cell delivery. After a second dose of naphthalene injury in the secondary recipients, a significant increase in donor DNA was detected in the right lung 20 days later which confirms at least the short-term self-renewal potential of these cells. The ability of CCSP⁺Sca-1⁺ cells derived from secondary recipients to reconstitute the airway epithelium of tertiary recipients is currently in progress.

Identifying the specific BMC subpopulation that is involved in tissue repair and regeneration is important for cell replacement strategies. Since stem cells are by definition plastic cells, it may be optimal in cell replacement strategies to use therapeutic cells that most resemble the target tissue cells. Undifferentiated embryonic stem cells delivered into the heart were shown to give rise to teratomas instead of regenerating the cardiac tissue (Nussbaum et al., 2007). Identification of bone marrow progenitor cells expressing early cardiac and neuronal markers have previously been identified (Kucia et al., 2004a; Kucia et al., 2006b). Furthermore a possible large airway circulating cell population expressing cytokeratin-5 was also shown to exist in the mouse marrow (Gomperts et al., 2006). This study is the first to identify a potential epithelial progenitor cell in the bone marrow expressing CCSP that can repopulate the airway epithelium. We have not examined their contribution to regeneration of epithelia of other tissues such as skin and GI tract. This population contains a rare stem cell that exhibits stem cell characteristics such as self-renewal and multi-lineage differentiation potential. Importantly, this stem cell can also restore lung expression of CCSP after airway injury. We remain optimistic that the bone marrow does play a role in tissue repair and remodeling whether it is by phenotypic conversion of the cells into tissue cells or by

modulating the local inflammatory or stem cell niche environment to support local stem cell repair.

5.2 Future Directions

5.2.1 Characterization of the CCSP gene expressed by bone marrow cells.

We recognize that there are many unaddressed questions surrounding the existence of the CCSP-expressing BMC. First, it would appear inefficient to harbour tissue progenitor cells in the marrow when it may serve no obvious purpose in the marrow other than to provide a resource of cells for the tissues. Why would these BMC express an epithelial protein? Is the CCSP gene expressed by BMC simply paralogous to the CCSP gene expressed in the airway and kidney epithelium and as such detectable by antibodies to CCSP protein in the BMC but may have a completely different function in the marrow? Sequencing the gene and determining the protein structure may provide insight into the function of the protein for BMC through nuclear magnetic resonance studies.

5.2.2. Determination of the anti-inflammatory role of CCSP⁺ cells in lung repair and/or regeneration.

As discussed in Chapter 1, the CCSP protein plays a role in regulating local airway inflammatory responses. Soluble airway CCSP appears to have an anti-inflammatory role (Dierynck et al., 1996; Harrod et al., 1998; Hayashida et al., 2000; Yoshikawa et al., 2005). CCSP has been associated with various human lung diseases

such as asthma (de Burbure et al., 2007; Martin et al., 2006; Sengler et al., 2003) and bronchiolitis obliterans (Mattsson et al., 2005; Nord et al., 2002). Therefore the question is whether bone marrow CCSP⁺ cells have an immunomodulatory or anti-inflammatory role? CCSP knockout animals can better clear airway infections (Watson et al., 2001). Treatment of lung injury in CCSP null animals with wild-type BMC versus BMC from knockout animals would address the role of the CCSP protein expressed by bone marrow cells in immunomodulation. Another approach would be to use soluble CCSP protein derived from ex vivo expanded cultured CCSP⁺ BMC to ameliorate lung injury. The level of anti-inflammatory cytokines (IFN- γ , TNF- α , IL-1 β , IgA), lung histology, and other parameters of lung injury could be compared to mice that received exogenous bone marrow-derived CCSP protein or CCSP⁺ cells to determine the efficacy of the BMC-derived CCSP protein in modulating lung injury.

5.2.3 Determination of the origin of the CCSP⁺ bone marrow cells.

Another obvious question is what is the origin of the CCSP⁺ BMC? This study shows that these cells express both hematopoietic and mesenchymal cell markers but our initial attempts at examining the mRNA pools from known lineages of the hematopoietic cell hierarchy showed no evidence of CCSP gene expression in the studied hematopoietic cell lineages suggesting that these cells are not part of the known hematopoietic cell lineages. Although these cells are co-isolated with the MSC population, they did not morphologically resemble the classical fibroblastic cultures of MSC. It is possible that these cells could still be hematopoietic or mesenchymal cells that have not been identified. A study by Dr. Darwin Prockop's group demonstrated that plastic-adherent

cultures of MSC contain small rapidly self-renewing stem cells (Colter et al., 2000; Colter et al., 2001; Prockop et al., 2001). It is possible that the CCSP⁺ cells are these small rapidly self-renewing stem cells or a subpopulation of these cells. Future work will need to address this possibility and compare the phenotypic profile of the CCSP⁺ cells with the reported small rapidly self-renewing stem cells.

Could it also be possible that expression of CCSP and CD45 in the BMC is an artifact caused by removal of these cells from their protective marrow niche/microenvironment? These cells could gain expression of markers that are not reminiscent of their true potential in vivo. Double-staining for CCSP and CD45 in situ would address this question. A possible in vitro experiment to determine the ability of the mesenchymal cells to acquire hematopoietic surface markers is to isolate CCSP⁺ cells from early “MSC” and assess CD45 expression as they are cultured in vitro. An in vivo experiment is to create chimeric mice by bone marrow transplantation of a single Sca-1⁺CCSP⁺CD105⁺CD90⁺CD45⁻ “MSC” cell from beta-actin GFP mice or use retroviral insertion site analysis following infection with a retrovirus into wild-type animals and follow the expression of CD45 on the progenies of cells as the cell proliferate and differentiate in vivo.

While it would not address the origin of these cells, we are currently performing bone marrow transplantation studies using a single GFP⁺CCSP⁺Sca-1⁺ cell as a donor to address whether these cells could give rise to hematopoietic and mesenchymal cells in vivo.

It also remains unclear whether the CCSP⁺Sca-1⁺ cells give rise to CCSP⁺Sca-1⁻ cells or vice versa. Pilot studies using coculture studies of CCSP⁺Sca-1⁺ cells with CCSP⁻

cells suggested that the double positive cells could give rise to the CCSP⁺Sca-1⁻ cells. If the CCSP protein is secreted by the CCSP⁺ cells then it is possible that the CCSP⁻ cells absorb the protein and result in CCSP⁺Sca-1⁻ or CCSP⁺Sca-1⁺ cells. Culturing CCSP⁻Sca-1⁻ and CCSP⁻Sca-1⁺ cells with media from CCSP⁺ cells would address this question. Future studies need to address the developmental hierarchy of the CCSP⁺ fraction and the surface markers these cells gain or lose during development and cell cycle progression and in response to specific tissue injury perhaps through gene arrays.

In identifying a pluripotent stem cell within the CCSP⁺Sca-1⁺ cell population, it appears that not all CCSP⁺Sca-1⁺ cells express the pluripotent markers Oct4 and nanog suggesting further work needs to be done to purify the stem cell population within the CCSP⁺Sca-1⁺ fraction and determining the contribution, if any, to tissue regeneration.

5.2.4 Determine the lineage relationship between CCSP⁺ BMC with other reported stem cell populations

The relationship between CCSP⁺ BMC with reported bone marrow-derived stem cells remains to be determined. We have preliminary evidence that CCSP⁺ cells are derived from a hematopoietic precursor suggesting CCSP⁺ cells are not mesenchymal cells but are co-isolated and adhere to plastic with marrow stromal cells. This is in contrast to reports that have identified “early MSC progenitors” from plastic-adherent cultures or grown in media enriched with growth factors that support the growth of the multipotent adult progenitor cells (MAPC) (Jiang et al., 2002a). A recent study identified a novel primitive SSEA1⁺ MSC population that are capable of giving rise to different mesenchymal, endothelial and hepatic cells in vivo (Anjos-Afonso and Bonnet, 2007).

These SSEA1⁺ cells are coisolated with the plastic-adherent mesenchymal stromal cells and are phenotypically Lin⁻CD45⁻CD31⁻Sca-1⁺CD105⁺CD73⁺CD44⁺vimentin⁺. Surprisingly, these cells can also give rise to CD45⁺ hematopoietic cells following marrow transplantation. While SSEA1⁺ cells appear to have multipotent stem cell characteristics, it remains to be elucidated whether culturing the cells in MAPC growth media (Jiang et al., 2002b) may have favored a multipotent phenotype. Previous reports by Colter et al have identified a small (~7 μm) and agranular recycling stem cell (RS1) population within the MSC compartment that account for up to 30% of the 5-day plastic-adherent marrow cell population (Colter et al., 2000). Like CCSP⁺ cells, RS1 cells expressed CD90 and ckit but do not express CD45 and CD34. Furthermore, RS1 cells appear to expand in culture at low plating density (1.5-3.0 cells/cm² compared to 5000 cells/cm² for “plastic-adherent MSC” as previously reported (Bruder et al., 1997)) and give rise to mesenchymal cells (Colter et al., 2001). It also does not appear that the CCSP⁺ cells are the very small embryonic-like (VSEL) stem cells identified by Kucia et al (Kucia et al., 2006a). VSEL are much smaller (2-4 μm) in size compared to CCSP⁺ cells and have pluripotent differentiation potential similar to embryonic stem cells. It may be possible that CCSP⁺ cells are derived from the VSEL cells. Future work will be done to identify early progenitors of the CCSP⁺ fraction and their relationship to VSEL, RS1 MSC, SSEA⁺ MSC and previously reported HSC.

The ability of a marrow cell to convert into cells with a lung phenotype in vivo may be associated with its cell cycle state. A recent publication demonstrated that GFP⁺CD45⁻ stromal cells induced to enter the G1/S phase of the cell cycle with specific cytokines (IL-3, IL-6 and IL-11) resulted in a three-fold increase in conversion to

GFP⁺cytokeratin⁺ pulmonary epithelial cells and lung homing potential (Dooner et al., 2008). The cell cycle state of the CCSP⁺ BMC when cultured under air liquid interface and during bone marrow transplantation is unknown however since CCSP⁺ cells can expand rapidly in vitro, it may be possible these cells are in G1/S phase of the cell cycle. Future work will need to be done to determine the effect of cell cycle status on the differentiation potential of the CCSP⁺ population.

5.2.5 Determine the contribution of CCSP⁺ BMC in lung regeneration in the absence of local progenitor cells

Future studies examining the contribution of bone marrow CCSP⁺Sca-1⁺ in lungs with defects or deficits in local lung progenitors would be useful in understanding the significance of these cells in the lung. One strategy is to perform bone marrow transplantation of male wild-type CCSP⁺Sca-1⁺ BMC into female transgenic animals that express the herpes simplex virus thymidine kinase under control of the CCSP promoter (kindly provided by Dr. Barry Stripp). Treatment of these mice with ganciclovir should eliminate all CCSP-expressing airway cells and theoretically eliminate bone marrow-derived recipient CCSP⁺ cells. Therefore it will allow the assessment of donor CCSP⁺Sca-1⁺ BMC in lung regeneration as well as the ability of these cells to repopulate the local lung stem cell niche. The anticipated result is there will be male (derived from the bone marrow) CCSP⁺ cells in the airway lining of the female recipients following ganciclovir-mediated depletion of airway CCSP⁺ cells. If the male BMC engraft as BASC, then we would expect these cells to localize to the BADJ as previously described (Kim et al., 2005). If these experimental mice are recovered from the lethal phenotype

after ganciclovir administration further assessment of the airway and alveolar epithelial regeneration can be done with specific lung injuries such as naphthalene and bleomycin-induced injuries respectively. To confirm the contribution of CCSP⁺ BMC in lung regeneration, treatment with the chemotherapeutic agent busulfan may be needed to suppress the marrow cell response.

5.2.6 Determine the signaling pathway involved in recruitment of bone marrow CCSP⁺ cells to the lung

It is important to delineate the signals involved in recruiting bone marrow CCSP⁺ cells to the injured lung since manipulation of the signaling pathway may have therapeutic implications for future regenerative purposes. This study has tried to show that the SDF-1/CXCR4 biological axis may play a significant role in recruiting CCSP⁺ cells to the lungs. Naphthalene-injured lungs expressed elevated levels of SDF-1 while a population of CCSP⁺ cells expressed the cognate receptor CXCR4. In an in vitro chemotaxis assay, CCSP⁺ cells could migrate in response to SDF-1 chemoattraction and this migration could be partially inhibited with antibodies against CXCR4. Future in vivo studies need to delineate whether this signaling pathway is important in mobilizing bone marrow CCSP⁺ cells from the marrow to the blood and eventually to the injured lungs. To do this, a method is to use a blocking strategy where neutralizing antibodies against SDF-1, soluble truncated CXCR4 receptor antagonist, or siRNA knockdown of CXCR4 receptor on the CCSP⁺ cells could be used to assess whether lung engraftment of the cells is prevented. If so, it would suggest a role of this signaling pathway in regenerating the lung. Furthermore, pilot PCR assessment of the CCSP⁺ cells detected SDF-1 mRNA in

these cells suggesting that CCSP⁺ can make SDF-1 which can bind to the receptor on the cell surface and induce an autocrine signaling pathway that may be involved in progenitor cell cycling/quiescence as has been previously shown in hematopoietic precursors (Chabanon et al., 2008; Nie et al., 2008). A recent study has shown that the SDF-1/CXCR4 pathway may be involved in inducing differentiation of hematopoietic progenitors (Tavor et al., 2008). AMD3100, the CXCR4 receptor antagonist has recently been shown to play a role in mobilizing long-term repopulating cells (Devine et al., 2008; Pulliam et al., 2008). This drug could also be used to mobilize CCSP⁺ cells from the marrow and determine the SDF-1/CXCR4 pathway in homing of the cells to the lung.

In addition to SDF-1/CXCR4 signalling, other pathways known to recruit bone marrow tissue progenitors such as HGF/c-met and SCF/ckit. Since only 25% of CCSP⁺ cells express CXCR4, it may be that other signaling pathways may be involved in recruiting CCSP⁺ cells to the lung.

5.2.7 Determine the regenerative potential of bone marrow CCSP⁺ cells in clinically relevant models of lung diseases

This study focused on naphthalene-induced airway injury exclusively. In Chapter 2, the goal was use a conditioning regimen with naphthalene that specifically depletes airway Clara cells in hopes that delivery of exogenous BMC would better remain in the airway “niche”. In Chapter 3 and 4 with the identification of the CCSP⁺ BMC, naphthalene injury was used to deplete the airway epithelium, since the mouse airways is predominantly CCSP-expressing Clara cells, to determine the repopulation potential of the CCSP⁺ cells. Future studies however will need to determine the regenerative potential

of CCSP⁺ BMC in clinically relevant models such as murine CF models, enzyme-induced emphysema, bleomycin-induced alveolar injury, heterotopic/orthotopic tracheal transplantation models of bronchiolitis obliterans and lung transplantation models. Approaches to regenerate the lung can be cell replacement, cell-based gene therapy and/or progenitor cell tissue engineering strategies.

5.2.8 Determine the role of bone marrow CCSP in human lung diseases

Finally, the question of particular significance is the role of CCSP⁺ BMC in lung repair or the development of human lung diseases. *K-ras* mutated CCSP⁺ expressing BASC have been associated with adenocarcinoma development (Kim et al., 2005) while decreased levels of airway CCSP is associated with bronchiolitis obliterans (Nord et al., 2002). Furthermore, polymorphism of the CCSP gene has been associated with asthma (Benson et al., 2007; Choi et al., 2000; Martin et al., 2006; Sengler et al., 2003) and sarcoidosis (Ohchi et al., 2004). Low levels of CCSP protein has been associated with tumor development (Chen et al., 2008; Yang et al., 2004). Assessment of bone marrow and blood samples from patients with and without end-stage lung diseases and various timepoints post-lung transplantation would give insight into the possible contribution of these cells, if any, to human lung repair/diseases.

5.3 Conclusions

The contribution of bone marrow stem cells in lung repair remains controversial in part because of the uncertainty of the specific subpopulation involved. We have identified cells in the bone marrow of both mice and humans with characteristics of airway epithelial progenitors expressing CCSP that has greater propensity to repopulate the lung. These cells increase in marrow and blood after airway injury and home to the injured lung and contribute to the airway epithelium. These cells express CXCR4 and can respond to SDF-1-induced migration implicating a potential role of the SDF-1/CXCR4 biological axis in recruiting these cells from the marrow to the injured lung. These cells can also express other epithelial genes under air liquid interface cultures suggesting they are epithelial progenitor cells. A subset of these cells has stem cell characteristics including self-renewal and multi-epithelial lineage differentiation potential. Phenotypically, both hematopoietic and mesenchymal cell markers are expressed in a subset of CCSP⁺ cells and may partly explain the controversy of the specific cell type involved in lung regeneration. Future work will need to determine the mechanism in which CCSP⁺ BMC contribute to lung regeneration. Overall, our data shows a novel discovered CCSP⁺ BMC subset that may play an important role in the bone marrow contribution to lung recovery and is very likely an ideal candidate for cell-based therapy for lung diseases.

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