The University of Alberta

# Mesenchymal Stem Cells Protect the Lung From Oxygen-Induced Experimental Bronchopulmonary Dysplasia.

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of
Master of Science
in

Medical Science- Pediatrics

Edmonton, Alberta

**Fall 2007**

*Your file Votre référence*
*ISBN: 978-0-494-33364-8*
*Our file Notre référence*
*ISBN: 978-0-494-33364-8*

**Abstract**

The role that mesenchymal stem cells (MSCs) play in the protection and repair of the lung is unknown. We hypothesized that bone marrow-derived MSCs protect the developing lung from oxygen-induced lung injury. Newborn rats exposed to hyperoxia (95% oxygen) from birth to postnatal day (P) 14 had stunted lung growth characterized by simplified alveoli and capillary rarefaction. Intratracheal MSC given at P4 engrafted and expressed the type II alveolar epithelial cell specific marker surfactant protein-C (SP-C). MSCs but not control cells increased survival prevented alveolar and lung vascular injury, and associated pulmonary hypertension. *In vitro*, MSCs exposed to lung tissue express SP-C and have characteristic lamellar bodies shown by electron microscopy. We conclude that exogenous MSCs are able to adopt the phenotype of type II AECs *in vitro* and *in vivo*, they contribute to the alveolar epithelium, and improve survival, alveolarization and pulmonary vascular morphology in experimental BPD.

# ACKNOWLEDGEMENTS


- To Dr. Bernard Thebaud
- To Dr. Stephen Archer and Dr. Evangelos Michelakis
- To Dr. Po-Yin Cheung, Dr. John Greer, and Dr. David Olson
- To Dr. Jayan Nagendran, Dr. Irene van der Horst, Dr. Debby Koonen and Dr. Sebastien Bonnet
- To Dr. John Akabutu, Dr. Ming Chen, and Dr. Pat Mayo
- To Rohit Mougdil, Gopi Sutendra, Anita Chan, Jono Cena, Anna Lam, Cliff Folmes, Haley Cleary, Tamara Skene, and Helen Coe
- To Farah Eaton, Kyoko Hashimoto, Al Haromy, Gwenyth Harry, Angie Jansen, Sandra Bonnet, and Bronwyn Lewis
- To Vikram Gurtu, Monika Sawicka, Roisin Byrne, Faruqa Ladha, Nick Etches, Christina Luong, and Nathan Myhill

# DEDICATION

To my parents, Gerry and Debbie;
For never demanding that I get a real job.

# QUOTATIONS

"As you set out for Ithaka hope your road is a long one, full of adventure, full of discovery."
*C. P. Cavafy (1894)*

"Always remember, Tim, you're doing research God never intended."
*Dr. Stephen L. Archer (2005)*

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# LIST OF ABBREVIATIONS

AEC- Alveolar epithelial cell

BMSC- Bone marrow-derived stem cell

BPD- Bronchopulmonary Dysplasia

eGFP- Enhanced Green Fluorescent Protein

ELISA- Enzyme Linked Immunosorbent Assay

ES cell- Embryonic stem cell

FISH- Fluorescence in situ hybridization

GFP- Green fluorescent protein

HSC- Hematopoietic stem cell

Il- Interleukin

MSC- Mesenchymal stem cell

SP cell- Side population cell

SP-C- Surfactant protein C

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# Chapter I: Introduction

## 1. Introduction

The lung is a remarkably complex and robust organ. The lung is composed of more than forty different cell types and is one of only two organs in continual contact with the outside environment. Furthermore, the lung undergoes numerous stages of development that begin on the 26$^{th}$ day of gestation and continue through 18 months of post-natal life. All of these events are necessary to ensure that the lung develops correctly and is able to undertake its crucial task of blood-gas exchange.

However, there are instances where the normal progression of lung development is disrupted. Bronchopulmonary dysplasia (BPD) is the chronic lung disease of prematurity that follows ventilator support. BPD is characterized by an arrest in alveolar and vascular growth (Jobe 1999; Abman 2001). The long-term consequences of this halted lung growth are still unknown.

Recent evidence suggests that stem cells derived from adult sources, including the bone marrow, can be used to regenerate tissues that were previously thought to be beyond repair. Therefore, the potential of a stem cell to restore or protect the alveolar epithelium is very attractive as this may reduce the morbidity and mortality associated with BPD.

The following will discuss the normal sequence of lung development, the disruptive consequences of BPD, and the potential and hope that adult-derived stem cells hold for repairing the injured lung.

### 1.1 Prenatal Growth and Development of the Lung

The human lung has a number of developmental stages, which are broadly categorized as occurring either a "prenatal" or "postnatal" environment. Prenatal lung development

encompasses numerous developmental stages: organogenesis, the pseudoglandular stage, the canalicular stage, and the saccular stage.

### 1.1.1 Organogenesis

For such an incredibly complex organ, the lung has a very humble beginning. On the 26$^{th}$ day of gestation, the first indication of the lung appears as a ventral out-pouching of the foregut (Burri 1997). It is not until approximately 4.5 weeks into gestation that the first distinctive structures of the lung, five saccules, appear. These five saccules, two on the left side and three on the right, are pre-forming what will eventually develop into the future bronchi and corresponding lobes (Burri 1997). Through a number of successive, dichotomous divisions this bronchial tree rapidly expands that is "strongly determined by the interactions of the endodermally derived epithelial tubes and the mesenchymal structures which are of mesodermal origin." (Burri 1997)

During this early period, the vascular connections are also established. The pulmonary arteries are derived from the sixth pair of aortic arches (Burri 1997) and the pulmonary vein first appears as a small tubule extending from the left atrial portion of the heart (Burri 1997). After approximately seven weeks, the lung then enters the next stage of development: the pseudoglandular stage.

### 1.1.2 The Pseudoglandular Stage

The pseudoglandular stage, sometimes referred to as the bronchial stage of development, is indicated by the presence of twenty divisions of the bronchial tree (Burri 1997). This stage of lung development encompasses the formation of all the structures required for gas conduction. Furthermore, during the pseudoglandular stage there is the first appearance of the ciliated, goblet,

and basal cells; which develop in the central airway and spread to the peripheral tubules (Burri 1997). However, the epithelium in these peripheral tubules is maintained in an undifferentiated state until alveolarization begins.

During this stage, the arterial tree branches along side with the branching airway. Supernumery arterial branches also form, these small branches function to supply blood to alveolar regions adjacent to the walls of the airway (Burri 1997). At the end of the pseudoglandular stage the hierarchal patterning seen in the preacinar airways and blood vessels corresponds to that of the adult lung (Burri 1997).

### 1.1.3 The Canalicular Stage

The canalicular stage is initiated by the "birth of the acinus" (Boyden 1977; Burri 1997). This is also the first time that the prospective gas exchange surfaces are visible with light microscopy; the acinus appearing as an "airway stem and a spray of short tubules arranged in a cluster" (Burri 1997). As the future airways expand, the vessels come in closer proximity with the epithelial layer and rearrange to form the peritubular network.

During the canalicular stage, the glycogen rich, cuboidal epithelial cells that line the tubules begin to flatten out allowing for the formation of regions with a thin air-blood barrier. This marks the first appearance and differentiation of the two classifications of alveolar epithelial cells (AECs): type I AECs and type II AECs. It is during this stage that the type II AECs begin to accumulate lamellar bodies for the storage of surfactant (Burri 1997). Interestingly, the production and storage of surfactant occurs in humans when gestation is approximately 60% complete, whereas this does not begin until 80-85% gestational age in most other species (Burri 1997).

### 1.1.4 The Saccular Stage

During the saccular stage of development the lung parenchyma undergoes a great increase in size. This serves to lengthen and widen each airspace generation (Burri 1997) so each terminal sac will eventually give rise to three generations of alveolar ducts and one generation of alveolar sacs (Boyden 1977). This lengthening and widening of the airspaces further compresses the interstitial tissue. This compression profoundly alters the three dimensional structure of the pulmonary capillary bed (Burri 1997) initiating the double capillary network that surrounds the prospective alveoli.

## 1.2 Postnatal Development of the Lung (Alveolarization)

Postnatal lung development in humans is dominated by alveolarization. Alveolarization occurs as the saccules thin out to form septa, this process is closely linked with an increased deposition of elastin in the lung (Burri 1997; Massaro 2000) as well as a remodeling of the double capillary system into a single capillary network. There is a marked thinning of the alveolar walls, accompanied by a change in the cellular composition of the AECs (Massaro 2000). From alveolarization in the rat, it has been demonstrated that the alveolar thickness decreases by 20%, the air-gas barrier distance decreases 25%, the volume density of type I AECs increases 45%, and the volume density of the interstitial fibroblasts decreases 22% (Massaro 2000). The new alveoli are formed by the alternate upfolding of the two capillary layers on both sides of the primary septa (Burri 1997).

This method of alveolarization allows the gas exchange surface area to scale linearly with the total oxygen consumption ($VO_2$) whereas the alveolar size varies inversely with the species body

mass specific $VO_2$ (Tenney and Remmers 1963; Massaro 2000). This partitioning (smaller and more numerous alveoli) allows for the achievement of a much larger surface area than would occur via increasing lung volume alone (Tenney and Remmers 1963; Massaro 2000).

### 1.3 Bronchopulmonary Dysplasia (BPD)

Bronchopulmonary dysplasia (BPD) arises when premature birth occurs in an infant that is still in the late canalicular or earlier saccular stage, thereby disrupting the normal sequence of lung development leading to alveolarization. BPD has evolved in its severity, incidence, and pathogenesis since the original description in 1967 (Northway, Rosan et al. 1967). This "old BPD" was defined in premature infants with respiratory distress syndrome (RDS) requiring supplemental oxygen and mechanical ventilation, with an abnormal chest radiograph demonstrating hyperextension, bullae, and the presence prominent fibroproliferation (the classic "ground glass" appearance) (Chess, D'Angio et al. 2006; Kinsella, Greenough et al. 2006). The advent of new treatment options and technology, including surfactant replacement therapy, new ventilatory strategies, the usage of continuous positive airway pressure (CPAP), oxygen saturation monitors, the administration of antenatal steroids, and changes in nutrition have shifted the clinical presentation to what is now called "new BPD" (Chess, D'Angio et al. 2006; Coalson 2006; Kinsella, Greenough et al. 2006).

"New BPD" develops in 77% of preterm infants weighing less than 1000 g, born at less than 32 weeks postmenstrual age, and receiving respiratory support, mechanical ventilation, and prolonged oxygenation (Ehrenkranz, Walsh et al. 2005; Chess, D'Angio et al. 2006; Coalson 2006). While previous reports on the incidence of "new BPD" may have been misleading (ranging from 5-85% based on birth weight) due to the lack of a universally accepted definition

(Lemons, Bauer et al. 2001; Allen, Zwerdling et al. 2003), a consensus on the definition for "new BPD" has only recently been published (Ehrenkranz, Walsh et al. 2005), BPD is still the most common cause of chronic lung disease in infants (Bhandari and Bhandari 2003; Bland 2005).

### 1.3.1 Pathogenesis of New BPD

The hallmark of new BPD is an arrest of alveolarization (Jobe and Bancalari 2001), whereas old BPD had a more severe phenotype that displayed central and peripheral airway injury, airway inflammation, and parenchymal fibrosis. The main risk factors that will predispose an infant to BPD are prematurity, oxygen therapy, mechanical ventilation, and infection (Chess, D'Angio et al. 2006).

Premature birth at 24-26 weeks gestational age interrupts the lung in the canalicular stage of development, whereas at 30-32 weeks the lung is in the saccular stage. Between week 24 and week 32 a substantial portion of lung development occurs; including extensive vasculogenesis within the evolving terminal saccules and the extensive interstitial extracellular matrix remodeling as secondary crests are formed (Coalson 2006). Therefore, pulmonary gas exchange is initiated within the lung well before alveolarization has begun, disrupting normal development of both the alveoli and the distal vasculature.

Supplemental oxygen, while not an absolute requirement for the development of BPD (Chess, D'Angio et al. 2006), is highly associated and one of the "prime sufficient conditions" (Chess, D'Angio et al. 2006) required for the development of BPD. Interestingly, hyperoxic exposure alone is sufficient to induce a BPD-like phenotype experimentally in numerous animal models (Bonikos, Bensch et al. 1976; Wilson, Mullen et al. 1985; D'Angio, Finkelstein et al. 1997). And even though the direct role that oxygen exposure plays in the development of BPD has been

described as "circumstantial" (Chess, D'Angio et al. 2006), even brief exposure to oxygen has been shown to disrupt the balance between oxidized and reduced glutathione, one of the major antioxidant systems within the lung (Vento, Asensi et al. 2001; Vento, Asensi et al. 2003; Saugstad, Ramji et al. 2005).

Mechanical ventilation is an intervention with a dual nature. It is a lifesaving intervention, however infants undergoing mechanical ventilation have tissue disruption, chronic inflammation and edema all of which can exacerbate the chronic lung disease (Bland 2005). Coupled with this quandary is that the optimal modes of ventilation, ventilator settings, and target blood gases remain presently unknown (Attar and Donn 2002; Thome, Carlo et al. 2005). Moreover, the chronic inflammation and edema can further lead to surfactant inactivation (Chess, D'Angio et al. 2006). Therefore the exogenous administration of surfactant, a naturally occurring substance within the lung that prevents alveolar collapse and reduces surface tension, has been successful in reducing symptoms and improving survival in infants with RDS, the efficacy of surfactant replacement therapies on the subsequent incidence and severity of BPD is not fully known (Kendig, Notter et al. 1991; Chess, D'Angio et al. 2006).

Oxygen supplementation and ventilator trauma cause a pro-inflammatory state in the premature lung through direct cellular injury, the oxidation of DNA, lipids, and proteins, the induction of chemokines and cytokines, and the recruitment of neutrophils and macrophages to the lung (Chess, D'Angio et al. 2006). Moreover, chorioamnionitis is a major risk factor for preterm birth; being detected in 87% of deliveries before 27 weeks gestation (Chess, D'Angio et al. 2006). Also the presence of sepsis or another nosocomial infection will further add to the inflammatory pressures placed on the lung. All of these factors influence the balance of numerous interleukins (Ils) including Il-1β, Il-6, Il-8 and tumor necrosis factor (TNF)-α (Chess,

D'Angio et al. 2006). And while inflammation on its own does not cause BPD, its presence definitely exacerbates the severity of BPD when it develops (Jobe and Ikegami 1998).

Each of these risk factors disrupts the normal coordinated development of both the distal epithelium and capillary network that is crucial for normal lung development. In the last 20% of gestation alone, the lung capillary surface area increases eightfold in the rat (Moschopulos and Burri 1993). Moreover the complex development of the alveoli heavily depends on epithelial-mesenchymal interactions. As stated by Chess and colleagues: "Because the lung epithelium likely affects capillary morphogenesis through elaboration of angiogenic factors, a potential regulatory role for the vasculature on epithelial development suggests that a complex interplay of epithelial-endothelial cells is required for normal lung morphogenesis" (Chess, D'Angio et al. 2006). Indeed the crucial role that lung vascular endothelial growth factor (VEGF) plays in normal lung development (Bhatt, Amin et al. 2000; Galambos, Ng et al. 2002) and it's potential as an experimental therapeutic approach for BPD (Kunig, Balasubramaniam et al. 2005; Thebaud, Ladha et al. 2005) are currently being studied in depth and exploited.

### 1.4 Stem Cells for Lung Injury

The current promise that stem cells hold for tissue regeneration is immense. The belief that once irreversibly damaged tissue can be restored to a normal functional capacity is providing great optimism for novel treatments in many diseases. However, this research is not without its controversies. There are ethical and moral issues surrounding the use of stem cells derived from embryos. Recent research is starting to unravel the potential for adult derived stem cells that was initially described more than 20 years ago. While the initial results from numerous disease models outside the lung show great potential for a cell replacement therapy, there is heated

debate over numerous concerns within the stem cell field. The mechanism of action, the plasticity, the source, the phenotype, and the "stemness" of the stem cells used are all contentious issues. Furthermore, stem cell research has been markedly slower in lung diseases due to the complexity of the lung. The lung possesses numerous anatomical areas (upper airways, bronchioles, alveoli and their underlying capillaries) each of which contains a unique cellular population with drastically different functions.

### 1.4.1 Definition of Stem Cells

Stem cells may be artificially classified into three categories: embryonic stem (ES) cells, adult bone marrow derived stem cells (BMSC), and tissue progenitor cells (Table 1). *ES* cells are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos (Evans and Kaufman 1981). They are able to form tissue within each of the germ layers (the endoderm, mesoderm, and ectoderm) and have thus enormous therapeutic potential to regenerate any damaged tissue; but ES cells are also mired in great controversy due to the present need of destroying an embryo to harvest pluripotent cells (McLaren 2001). Conversely, *progenitor cells* are thought to reside within a tissue and are stimulated for repair after injury. It is these tissue progenitor cells that give the liver (the hepatic oval cell) its robust regenerative ability (Michalopoulos and DeFrances 1997; Hatch, Zheng et al. 2002). In the lung, type II alveolar epithelial cells (AEC) are the putative distal progenitor cells responsible for repair after injury (Mason, Williams et al. 1997; Warburton, Wuenschell et al. 1998). Side population (SP) cells, identified by their ability to efflux Hoechst dye, and tracheal epithelial basal cells, are putative progenitor cells in the upper airways (Pitt and Ortiz 2004). While the pluripotency of ES cells and further characterization of resident lung stem cells (Driscoll, Buckley et al. 2000; Reddy,

Buckley et al. 2004) hold promise for novel therapeutic approaches for lung diseases, we will focus on the *adult bone-marrow derived stem cell* and their exogenous administration in various models of lung diseases.

The bone marrow contains several population of primitive cells: (i) Hematopoeitic stem cells (HSCs), (ii) mesenchymal stem cells (MSCs), (iii) endothelial stem/progenitor cells (EPC), (iv) SP cells, and (v) multipotent adult progenitor cells (MAPC). Among these, the HSCs are the best characterized of the adult stem cells (Wagers and Weissman 2004). HSCs are multipotent and have the ability to maintain or restore the mature circulating blood cells (erythrocytes and leukocytes). Recent evidence suggest that bone-marrow derived cells have the ability to cross lineage barriers and generate differentiated tissue beyond their own tissue boundaries to form functional components of other tissues, expressing tissue-specific proteins in organs such as heart, liver, brain, skeletal muscle, and vascular endothelium (Blau, Brazelton et al. 2001). A true potential for adult derived stem cells lies with the multipotent MSCs and these cells have been extensively used in lung stem cell research. This potential was first proposed in the 1970's by Friedenstein et al (Friedenstein, Gorskaja et al. 1976). This initial report showed that plastic adherent cells, previously believed to be committed to a specific tissue type (i.e. bone marrow), had the ability to form cells within the osteogenic, chondrogenic, and adipogenic lineages (Friedenstein, Gorskaja et al. 1976). There have been numerous reports characterizing the phenotype of these MSCs in both humans (Jones, Kinsey et al. 2002; Gronthos, Zannettino et al. 2003) and mice (Friedenstein, Gorskaja et al. 1976; Tropel, Noel et al. 2004). However, it was not until over 20 years after Friedenstein's first report that the true potential of these cells were made apparent. In 2001, Krause *et al.* reported that a single bone-marrow derived stem cell had the ability to engraft into numerous organs as well as having a "tremendous differentiative

capacity" by adopting the phenotype of epithelial cells within the liver, lung, GI tract, and skin (Krause, Theise et al. 2001).

This initial report from Krause has led to extensive study of these cells in the heart (Orlic, Kajstura et al. 2001; Amado, Saliaris et al. 2005; Kajstura, Rota et al. 2005; Yoon, Wecker et al. 2005), liver (Petersen, Bowen et al. 1999; Theise, Badve et al. 2000; Jang, Collector et al. 2004), brain (Kopen, Prockop et al. 1999), kidney (Kale, Karihaloo et al. 2003), endothelium (Zhao, Courtman et al. 2005), and pancreas (Lee, Seo et al. 2006). And while these initial results show great promise in providing new therapies for numerous diseases, these studies have also generated great controversy and highlight the discrepancies that exist within adult stem cell research. These controversies include the source and phenotype of the stem cells used, the experimental model of injury, the mechanism of effect, and the methods used to determine efficacy. While stem cell research in the lung has been less prolific than in cardio-vascular or neurologic diseases, the same controversies apply and will be discussed below.

### 1.4.2 Indications for Stem Cell Therapies to Prevent Lung Injury

The 2001 report from Krause *et al.* opened the door to the potential that there may be an ethically appealing option in using stem cells for tissue replacement. Based on their previous work showing the potential of $CD34^+$ $lin^-$ for regenerating hepatocytes (Theise, Badve et al. 2000), Krause et al extended their observation to the lung. Myelo-ablation was induced in female mice by total body irradiation of 1050-1100 cGy (an otherwise lethal dose if the bone marrow is not reconstituted; in most cases of irradiation the lungs are also injured). $CD34^+$ $lin^-$ cells from male donor mice were administered intravenously (i.v.) (Krause, Theise et al. 2001). Fluorescent in situ hybridization (FISH) to track the Y-chromosome showed 20% of AEC being derived from

a donor animal. Donor derived BMSC persisted up to 11 months post-transplant (Krause, Theise et al. 2001).

Consistent with this first observation, Kotton *et al.* reported that plastic adherent MSCs (1-2x$10^6$ injected i.v.) from transgenic mice overexpressing *lacZ*, allowing tracking of the cells via X-gal staining, were able to engraft and form type I AEC in experimental lung fibrosis induced by intratracheal (i.t.) bleomycin (Kotton, Ma et al. 2001). Bleomycin is one of the most extensively studied and reproducible experimental models for lung fibrosis. When bleomycin is given into the airway, it produces lung epithelial injury, followed by an inflammatory response over several days that is followed by lung fibrosis that eventually resolves (Jones and Reeve 1978). Engrafted cells had morphological features of type I AEC (i.e. flattened with ovoid nuclei bulging into alveolar lumen and adjacent to type II AEC) and expressed type I AEC specific markers such as *Lycopersicon esculentum* lectin and T1α (Kotton, Ma et al. 2001). The proportion of donor derived MSCs contributing to the type I AEC population was not assessed. Interestingly however, all bleomycin injured-mice (4/4) showed engraftment when compared to PBS injected controls (2/9), suggesting that prior injury amplifies stem cell engraftment, as has been observed in other tissues (Hatch, Zheng et al. 2002). Furthermore, there were no donor-derived type II AECs, even in lungs harvested 1 and 2.5 days post-injection. *In vitro*, 10% of plastic adherent donor bone marrow cells cultured for one week expressed T1α and aquaporin 5, used as markers for type I AEC, (but are not solely confined to these cells) (Rishi, Joyce-Brady et al. 1995; Krane, Fortner et al. 2001; Williams 2003), but not surfactant protein C (SP-C), a type II AEC specific marker (Horowitz, Watkins et al. 1991) suggesting that cultured bone marrow cells can serve as type I AEC precursors (Kotton, Ma et al. 2001).

These two reports form the prototypes for most of the experiments in BMSC in the lung that have followed. In 2002 Theise *et al.* reported that donor-markers for either whole bone marrow or $CD34^+$ $lin^-$ cells were detected in 14% of type II AEC after 1200 cGy total body irradiation (Theise, Henegariu et al. 2002). Engraftment was still present 6 months later (for total bone marrow injection) and 8 months later for $CD34^+$ $lin^-$ cells, detectable by FISH. In another study, administration of MAPC to non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice following mild irradiation of 250 cGy accounted for 4% of the alveolar epithelium assessed by β-galactosidase immunofluorescence (Jiang, Jahagirdar et al. 2002). In another elegant study, Abe et al generated parabiotic mice by surgically joining green fluorescent protein (GFP) transgenic mice and wild-type littermates (Abe, Lauby et al. 2003). These mice develop a common circulation (approximately 50% green cells in blood) by 2 weeks after surgery. The wild-type mouse was either uninjured or irradiated or received i.t. elastase (a well accepted emphysema model) or the combination of radiation with i.t. elastase injection. Radiation or the combination of radiation with elastase significantly increased the proportion of bright green cells in the lungs of wild-type mice. These cells resembled morphologically, interstitial monocytes/macrophages, subepithelial fibroblast-like interstitial cells, and type I AEC. Approximately 5 to 20% of lung fibroblasts primary cultured from injured wild-type mice expressed GFP.

Ortiz *et al.* used plastic adherent and purified, magnetic bead immunodepleted MSCs from male bleomycin (BLM)-resistant BALB/c mice expressing CD34, CD45, and CD11b in bleomycin (4 U/kg)-induced pulmonary fibrosis in female BLM-sensitive C57BL/6 recipients (Ortiz, Gambelli et al. 2003). The purified MSCs ($5x10^5$) were injected into the jugular vein. Lung engraftment quantified by real-time PCR showed that male DNA accounted for 2.21 x $10^-$

[5]% of the total lung DNA in control-treated mice but prior injury with bleomycin increased engraftment by 23-fold. FISH revealed that engrafted male cells were localized to areas of BLM-induced injury and exhibited an epithelium-like morphology, suggesting that stem cells homed specifically to sites of injury. Furthermore, immediate administration of MSCs was able to protect the lung from the bleomycin-induced inflammation, as assessed by collagen deposition, and matrix metalloproteinase (MMP) activation (Ortiz, Gambelli et al. 2003) as compared with animals that received MSCs one week following bleomycin, indicating the importance of timing of stem cell therapy, as observed in myocardial infarct (Vandervelde, van Luyn et al. 2005). The low numbers of donor-derived cells engrafting the lung did not appear sufficient to account for the therapeutic response, suggesting that donor stem cells may have other local effects.

Rojas *et al.* used a model in which prior to bleomycin injury, the animals also underwent myelosuppression via a single administration of busulfan (Rojas, Xu et al. 2005). Myelosuppression increased the susceptibility to bleomycin injury, suggesting that an intact bone marrow serves to limit the extent of lung injury. After this suppression of the bone marrow, plastic adherent and immuno-depleted MSCs were given i.v. MSCs administration improved survival when compared to untreated animals. Furthermore, within the MSC administered group, there were a "substantial number" of donor cells within the lungs 2 weeks following bleomycin insult with characteristics of AEC I and II, fibroblasts, and endothelial cells. In the bleomycin+busulfan group, 29% of cells were derived from donor MSCs. Interestingly, circulating levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) (factors known to stimulate stem cell mobilization from the bone marrow), were higher in bleomycin-injured animals receiving MSC. This suggests that, besides lung engraftment, humoral factors may also contribute to stem cell-

induced tissue protection. This is consistent with the greater protection conferred by stem cell transplant in bone marrow sufficient (no busulfan) animals.

These studies demonstrate the potential of BMSC to home to the injured lung, engraft, and adopt the phenotype of one or many of the lung cells. These reports also illustrate the numerous methods that exist in culturing and selecting BMSC (Table 2). The differences in experimental design and the numerous techniques to determine treatment efficacy (engraftment, differentiation, improvement in lung histology) further render the interpretation of these results difficult.

### 1.4.3 Failures of Stem Cells to Repair the Injured Lung

Furthermore, there are numerous studies that do not support the plasticity of adult stem cells in different organs. Wagers *et al.* attempted to determine the fate of "prospectively isolated, long-term reconstituting HSCs" by using either a chimeric animal, produced via bone marrow ablation and reconstitution with GFP HSCs, or using a parabiotic model, joining the circulation of a transgenic GFP mouse with the circulation of a wild type mouse (Wagers, Sherwood et al. 2002). While the BM ablated group showed robust reconstitution of circulating HSCs, and the parabiotic animals had robust hematopoietic chimerism, there was little evidence for stem cell engraftment and transdifferentiation. Only one cell of $13.2x10^6$ cells examined within the brain, and 7 cells of $4.7x10^5$ cells examined within the liver co-expressed the donor marker GFP as well as a tissue specific marker (albumin for hepatocytes) in the BM ablation group (Wagers, Sherwood et al. 2002). There was no evidence for engraftment and transdifferentiation in the lung. Moreover, the parabiotic model had no indication of HSC engraftment and transdifferentation in any of the tissues analyzed (Wagers, Sherwood et al. 2002).

In addition, two recent reports have challenged the engraftment potential of adult-derived stem cells in the lung. These two studies take advantage of transgenic mouse that express a marker protein ubiquitously or under the control of the type II AEC specific promoter SP-C (Chang, Summer et al. 2005; Kotton, Fabian et al. 2005). Chang et al transplanted bone marrow ($2.0x10^7$ cells) from transgenic mice expressing the LacZ or eGFP gene ubiquitously (under the control of the actin promoter), or under the control of the human SP-C promoter into irradiated, neonatal mice. The lungs of recipients transplanted with bone marrow from transgenic mice that ubiquitously express eGFP or LacZ showed cells whose morphology and location were compatible with type II AEC (Chang, Summer et al. 2005). These green cells also co-localized with pro-SP-C. These initial results indicated that the eGFP BMSC were contributing to the alveolar epithelium. Flow cytometric analysis of SP-C immunostained cells with eGFP indicated that eGFP-fluorescent cells accounted for 50-55% of all lung cells, as well as 70% of type II AECs 2 weeks after bone marrow transplantation (Chang, Summer et al. 2005). One month post-transplantation, eGFP+ cells only comprised 2-25% of total lung cells with 0-8% of type II AECs being donor-derived. However, upon analysis with cyto-centrifugation, the phenotype of the eGFP+ SP-C+ cells displayed a small, round cell type with a scant cytoplasm which is not congruent with the normal type II AEC phenotype of larger, cuboidal cells with an abundant cytoplasm (Shannon, Mason et al. 1987). Moreover, mice that received SP-C-eGFP bone marrow transplant showed no eGFP expression, indicating that the transplanted cells are not adopting the type II AEC phenotype (Chang, Summer et al. 2005). This result led to retrospectively analyze the lungs from mice receiving actin-eGFP bone marrow transplantation using deconvolution microscopy. Deconvolution microscopy was able to generate a three dimensional image of the lung allowing to determine that the co-expression of SP-C and eGFP was a false positive: the

pro-SP-C signal resided just outside the eGFP+ cells, with a difference between the two signals less than 300 nm, and this phenomenon was not apparent until the three dimensional image was viewed from multiple angles (Chang, Summer et al. 2005).

Kotton *et al.* also utilized SP-C-eGFP mice, but investigated both unfractionated BM as well as BM-derived SP cells (Kotton, Fabian et al. 2005). SP cells were purified from the femurs, tibias, and iliac crests and identified using a model of Hoechst dye efflux. These cells are enriched for HSC activity. Adult mice underwent myeloablation via either a single dose of 11 or 12 Gy radiation or 2 doses of 7 Gy (total of 14 Gy). The bone marrow was then reconstituted with either a whole bone marrow transplant or transplantation of the highly enriched HSCs SP cells from SP-C-eGFP donor mice. Three months post-transplantation, with robust blood reconstitution, the mice were challenged with a bleomycin injury (0.05 units) and engraftment was assessed 1 month post-transplant. Using 3 antibody independent assays, fluorescent activated cell sorting (FACS), fluorescent microscopy, and real time PCR, the authors showed no evidence of donor cells becoming type II AECs (Kotton, Fabian et al. 2005).

In humans, BMSC repopulation also seems to contribute minimally to the type II AECs after cross-gender lung transplantation (Zander, Baz et al. 2005). Sequential immunohistochemistry and FISH performed on lung biopsy specimens from male recipients of transplanted lungs from female donors showed Y-chromosome–containing type II AEC in 9 of 25 biopsy specimens from 5 of 7 gender mismatched male lung transplant recipients, that accounted for 0% to 0.553% of type II AEC (Zander, Baz et al. 2005). The number of type II AEC of male karyotype showed a statistically significant relationship to the cumulative number of episodes of acute cellular rejection. This study also suggests that BMSC contribute minimally to the type II AEC

proliferation that is often present in these patients as a sequela to alveolar injury (Zander, Baz et al. 2005).

### 1.5 Hypothesis

We hypothesize that bone marrow derived mesenchymal stem cells, when given intratracheally, will prevent the progression of bronchopulmonary dysplasia, and associated morbidities and mortality, in an experimental rodent model.

### 1.6 Specific Aims

1. To isolate and characterize bone marrow mesenchymal stem cells derived from the bone marrow of adult rats.

2. To quantify the affect of hyperoxic exposure on the proportion of circulating and lung resident mesenchymal stem cells.

3. To administer MSCs in an experimental model of BPD, and determine any beneficial affect on lung histology and pulmonary vascular development.

4. To track the engraftment potential of IT administered MSCs.

5. To determine if MSCs are able to adopt the phenotype of the type II alveolar epithelial cell *in vitro* and *in vivo*.

**Table 1.1- Stem Cell Terminology**

| Term | Definition | Example |
|---|---|---|
| Totipotent | Able to form any cell type, includes the embryo and the trophoblast of the placenta | Fertilized oocyte, zygote |
| Pluripotent | Able to form cells within any of the germ layers: the endoderm, ectoderm, and mesoderm | Embryonic Stem Cells |
| Multipotent | Able to differentiate into a select range of cell types, heavily influenced by the microenvironment | Mesenchymal Stem Cells, Hematopoietic stem cells, Certain tissue-specific stem cells, Side-population cells |

**Table 1.2- Characteristics of Adult Bone Marrow-Derived Cells in Lung Stem Cell Biology**

| Cell type | Phenotype |
|---|---|
| Hematopoietic Stem Cells | $CD34^{+/-}$, Stem cell antigen (SCA)-$1^{+}$, $Thy1^{+}$, c-$kit^{+}$<br>Lineage $(lin)^{-}$ - this is the absence of numerous markers for mature lymphocytes (CD45, CD3, CD4, CD8), myeloid (CD11b/Mac-1), and erythroid (TER-119) cells |
| Mesenchymal Stem Cells | - no unique phenotype<br>- adherence to tissue culture plastic main isolation aid<br>- $CD34^{+}$ $lin^{-}$ MSCs (Krause, Theise et al. 2001)<br>- Plastic adherent MSCs (Kotton, Ma et al. 2001)<br>- $CD34^{+}$ $lin^{-}$ MSCs (Theise, Henegariu et al. 2002)<br>- Multi-potent adult progenitor cell (MAPC) (Jiang, Jahagirdar et al. 2002)<br>- Side Population Cells (Hoechst dye efflux) (Abe, Lauby et al. 2003)<br>- MSCs immunodepleted for CD34, CD45, and CD11b (Ortiz, Gambelli et al. 2003)<br>- MSCs immunodepleted for CD34, CD45, and CD11b (Rojas, Xu et al. 2005)<br>- Total Bone Marrow, MSCs not isolated (Chang, Summer et al. 2005)<br>- Total Bone Marrow, and HSC side population cells (Hoechst dye efflux) (Kotton, Fabian et al. 2005) |

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# Chapter II: Materials and Methods

## 2.1 Animal Model

All procedures and protocols were approved by the Animal Health Care Committee of the University of Alberta. Experimental BPD was induced as previously described (Thebaud, Ladha et al. 2005). Sprague-Dawley (Charles River) rat pups were exposed to normoxia (21% oxygen, control group) or hyperoxia (95% oxygen, BPD model) from birth to postnatal day 14 (P14) in sealed plexiglass chambers with continuous oxygen monitoring (BioSpherix, Redfield, NY). Litters were normalized so all cages had an equal number of pups at P0 to control for the effects of litter size and nutrition on growth. Dams were switched between normoxic and hyperoxic chambers every 48 hours to prevent damage to the dam's lungs. The hyperoxic exposed animals were allowed to recover in normoxia for 7 days. Rat pups were euthanized at P21 via an intraperitoneal injection of pentobarbital. A subset of animals were allowed to reach the age of 50 days, to allow adequate measurements of exercise capacity on a treadmill.

## 2.2 Mesenchymal Stem Cell Harvest and Cell Culture

Bone marrow was harvested from adult Sprague-Dawley rats by excising the femur and tibia and flushing the marrow with Dulbecco's Modified Eagle Medium (DMEM; Gibco Canada, Burlington, ON, Canada). The isolated marrow was dissociated with a 21 gauge needle and plated into a tissue culture flask. After overnight adherence, the media was aspirated and adhered cells were rinsed with PBS, and the media replenished. Adhered cells were allowed to grow to ~75% confluency, then trypsinized and reseeded at a density of $10^5$ cells/cm$^2$. This procedure was performed for 2 passages. Pulmonary artery smooth muscle cells, used as control cells, were

obtained from 20 week old Sprague-Dawley as previously described (Bonnet, Michelakis et al. 2006).

### 2.3 Fluorescence-Activated Cell Sorting of Cell Surface Markers

Phycoerythrin labeling for rat monoclonal antibodies against CD31 (PECAM-1), CD34, CD44 (H-CAM), CD45 (leukocyte common antigen (LCA)), CD54 (ICAM-1), CD73 (SH3, SH4) and CD90 (Thy-1) (Santa Cruz, Santa Cruz, California, USA) were utilized according to the manufacturer's protocol. These antibodies were selected in accordance with the position statement for the minimal criteria to define a mesenchymal stem cell from the International Society for Cellular Therapy (Dominici, Le Blanc et al. 2006). Passage two MSCs formed a single cell suspension and were incubated for 60 minutes at 4°C with monoclonal antibodies against rat antigens including CD31, CD44, CD45, CD54, CD73 and CD90 (each in a separate vial). Irrelevant isotype-identical antibody, immunoglobulin-G (IgG), served as negative control for auto-fluorescence. Specific and unspecific antibody binding was detected with a secondary phycoerythrin-labeled anti-mouse antibody. Samples were analyzed by collecting 10,000 cellular events. Gating was set to remove cellular debris and doublet cells. Cells were analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) and CellQuest Software.

### 2.4 Stem Cell Lineage Differentiation Assay

The lineage differentiation assay was performed in accordance with the position statement for the minimal criteria to define a mesenchymal stem cell from the International Society for Cellular Therapy (Dominici, Le Blanc et al. 2006).

Passage two bone marrow-derived MSC differentiation potential was evaluated as follows:

*Adipogenic induction.* Cells were cultured for 14 days in α-MEM (minimal essential media) containing 10% (v/v) FCS (fetal calf serum), 100μM isobutyl methylxanthine, 60μM indomethacin, 1μg/ml insulin and 0.5μM hydrocortisone (Sigma Canada, Oakville, ON, Canada). Media was changed every 3 days. Adipogenic differentiation was shown by cellular accumulation of large-sized (≈5μm in diameter) lipid vacuoles that were stained with Oil-red O and counterstained with DAPI (Pittenger, Mackay et al. 1999).

*Osteogenic induction.* Cells were cultured for 21 days in α-MEM containing 20% (v/v) FCS, 0.1μM dexamethasone, 2mM β-glycerophosphate and 150μM ascorbic acid; medium was changed every 3 days. Mineralization areas were revealed by Alizarin red staining (Sigma Canada, Oakville, ON, Canada) (Pittenger, Mackay et al. 1999).

*Chondrogenic induction.* Cells at 80% confluency were trypsinized with 0.05% (v/v) trypsin-EDTA and resuspended in low-glucose DMEM containing 1mM dexamethasone, 1mM sodium pyruvate, 1X insulin-transferrin-selenium, 17mM ascorbic acid, 35mM proline and 10ng/ml transforming growth factor β1. Viable cells were counted and seeded at a density of $5x10^5$ cells per pellet in 15-$cm^3$ conical tubes. Cells were gently centrifuged to the bottom of the tubes and allowed to form compact cell pellets, then incubated in a humidified atmosphere at 37°C with 5% $CO_2$ with medium changes every 3 days. After 21 days in culture, pellets were embedded in paraffin. Cartilage glycosaminoglycans were detected by staining with Safranin O (Pittenger, Mackay et al. 1999).

*All chemicals were purchased from Sigma (Sigma Canada, Oakville, ON, Canada).*

## 2.5 RT-PCR Analysis for Lineage Conversion

Total RNA was extracted from undifferentiated (control) and differentiated BM-derived MSCs and analyzed by RT-PCR with primers specific for rat acidic ribosomal phosphoprotein P0 (Rplp0), lipoprotein lipase (Lpl), peroxisome proliferator activated receptor gamma 2 (Pparg2), bone gamma-carboxyglutamate protein (Bglap), runt-related transcription factor 2 (Runx2), pro-α1(II) collagen (Col2a1) and pro-α1(X) collagen (Col10a1) (table 1). Amplified cDNA fragments were electrophoresed through a 2% (w/v) agarose gel, stained by ethidium bromide, and photographed under an ultraviolet light transilluminator.

## 2.6 Colony Forming Unit- Fibroblast (CFU-F) Assay of Bone Marrow, Circulating and Lung Resident MSCs

MSCs were obtained from each of the tissues in various ways. The bone marrow was dissociated as per the above protocol. Circulating MSCs were obtained by pooling the blood (heparinized) of two litters in each of normoxia and hyperoxia. The circulating blood was diluted with sterile PBS (two fold dilution) and layered over 15 ml of Ficoll-Paque. This was then centrifuged at 2000 RPM for 30 minutes with the brake off. The mononuclear cells were then harvested from the interface between the PBS and Ficoll layers. Lung resident MSCs were obtained by enzymatically digesting the lung tissue with type-V collagenase. Each of these cellular pools were then adjusted to the following concentrations: $10^8$, $10^7$, $10^6$, $10^5$, $10^4$ cells/ml.

Following this dilution, the cells were plated in triplicate and colony forming units were analyzed a week later (with normal changes of culture media).

### 2.7 *In Vivo* Experimental Design

Newborn rat pups were randomized to four groups: (1) normoxia (21% oxygen, control group); (2) hyperoxia (95% oxygen, BPD group); (3) hyperoxia + MSCs (BPD treatment group); and (4) hyperoxia + pulmonary artery smooth muscle cells (PASMCs; BPD treatment control group). MSCs or PASMCs were administered at P4 via an IT injection ($1.0 \times 10^5$/cells per animal). Before administration, MSCs and PASMCs were labeled with the intra-vital green fluorescent dye 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) (Sigma Canada, Oakville, ON, Canada) according to the manufacturer's protocol. Oxygen exposure was ceased at P14, and all tissues were harvested at P21.

### 2.8 Lung Morphometry

Lungs were inflated and fixed via the trachea with a 4% formaldehyde solution at a constant pressure of 20 cm $H_2O$(Thebaud, Ladha et al. 2005). After tracheal ligation, the lungs were placed in fixative overnight. Lung volume was measured via water displacement. Lungs were processed and paraffin embedded. Four μm thick serial sections were taken along the longitudinal axis of the right middle lobe. The fixed distance between the sections was calculated to allow systematic sampling of 10 sections across the whole lobe. Lung sections were stained with hematoxylin and eosin. Alveolar development was quantified using the mean linear

intercept method (Thebaud, Ladha et al. 2005). This method quantifies the average distance between adjacent structures for blood-gas exchange.

### 2.9 MSC Engraftment

Lungs from P14 and P21 animals were inflated with Tissue-Tek Optimal Cutting Temperature (OCT) Media (Ted Pella, Inc. Redding, CA) and subsequently frozen in a block of OCT. Lungs were stained for surfactant protein-C (SP-C) and cellular nuclei (DAPI). Lung slices were imaged using a confocal microscope. Five longitudinal slices were used per animal and five random fields per slide were imaged. Total cellular number was based on DAPI staining, type II AECs were identified by the staining of SP-C, MSCs by the presence of green fluorescence (CFSE), and "transdifferentiated" MSCs were identified by the co-localization of SP-C with CFSE.

### 2.10 Barium Angiogram

Barium was instilled into the pulmonary vasculature as previously described (Thebaud, Ladha et al. 2005). The animals were anesthetized with a dose of pentobarbital. Once in surgical plane, with the heart still beating, the chest was opened and 5 μl of heparin was injected directly into the right ventricle. The pulmonary artery was cannulated with a 22 gauge cannula. The lungs were flushed with a 10% heparin 90% saline solution (1 ml). Barium sulfate (Polibar) was infused into the lungs via the pulmonary artery catheter until it flowed out of the left atrium. The barium was imaged with a rodent SPECT-CT (FLEX Pre-clinical platform) and the *Amira* software package (Gamma Medica, Northridge, CA, USA).

### 2.11 Mercox Vascular Casting and Scanning Electron Microscopy

Mercox catalyst and resin (Ladd Research Industries), at a 50:1 ratio, was infused into the pulmonary vasculature in the same manner as the barium angiogram. The soft tissue was dissolved by placing the lungs in 20% KOH for two days. The resulting vascular casts were rinsed with distilled water, air dried at 40C for one hour, and mounted on a stub. The casts were sputter-coated with gold (Edwards S150B; Edwards) and imaged with a Hitachi SEM S-2500 (Thebaud, Ladha et al. 2005).

### 2.12 Right Ventricular Hypertrophy

Right ventricular hypertrophy was assessed post-mortem. The atria and great vessels of the heart were dissected away. The right ventricle free wall was separated from the left ventricle and the septal wall. The tissue was allowed to dry overnight, and was weighed the following day (Bonnet, Michelakis et al. 2006).

### 2.13 Pulmonary Artery Acceleration Time

Pulmonary artery acceleration time, a valid measure of mean pulmonary arterial pressure in rodents (McMurtry, Bonnet et al. 2004; McMurtry, Archer et al. 2005), was assessed with Doppler echocardiography (Bonnet, Michelakis et al. 2006).

### 2.14 Exercise Capacity

Rats within the experimental protocol were allowed to age until P50, to allow for adequate assessment of exercise capacity. The animals were run with the following protocol: 5 minutes at 10 m/min., 5 minutes at 12 m/min., 10 minutes at 14 m/min., 15 minutes at 16 m/min., 10

minutes at 18 m/min., and 20 m/min. until exhaustion. Exhaustion was defined as the animal running exclusively on the lower third of the treadmill coupled with hitting of the shock panel twice within 30 seconds.

### 2.15 *In vitro* Co-Culture Assay

MSCs were seeded in the bottom of a 24-well culture plate in either DMEM (Gibco) or small airway growth media (SAGM, Clonetics). We exposed the MSCs to normal growth media (DMEM), small airway growth media (SAGM), SAGM + hyperoxic damaged lung tissue, DMEM + normoxic lung tissue, DMEM + hyperoxic damaged lung tissue, and DMEM + liver tissue via a modified Boyden chamber (Corning Inc, Corning, NY) with a 0.4 μm mesh separating the upper and lower chambers. The cells were stained for surfactant protein-C (SP-C) and cellular nuclei (DAPI). The cells were imaged with a confocal microscope. Total RNA from cells was obtained using an RNEasy mini kit (Qiagen). Primer for SP-C was obtained from Applied Biosystems. The TaqMan One-Step RT-PCR Master Mix reagent kit (Applied Biosystems, Foster City, CA, USA) was used to quantify the copy number of cDNA targets. The reaction used 50ng RNA in 50 μl using relevant (SP-C) primer (500nM), and TaqMan probe (200nM). The assay was performed using ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). Reverse transcription proceeded for 30 min at 48°C. AmpliTaq Gold activation occurred for 10 min at 95°C. Subsequently, 40 cycles of PCR were performed. Each cycle consisted of 15 seconds of denaturing (at 95°C) and 1 min of annealing and extension (at 60°C). $2^{\Delta\Delta Ct}$ is a ratio of the expression the gene of interest to the reporter18S ribsosome and SP-C messenger RNA (mRNA) levels were normalized to this housekeeping gene (Thebaud, Ladha et al. 2005).

## 2.16 RT-PCR Analysis of Candidate Growth Factors

Primers for vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor-7/keratinocyte growth factor (FGF-7/KGF), FGF10, and FGF18 were obtained from Applied Biosystems. The TaqMan one-step RT-PCR Master Mix reagent kit was used (Applied Biosystems, Foster City, CA, USA). The reaction used 50ng RNA in 50 μl using primer (500nM), and TaqMan probe (200nM). The assay was performed using ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). Reverse transcription proceeded for 30 min at 48°C. AmpliTaq Gold activation occurred for 10 min at 95°C. Subsequently, 40 cycles of PCR were performed. Each cycle consisted of 15 seconds of denaturing (at 95°C) and 1 min of annealing and extension (at 60°C). $2^{\Delta\Delta Ct}$ is a ratio of the expression the gene of interest to the reporter18S ribsosome and messenger RNA (mRNA) levels for candidate genes were normalized to this housekeeping gene.

**Table 2.1 Primers used for Lineage Differentiation RT-PCRs**

| | Target cDNA | Accession numbers | Primer sequence (5'-3') | Product size (bp) |
|---|---|---|---|---|
| House Keeping gene | Rplp0 | NM_022402 | 5': TTTGGGCATCACCACTAAAATCTCCAGAG<br>3': GCTCCCACCTTGTCTCCAGTCTTTATCAG | 87 |
| Adipogenic differentiation | Lpl | NM_012598 | 5': CAGGATGCAACATTGGAGAAGCCATTC<br>3': GGAGTCAATGAAGAGATGGATGGAGCG | 113 |
| | Pparg2 | NM_013124 | 5': CTCCTGTTGACCCAGAGCATGGTGC<br>3': TGATTCCGAAGTTGGTGGGCCAG | 114 |
| Osteogenic differentiation | Bglap2 | NM_013414 | 5': TCTGCTGGCCCTGACTGCATTCTG<br>3': CACCACCTTACTGCCCTCCTGCTTG | 109 |
| | Runx2 | XM_346016 | 5': CTCGCACTGGCGGTGCAACAAG<br>3': TCTCATCATTCCCGGCCATGACG | 107 |
| Chondrogenic differentiation | Col2a1 | NM_012929 | 5': GAACAAGGACCCAGAGGTGACCGTG<br>3': CAGGATTTCCAGGGGTACCAGGTTCTC | 97 |
| | Col10a1 | AJ131848 | 5': TTGGACCACCAGGTATCCCAGGATTC<br>3': GGGAGACCAGGCTCTCCAGTATGACC | 160 |

Rplp0, acidic ribosomal phosphoprotein P0; Lpl, lipoprotein lipase; Pparg2, peroxisome proliferator activated receptor gamma 2; Bglap2, bone gamma-carboxyglutamate protein 2; Runx2, runt-related transcription factor 2; Col2a1, pro-α1(II) collagen; Col10a1, pro-α1(X) collagen.

## References

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Dominici, M., K. Le Blanc, et al. (2006). "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement." Cytotherapy **8**(4): 315-7.

McMurtry, M. S., S. L. Archer, et al. (2005). "Gene therapy targeting survivin selectively induces pulmonary vascular apoptosis and reverses pulmonary arterial hypertension." J Clin Invest **115**(6): 1479-91.

McMurtry, M. S., S. Bonnet, et al. (2004). "Dichloroacetate prevents and reverses pulmonary hypertension by inducing pulmonary artery smooth muscle cell apoptosis." Circ Res **95**(8): 830-40.

Pittenger, M. F., A. M. Mackay, et al. (1999). "Multilineage potential of adult human mesenchymal stem cells." Science **284**(5411): 143-7.

Thebaud, B., F. Ladha, et al. (2005). "Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization." Circulation **112**(16): 2477-86.

# Chapter III: Results

# 3. Results

## 3.1 Flow Cytometric Analysis of Cell Surface Markers on Bone Marrow MSCs

MSCs extracted from bone marrow formed a homogeneous layer of cells by the second passage. The analysis of cell surface phenotype indicates that our MSC population is positive for CD44 (H-CAM), CD54 (ICAM-1), CD73 (SH3, SH4) and CD90 (Thy-1) while negative for CD31 (PECAM-1), CD34, CD45 (leukocyte common antigen (LCA)) (figure 3.1). This result is similar to other published studies (Prockop 1997; Pittenger, Mackay et al. 1999; Orlic, Kajstura et al. 2001; Rojas, Xu et al. 2005), and is inline with the position statement for the minimal criteria to define a mesenchymal stem cell from the International Society for Cellular Therapy (Dominici, Le Blanc et al. 2006).

## 3.2 Lineage Differentiation of Bone Marrow MSCs

Our MSCs demonstrate a degree of "multipotency." The MSCs are able to differentiate into three mesenchymal lineages when grown in specific media for each lineage.

Induction of the adipogenic phenotype is demonstrated by the staining of lipid vacuoles with Oil-red O. This conversion into adipocytes was further confirmed with RT-PCR expression of lipoprotein lipase (Lpl) and peroxisome proliferator activated receptor gamma 2 (Pparg2) (markers of adipocytes; figure 3.2 A.) with rat acidic ribosomal phosphoprotein P0 (Rplp0) used as a control.

Induction of the osteogenic phenotype is demonstrated by the staining of areas of mineralization with Alizarin Red. The osteogenic conversion was further confirmed via RT-PCR expression of peroxisome proliferator activated receptor gamma 2 (Pparg2) and bone gamma-carboxyglutamate protein (Bglap) (osteogenic markers; figure 3.2 B.) with rat acidic ribosomal phosphoprotein P0 (Rplp0) used as a control.

Induction of the chondrogenic phenotype is demonstrated by cartilage glycosaminoglycans staining with Safranin O. The chondrogenic conversion was further confirmed via RT-PCR expression of pro-α1 (II) collagen (Col2a1) and pro-α1 (X) collagen (Col10a1) (chondrogenic markers; figure 3.2 C.) with rat acidic ribosomal phosphoprotein P0 (Rplp0) used as a control.

Therefore, based on both cell surface marker phenotype and multipotency for the three mesenchymal lineages, we are confident that our harvested bone marrow cells are true mesenchymal stem cells (Dominici, Le Blanc et al. 2006).

### 3.3 Reduction of Circulating and Lung Resident MSCs Following Hyperoxia

Following two weeks of exposure to hyperoxia, colony forming unit- fibroblast (CFU-F) assays demonstrate a significant reduction in the proportion of MSCs in both the circulating blood and resident within the lung, with no effect on the presence of MSCs within the bone marrow (figure 3.3). Flow cytometric analysis of cell surface markers (figure 3.4) as well as lineage differentiation (figure 3.5) was performed on cells recovered from the CFU-F assay.

These recovered cells have the same characteristics of our harvested bone marrow MSCs (lineage differentiation and cellular phenotype).

### 3.4 Survival Analysis

Kaplan-Meier analysis demonstrates that IT MSC injection at P4 significantly ($p=0.007$, Log-Rank test) improves survival as compared to both the hyperoxic group and the hyperoxic + PASMC group (figure 3.6) in our model of experimental BPD.

### 3.5 Effect of Hyperoxia and IT MSC Therapy on Lung Architecture

Hyperoxia induced a histological pattern that is reminiscent of human BPD, air space enlargement with simplified and fewer alveolar structures (secondary septations) as compared with the normoxic controls (figure 3.7 A.).

The IT administration of MSCs significantly improved alveolarization, as demonstrated by the smaller alveoli and the increased number of secondary septations (figure 3.7 A.) in a pattern that more closely resembled the normoxic control group. Conversely, the IT administration of the control PASMCs had no protective affect on lung architecture from the hyperoxic exposure (figure 3.7 A.).

We were able to objectively quantify the affect that the IT administration of MSCs had on the lung architecture using the mean linear intercept (MLI) method (figure 3.7 B.) (Thebaud,

Ladha et al. 2005). We observed a significant ($p<0.001$) reduction of the IT MSC MLI as compared to both the hyperoxic and the hyperoxic + PASMC groups.

### 3.6 Engraftment of CFSE Labeled MSCs

To assess the engraftment potential of the MSCs, the engrafted cells were manually counted and compared with the entire cellular population of the lung. The CFSE labeled MSCs accounted for 3.7% ± 2.9% of the total number of lung cells (an average of 5 cells per high powered field) at P14 (10 days after initial administration) and 2.9% ± 2.6% of the total number of lung cells (an average of 3 cells per high powered field) at P21 (17 days after initial administration) (figure 3.8).

### 3.7 Engraftment of MSCs Obtained from Green Fluorescent Protein Over-Expressing Transgenic Rats

Bone marrow MSCs were obtained from rats that over-express green fluorescent protein (GFP). The presence of GFP was determined through PCR analysis of tail tissue from donor rats (figure 3.9 A.). A discrete band at 799 kb indicates a homozygous, wildtype (i.e. GFP negative) animal, whereas the presence of two bands (799 kb and 1050 kb) indicates an animal that is heterozygous for GFP over-expression.

To further confirm the presence of GFP expression, isolated MSCs were imaged with a confocal microscope (figure 3.9 B.). The GFP appears to be present throughout the entire cell, not localized to any particular structure.

At P21, there is a robust appearance of green signal within the lung (figure 3.9 C.). This engraftment potential appears to be much higher than the CFSE labeled MSCs. This discrepancy could be due to the loss of CFSE signal over time, whereas the genetic GFP is maintained within all daughter cells derived from the injected MSCs.

### 3.8 IT MSCs Decrease Pulmonary Hypertension in Irreversible, Oxygen-Induced BPD

Chronic hyperoxic exposure caused a significant ($p<0.01$) increase in right ventricular hypertrophy (RVH, a surrogate marker of pulmonary hypertension; figure 3.10 A.) and a significant ($p<0.01$) decrease in pulmonary artery acceleration time (PAAT; figure 3.10 C.) (McMurtry, Bonnet et al. 2004; McMurtry, Archer et al. 2005). Moreover, the waveform displayed the characteristic "notching" seen in pulmonary hypertension (figure 3.10 B.). The IT MSC therapy significantly ($p<0.01$) reduced the degree of RVH (figure 3.10 A.) and normalized the PAAT to near control levels (figure 3.10 C.), whereas the PASMCs had no effect on the RVH or PAAT (figure 3.10 A, B and C) as compared to the hyperoxic group.

### 3.9 IT MSCs Preserve Vascular Development in Irreversible, Oxygen-Induced BPD

Barium angiography demonstrates that the normoxic control lungs have a very dense pulmonary capillary system (figure 3.11 A.). Hyperoxic exposure causes a severe retardation in pulmonary capillary growth (figure 3.11 A.), a phenotype that is reminiscent of severe pulmonary hypertension and has been described as a "tree in winter" (Zhao, Courtman et al. 2006). The IT administration of MSCs promoted pulmonary vascular development (figure 3.11 A.), however the degree of vascular growth is still less than that of the normoxic control lung.

Scanning electron microscopy (SEM) of the mercox cast of the normoxic pulmonary capillaries reveales a vascular system with relatively large, rounded, smooth, and well organized vessels (figure 3.11 B.). Hyperoxic exposure caused a severe disruption of organization, thinning, and scaring of the vasculature (figure 3.11 B.). The pulmonary vasculature from lungs treated with IT MSCs demonstrated greater organization, as well as larger and rounder vessels, however a large degree of scaring was still present (figure 3.11 B.).

Using a barium-gelatin mixture, we were able to quantify the density of the pulmonary capillaries. Representative histological sections are shown in figure 3.12 A. Quantification demonstrates a sever blunting of the pulmonary vascular density in both the hyperoxic and hyperoxic + PASMC group (figure 3.12 B.); whereas the hyperoxic + MSC group shows a significant increase ($p<0.001$, Fisher's PLSD) in pulmonary vascular density compared to the two hyperoxic groups (figure 3.12 B.). However, this increase in vascular density is not a return to the same density of the normoxic animals (figure 3.12 B.).

### 3.10 IT MSCs Improve Functional Exercise Output

Animals treated with IT MSCs were able to run for longer periods of time and at higher intensities as compared to the hyperoxic and hyperoxic + PASMC groups (figure 3.13). This strongly indicates that the beneficial effect seen in both lung architecture, pulmonary vascular development, and pulmonary hypertension is translatable to an improvement in functional output as well.

### 3.11 MSCs Adopt the Type II Alveolar Epithelial Cell Phenotype *In Vivo* and *In Vitro*

*In vivo*, we labeled the MSCs using the intra-vital green fluorescent marker CFSE. Using confocal microscopy, we observed SP-C colocalizing with the CFSE labeled MSCs (figure 3.14). Furthermore, we also assessed the number of MSCs that "converted" to the AEC2 phenotype. At P14, 75.3±24.5% of the MSCs co-expressed SP-C (based on immunofluorescence) and at P21 72.2±28.0% of the MSCs co-expressed SP-C.

*In vitro*, MSCs were able to adopt the AEC2 phenotype after exposure to normoxic or hyperoxic lung in a modified boyden chamber. MSCs exposed to either culture media alone or to liver did not show any SP-C mRNA production. Cells exposed to SAGM + hyperoxic lung displayed SP-C mRNA expression (figure 3.15 A.). Immunofluorescence and confocal microscopy confirmed the absence of SP-C within the culture control groups, and SP-C expression reminiscent of AEC2s within the MSCs exposed to the hyperoxic lung (figure 3.15 A.).

Transmission electron microscopy (TEM) of freshly isolated, fetal AEC2s provided a reference for the appearance of lamellar bodies (figure 3.15 B.). MSCs grown under normal culture conditions are devoid of any structures that resemble lamellar bodies (figure 3.15 B.). MSCs exposed to lung tissue in the boyden chamber system had numerous structures that resembled lamellar bodies (figure 3.15 B.).

Therefore, we have strong evidence that the mesenchymal stem cell is able to adopt the phenotype of the AEC2 when exposed to the microenvironment of the lung in both *in vitro* and *in vivo* settings.

### 3.12 Exploration of a Potential Paracrine Mechanism in the Protection of Pulmonary Development

To determine if there was selective induction of known lung protective growth factors by IT MSC administration, or if there was a beneficial effect on the production of inflammatory cytokines, lung tissue was harvested from normoxic, hyperoxic, and hyperoxic + MSC treated animals at P4, P7, P10, P14, and P21.

Numerous growth factors have been implicated in the normal process of alveolarization, as well many have been shown to exert a protective effect on the developing lung. To this end, we selected numerous candidate growth factors that could also have been selectively induced through the administration of MSCs. We focused on vascular endothelial growth factor (VEGF) (Kunig, Balasubramaniam et al. 2005; Thebaud, Ladha et al. 2005), keratinocyte growth factor (KGF, also referred to as FGF-7) (Frank 2003), hepatocyte growth factor (HGF) (Padela,

Cabacungan et al. 2005), fibroblast growth factor 10 (FGF-10) (Benjamin, Smith et al. 2007), and fibroblast growth factor 18 (FGF-18) .

Temporal analysis of VEGF mRNA levels over the time course (figure 3.16) demonstrates no significant differences in VEGF levels except for a reduction at P14 in both the hyperoxia and hyperoxia + MSC groups compared with the normoxia group. However, there is no evidence that VEGF is selectively induced within the developing lung following IT MSC administration.

Temporal analysis of HGF mRNA levels over the time course (figure 3.17) demonstrates no significant differences in HGF. However, there is no evidence that HGF is selectively induced within the developing lung following IT MSC administration.

Temporal analysis of KGF mRNA levels over the time course (figure 3.18) shows a reduction of KGF levels at P10 and P14. However, there is no evidence that KGF is selectively induced within the developing lung following IT MSC administration.

Using enzyme linked immuno-sorbent assays (ELISAs), we assessed the inflammatory cytokines Il-1α, Il-6, and TNF-α. After four days of hyperoxic exposure, Il-1α was significantly increased (figure 3.21). This increase in Il-1α was sustained through P7.5, and diminished by P10.5. Interestingly, the MSC treatment group saw a similar induction of Il-1α at P7.5 (figure 3.21) however this increase was sustained at P10.5, and while not statistically significant there is still a trend to an increase at P14 (figure 3.21). Temporal analysis of Il-6 did not show any significant changes in Il-6 production between the three groups throughout the time course (figure 3.22). Temporal analysis of TNF-α shows a reduction of TNF-α production in the hyperoxic group as compared to the normoxic group (figure 3.23) at P7.5 and P10.5. However, there is no evidence that TNF-α is selectively affected by the IT administration of MSCs.

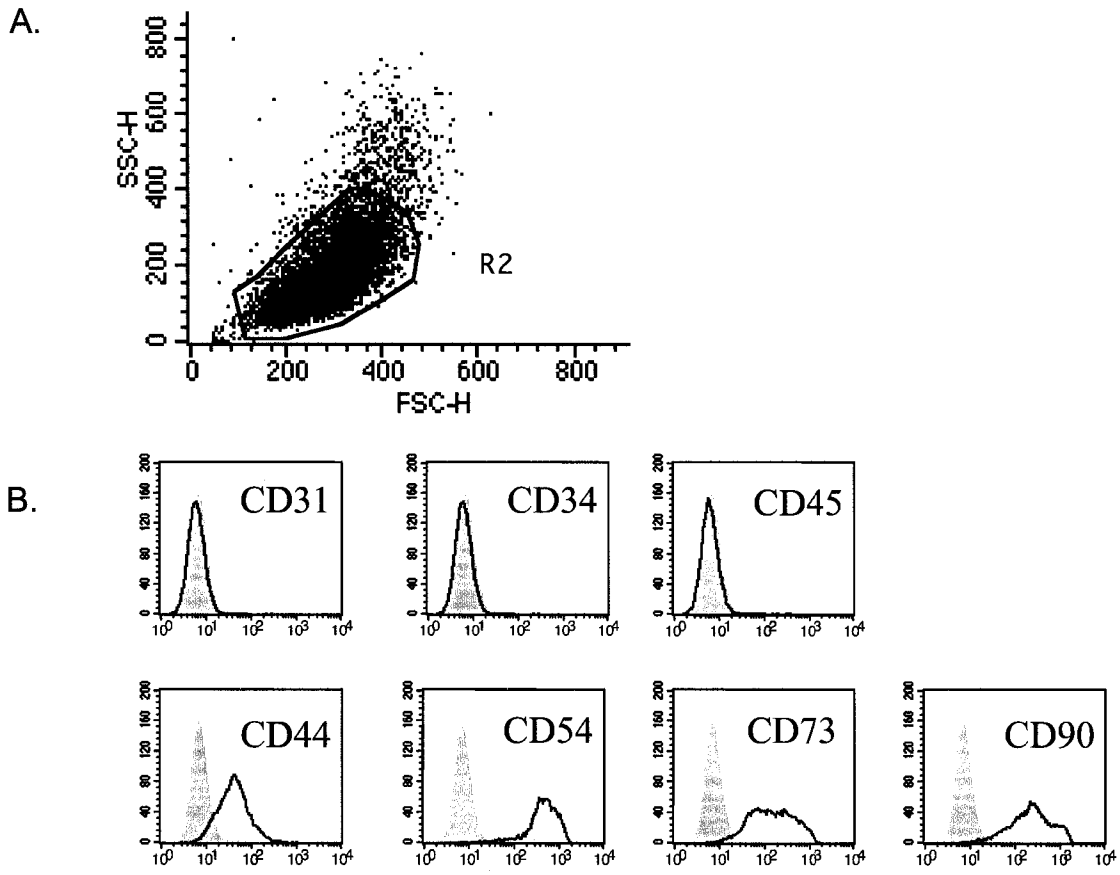


**Figure 3.1 Flow Cytometric Analysis of Cell Surface Markers on Bone Marrow MSCs**

A. Representative scatter plot obtained from the flow cytometer. Gating (R2) was used to exclude cell doublets and cellular debris as determined by side scatter (SSC) and forward scatter (FSC).

B. Representative histograms for surface markers of interest (CD31. CD34, CD44, CD45, CD54, CD73, and CD90). Isotype control antibody (shaded histogram) was used to set baseline autofluorescence. Fluorescence for each marker of interest is indicated by the red histogram. Our MSC population is CD31, CD34, CD45 negative, and CD44, CD54, CD73, and CD90 positive.

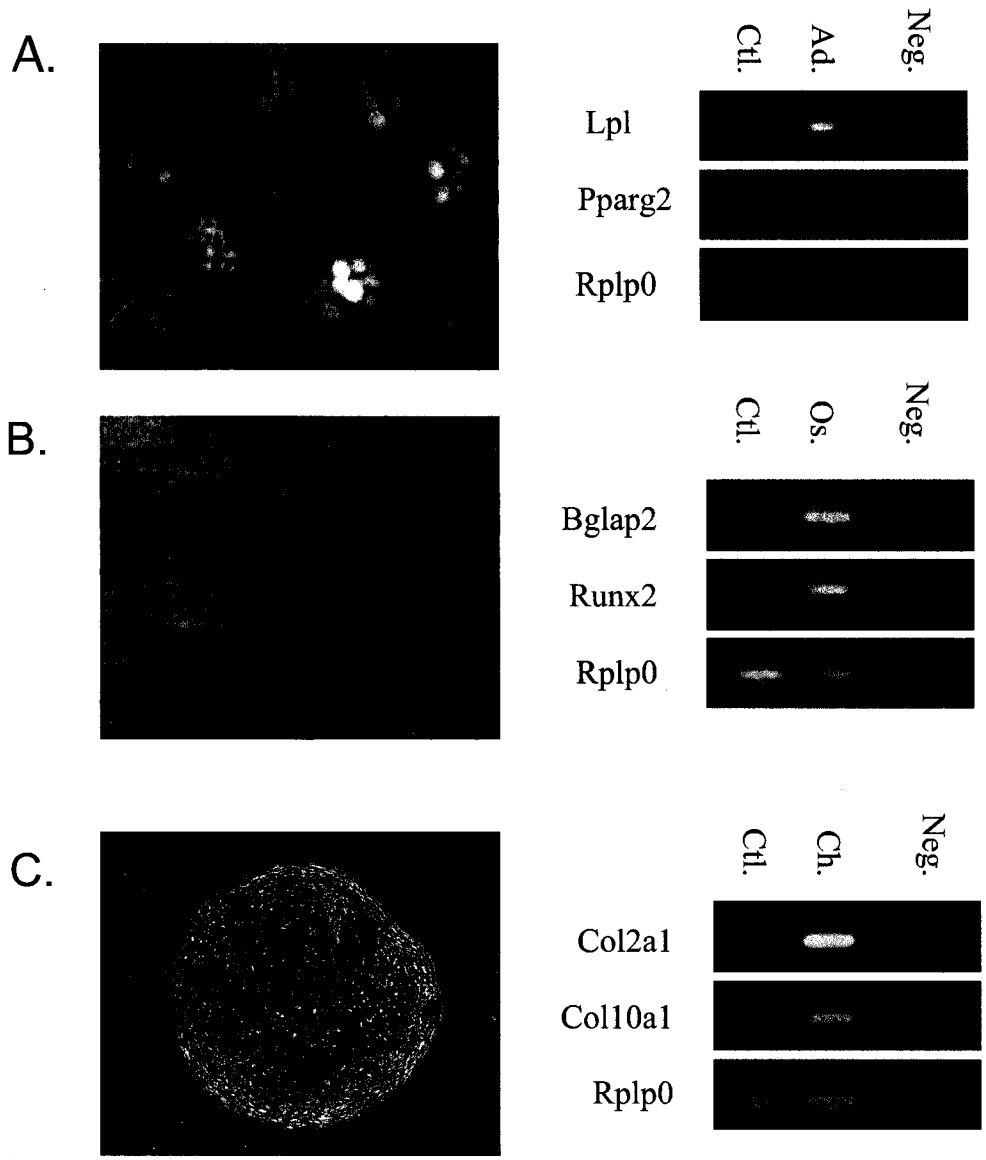


**Figure 3.2 Lineage Differentiation of Bone Marrow MSCs**

A. Conversion of MSCs to adipocytes is shown by the oil-red O staining of lipid vacuoles (left panel); cellular nuclei are stained blue with DAPI. This transformation was confirmed using RT-PCR (right panel) showing the presence of mRNA for lipoprotein lipase (Lpl) and peroxisome proliferator activated receptor gamma 2 (Pparg2) which are not shown in native (control) MSCs.

B. Conversion of MSCs to osteoblasts is indicated by areas of mineralization staining with alizarin red (left panel). This transformation was confirmed using RT-PCR (right panel) showing the presence of mRNA for bone gamma-carboxyglutamate protein (Bglap) and peroxisome proliferator activated receptor gamma 2 (Pparg2) which are not shown in native (control) MSCs.

C. Conversion of MSCs to chondrocytes shown by cartilage glycosaminoglycans staining with safrainin O (left panel). This transformation was confirmed using RT-PCR (right panel) showing the presence of mRNA for pro-α1 (II) collagen (Col2a1) and pro-α1 (X) collagen (Col10a1) which are not shown in native (control) MSCs.

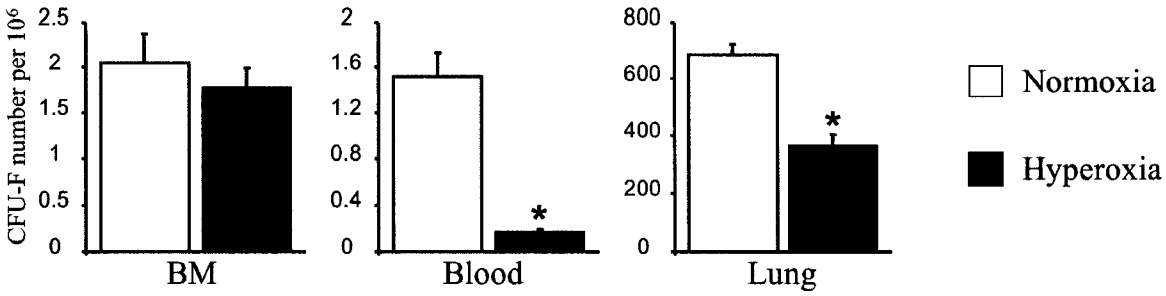


**Figure 3.3 Hyperoxia Decreases Circulating and Lung Resident MSCs**
Colony forming unit-fibroblast (CFU-F) assay indicates that 14 days of exposure to 95% oxygen significantly reduces the number of MSCs present in the circulating blood and within the lung itself, with no effect on the MSC population within the bone marrow (*$p<0.01$ vs. Normoxia, Student's T-Test, n=3/group).

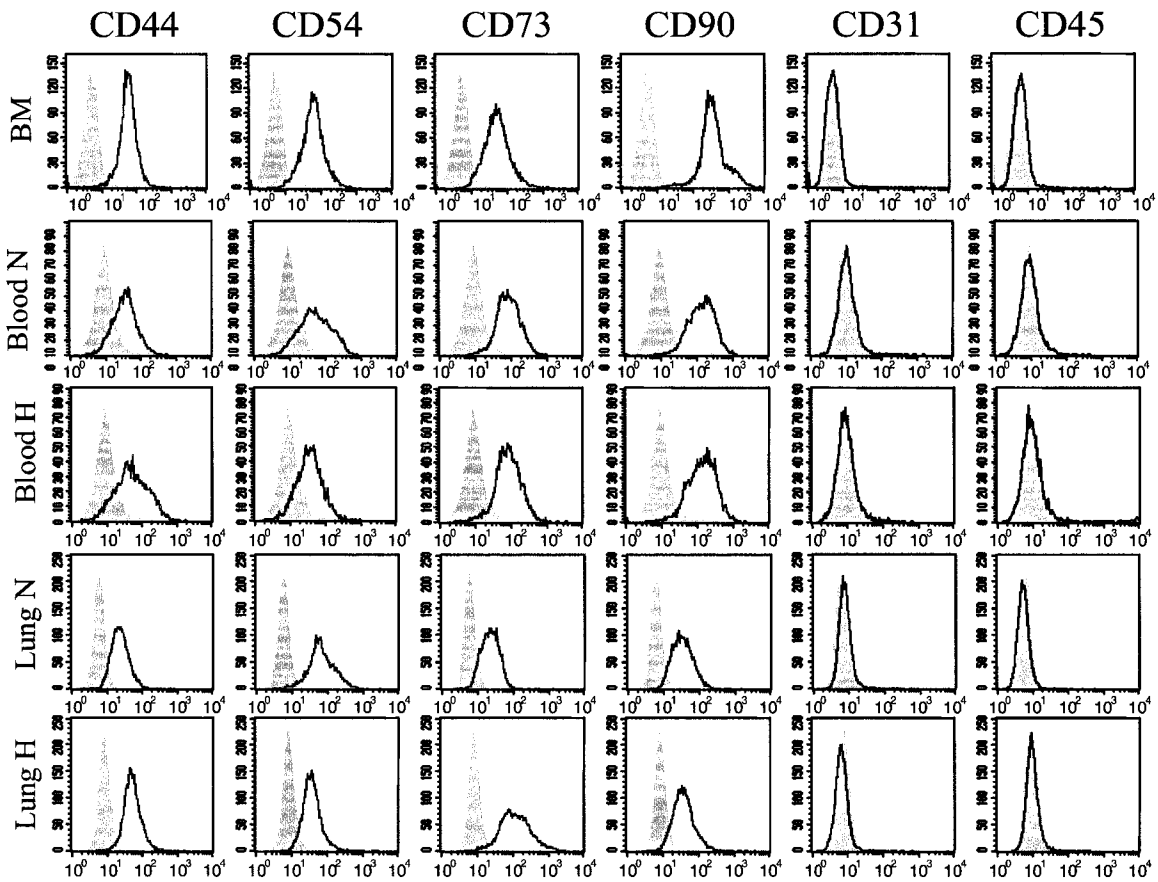


**Figure 3.4 Flow Cytometic Analysis of Cells Recovered from CFU-F Assay**
Representative histograms for surface markers of interest (CD31. CD34, CD44, CD45, CD54, CD73, and CD90). Isotype control antibody (shaded histogram) was used to set baseline autofluorescence. Fluorescence for each marker of interest is indicated by the red histogram. Similar to the bone marrow MSCs, the circulating and lung MSCs are also CD31, CD34, CD45 negative, and CD44, CD54, CD73, and CD90 positive.

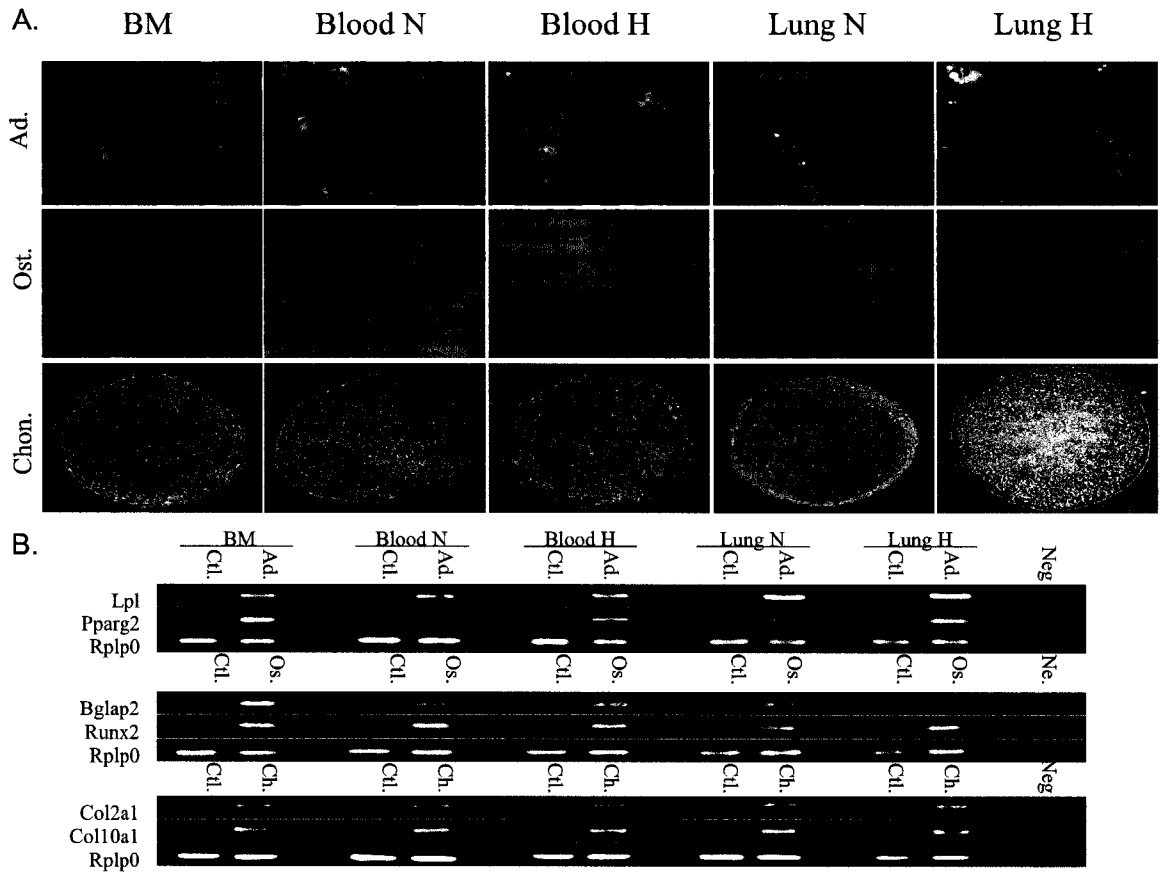


**Figure 3.5 Lineage Differentiation of Cells Recovered from CFU-F Assay**

A. Characteristic lineage differentiation of MSCs recovered from each tissue source into adipocytes, osteoblasts, or chondrocytes in a similar pattern as compared with our bone marrow MSCs.

B. RT-PCR confirmation of lineage differentiation of MSCs into adipocytes, osteoblasts, or chondrocytes in a similar pattern as compared with our bone marrow MSCs.

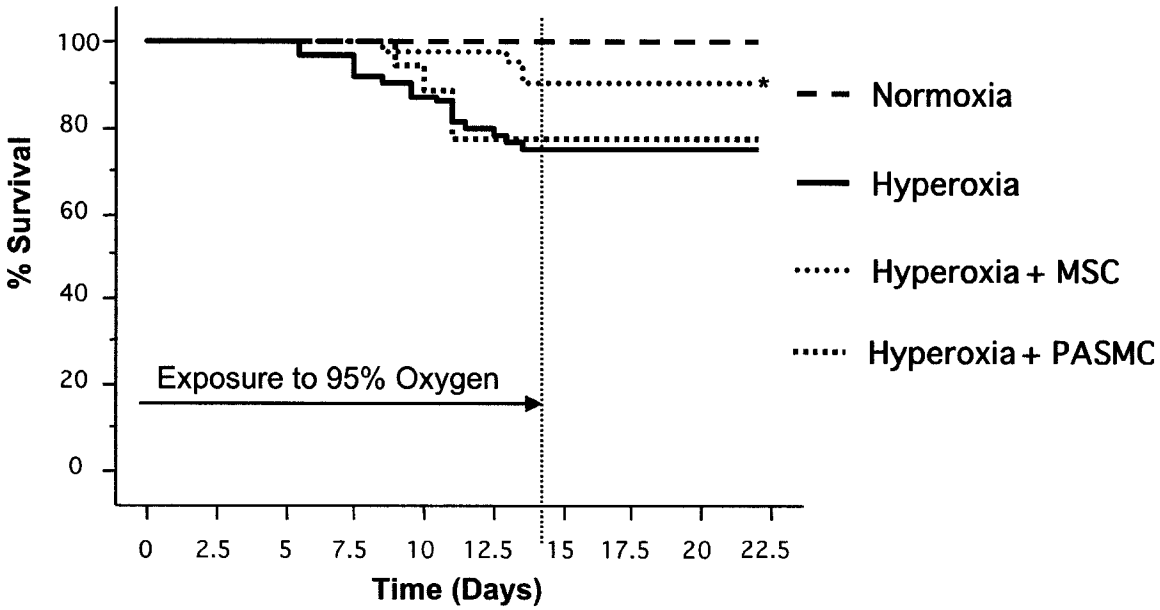


**Figure 3.6 Administration of MSCs Significantly Improves Survival During Hyperoxia** Compared to untreated hyperoxic animals or hyperoxic animals treated with PASMCs, the administration of MSCs significantly improves survival during hyperoxic exposure as determined by the Log-Rank test (*p=0.007 vs. hyperoxia, Log-Rank Test, n=40/group).

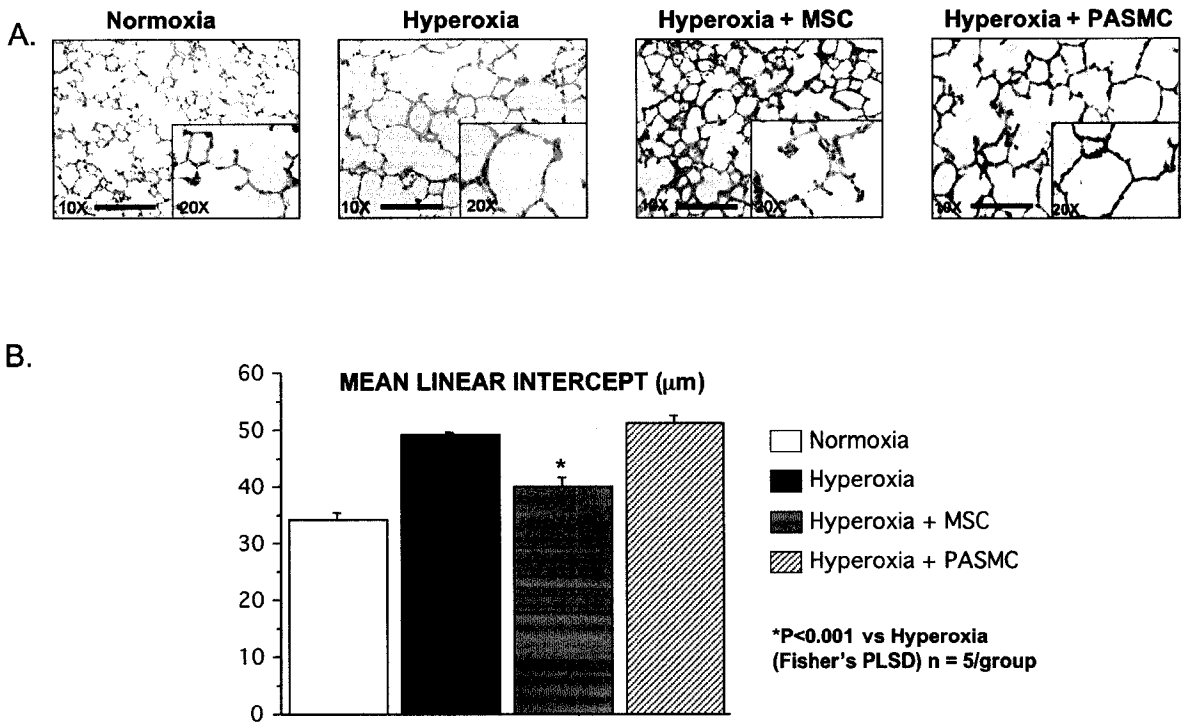


**Figure 3.7 MSC Administration Improves Lung Architecture**

A. Histological analysis shows normal alveolar development (relatively small alveoli and numerous secondary septations) in normoxic animals. This process is interrupted in animals exposed to hyeroxia as they develop with larger but fewer alveoli and decreased secondary septations. MSC administration restores the phenotype of smaller alveoli and secondary septations, whereas the administration of PASMCs did not prevent alveolar simplification.

B. Analysis of the mean linear intercept, a measure of adjacent structures for gas exchange, shows that MSCs improve alveolar development as compared to the hyperoxic or hyperoxic + PASMC groups (*$p<0.001$vs. hyperoxic, Fisher's PLSD, n=5/group).

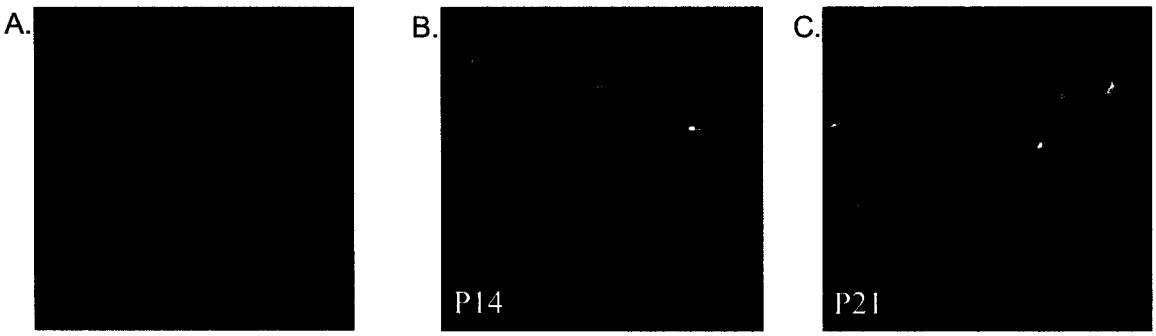


**Figure 3.8 Engraftment of CFSE Labeled MSCs**

A. Representative picture of a CFSE labeled MSC *in vitro*.
B. Engraftment of CFSE MSCs at P14 shows 3.7% of lung cells MSCs, compared with all DAPI positive cells (blue).
C. Engraftment of CFSE MSCs at P21 shows 2.9% of lung cells MSCs, compared with all DAPI positive cells (blue).

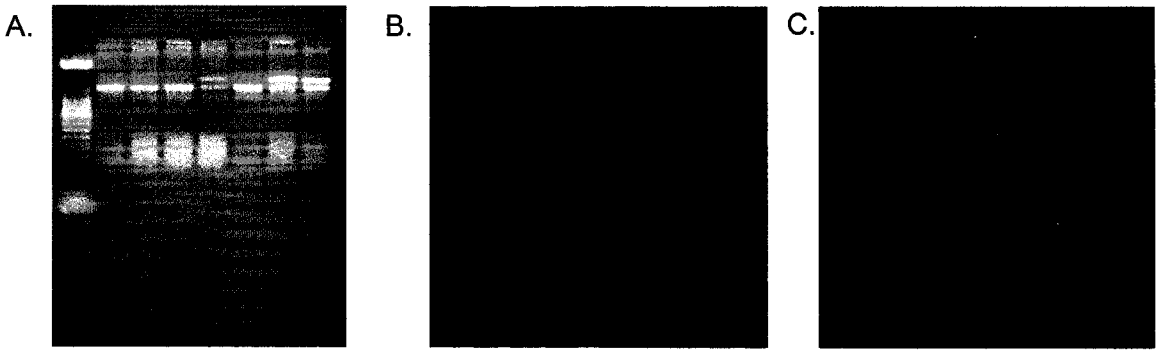


**Figure 3.9 Engraftment of MSCs From GFP-Transgenic Rats**

A. PCR confirmation for GFP indicated by the discrete bands at 799kb indicate animals homozygous for wildtype whereas the lanes with two bands indicate animals heterozygous for GFP.
B. Representative picture of GFP-MSCs *in vitro*, cellular nuclei stained blue by DAPI.
C. Engraftment of GFP-MSCs in the lung at P21. A much higher proportion of cells are GFP positive (normalized to DAPI positive cells) than compared to the CFSE engraftment.

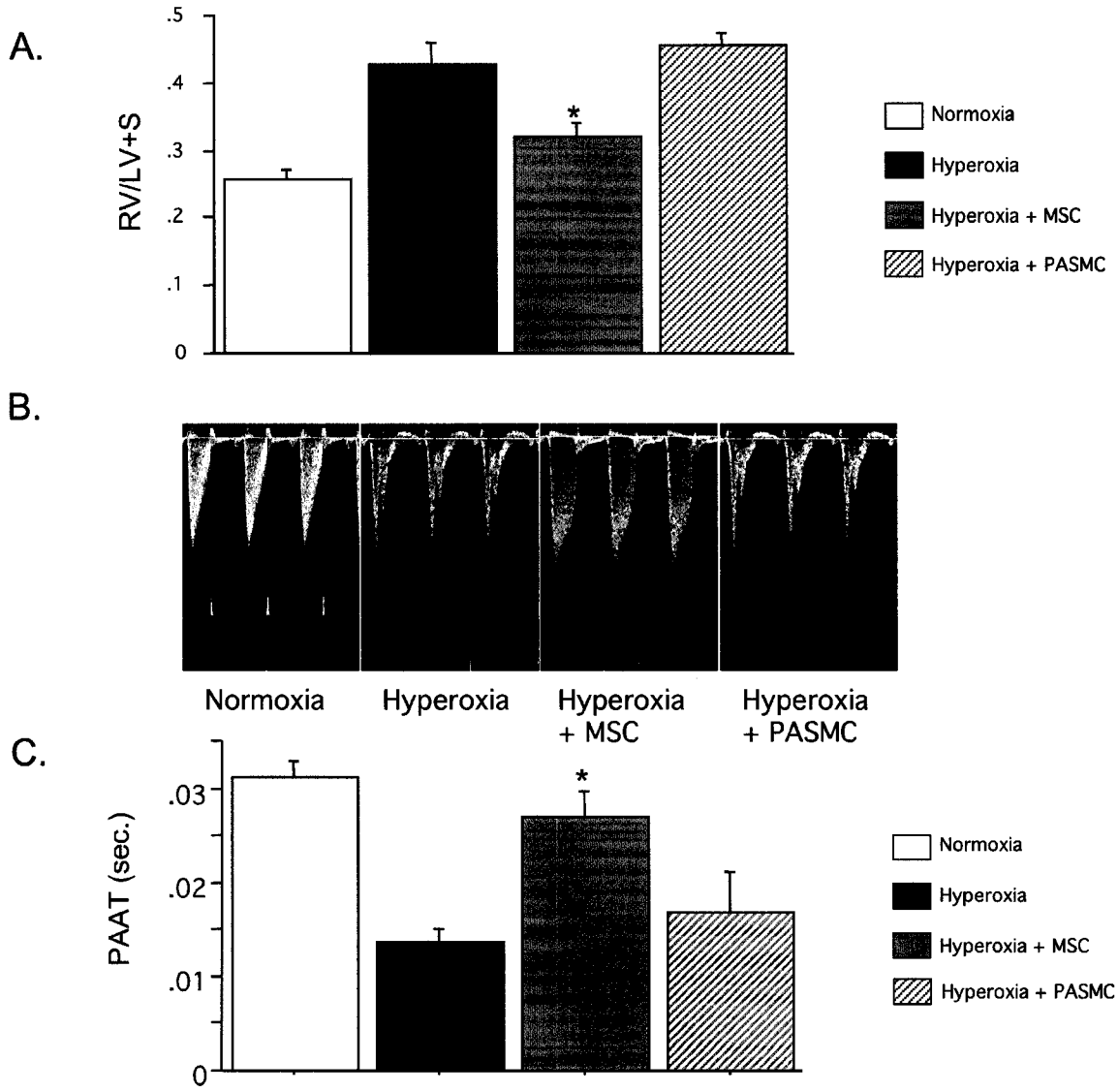


**Figure 3.10 MSC Administration Attenuates Pulmonary Hypertension**

A. Right ventricular hypertrophy, an indication of pulmonary hypertension, is significantly increased in the hyperoxic and hyperoxic + PASMC groups. MSC administration significantly improved right ventricular hypertrophy (*$p<0.01$ vs. hyperoxic, Fisher's PLSD, n=10/group).

B. Representative waveforms obtained from Doppler echocardiography. The hyperoxic and hyperoxic + PASMC groups demonstrate the characteristic notching associated with pulmonary hypertension, whereas the MSC waveform has an appearance more similar to the normoxic group.

C. The hyperoxic and hyperoxic + PASMC groups have an increase in PAAT, indicating higher pressures within the main pulmonary artery (*$p<0.01$ vs. hyperoxic, Fisher's PLSD, n=10/group). This increase in PAAT is ameliorated with the MSC treatment.

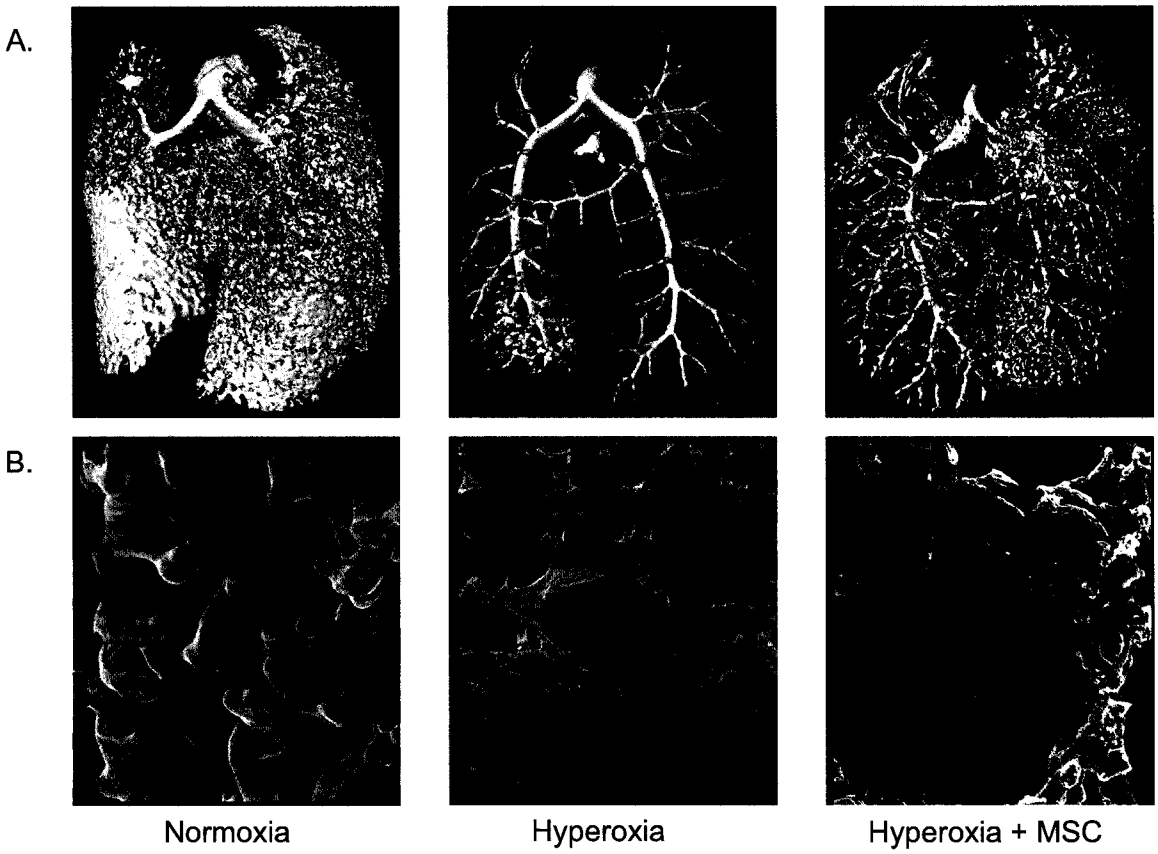


**Figure 3.11 MSCs Improve Lung Capillary Density and Capillary Morphology**

A. Small animal CT-scans reveal the dense vasculature present within a normal lung. There is a significant pruning of the vasculature within hyperoxic group, and this pruning is attenuated with the MSC treatment.

B. Scanning electron microscopy shows the normal capillaries having a relatively round and smooth phenotype, the hyperoxic vessels have a thinned and significantly scarred phenotype, whereas the MSC treated lungs have capillaries that are larger and rounder than the corresponding hyperoxic vessels but still demonstrate a significant degree of scarring from the hyperoxic injury.

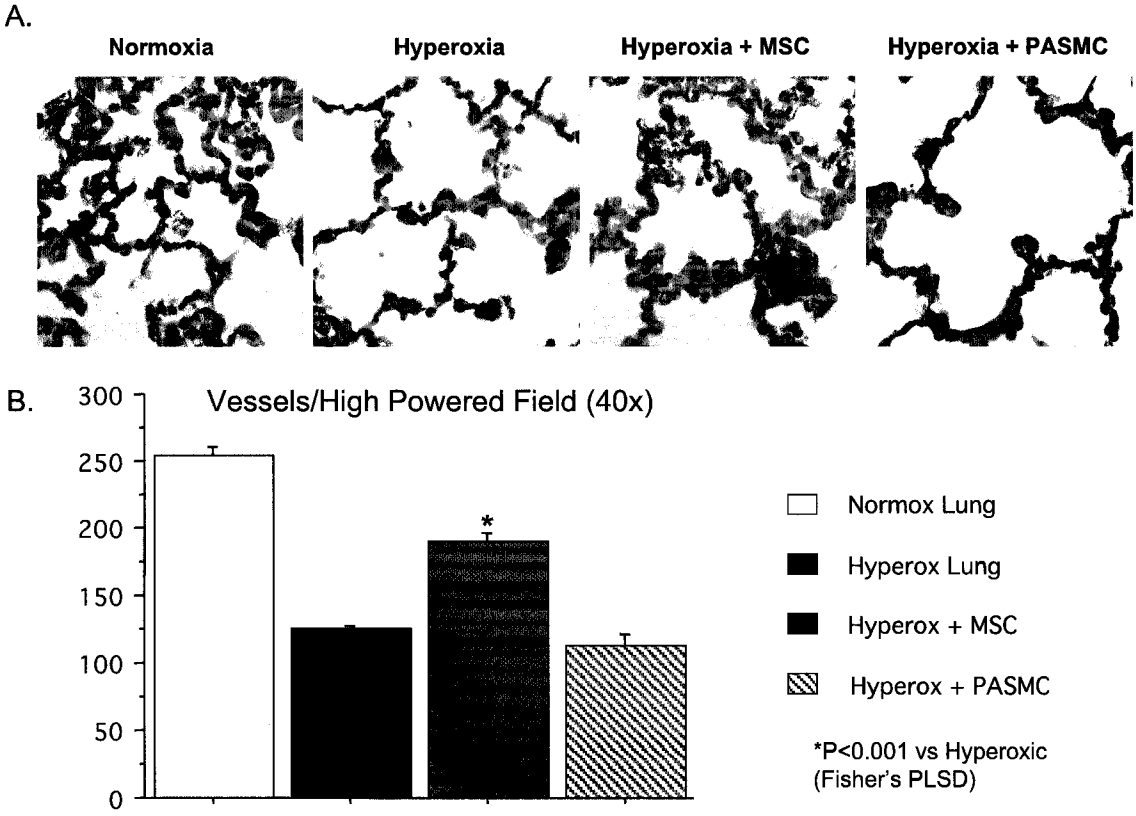


**Figure 3.12 Quantification of Pulmonary Capillary Density**

A. Representative histological sections from lungs with barium-gelatin inflated pulmonary arteries.

B. Quantification of barium filled vessels confirms the result obtained from CT-scanning; the hyperoxic and hyperoxic + PASMC groups have a significant decrease in pulmonary vascular growth, whereas the MSC treatment group has a significant increase in the density of the pulmonary vasculature (*$p<0.001$ vs. Hyperoxic, Fisher's PLSD, n=5/group).

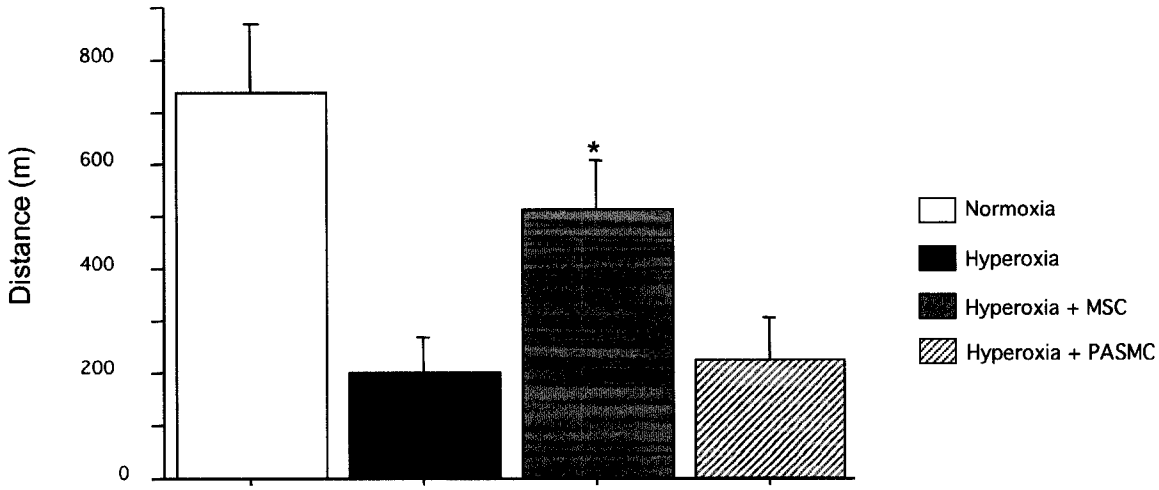


**Figure 3.13 MSCs Improve Functional Exercise Capacity**
Our results indicate that MSC treated animals are able to run greater distances, and at higher intensities, than those animals in the hyperoxic or hyperoxic + PASMC groups (*$p<0.001$ vs. Hyperoxia, Fisher's PLSD, n=5/group).

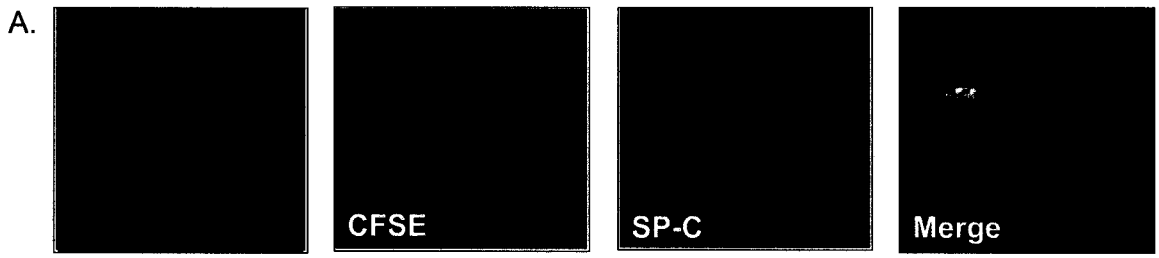


**Figure 3.14 *In Vivo* Demonstration of MSC Plasticity**
Engrafted MSCs, labeled with CFSE (green), co-localized with the type II alveolar epithelial cell specific marker Surfactant Protein-C (SP-C; red). This phenomenon occurred in 75.3% of the engrafted MSCs (normalized to cellular nuclei, DAPI (blue)).

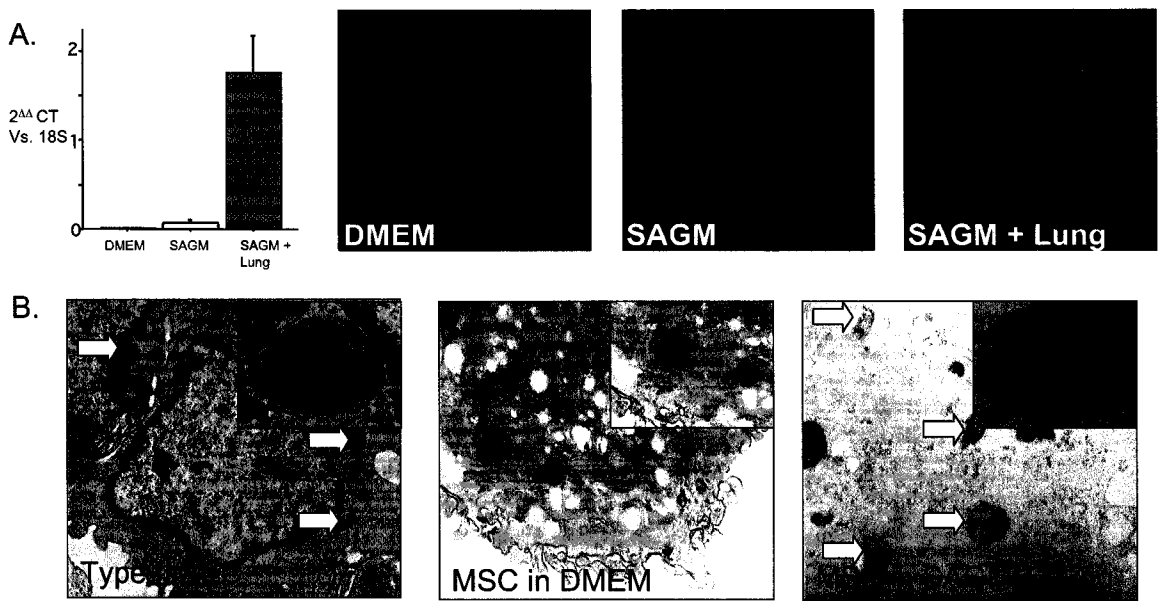


**Figure 3.15 *In Vitro* Plasticity of MSCs**

A. MSCs exposed to lung tissue begin to express surfactant protein C. MSCs grown in DMEM do not express SP-C natively, whereas MSCs exposed to lung tissue show production of SP-C mRNA as indicated by RT-PCR. Immunofluorescence imaging indicates that the SP-C (red) produced appears in a discrete, granular way and that there is no SP-C present within the 2 control groups.

B. Transmission electron microscopy of freshly isolated, fetal type II alveolar epithelial cells shows the presence of lamellar bodies containing surfactant (arrows). MSCs grown in normal culture media do not contain structures resembling lamellar bodies. MSCs exposed to lung tissue had numerous structures resembling lamellar bodies (arrows). However, the density of the lamellar bodies was not the same as those from freshly isolated AEC2s (inset).

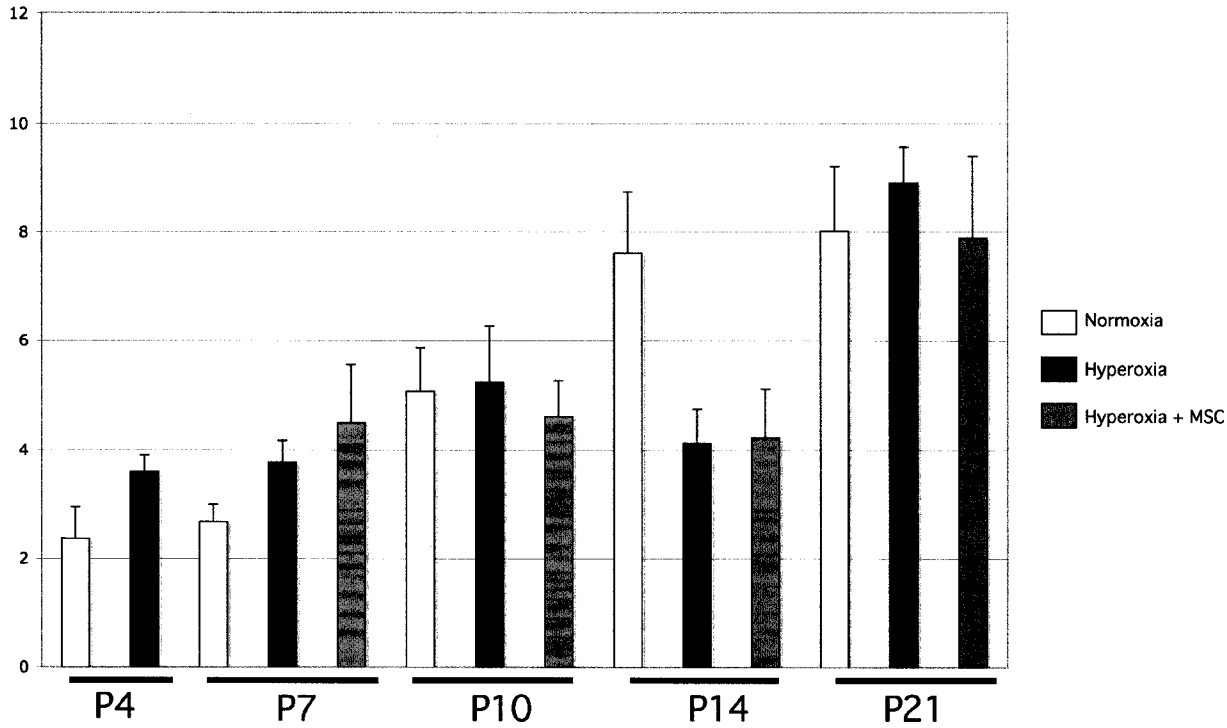


**Figure 3.16 RT-PCR Analysis of VEGF mRNA Expression**
Temporal expression of VEGF mRNA (normalized to 18S) did not reveal significant differences within mRNA expression between the three groups.

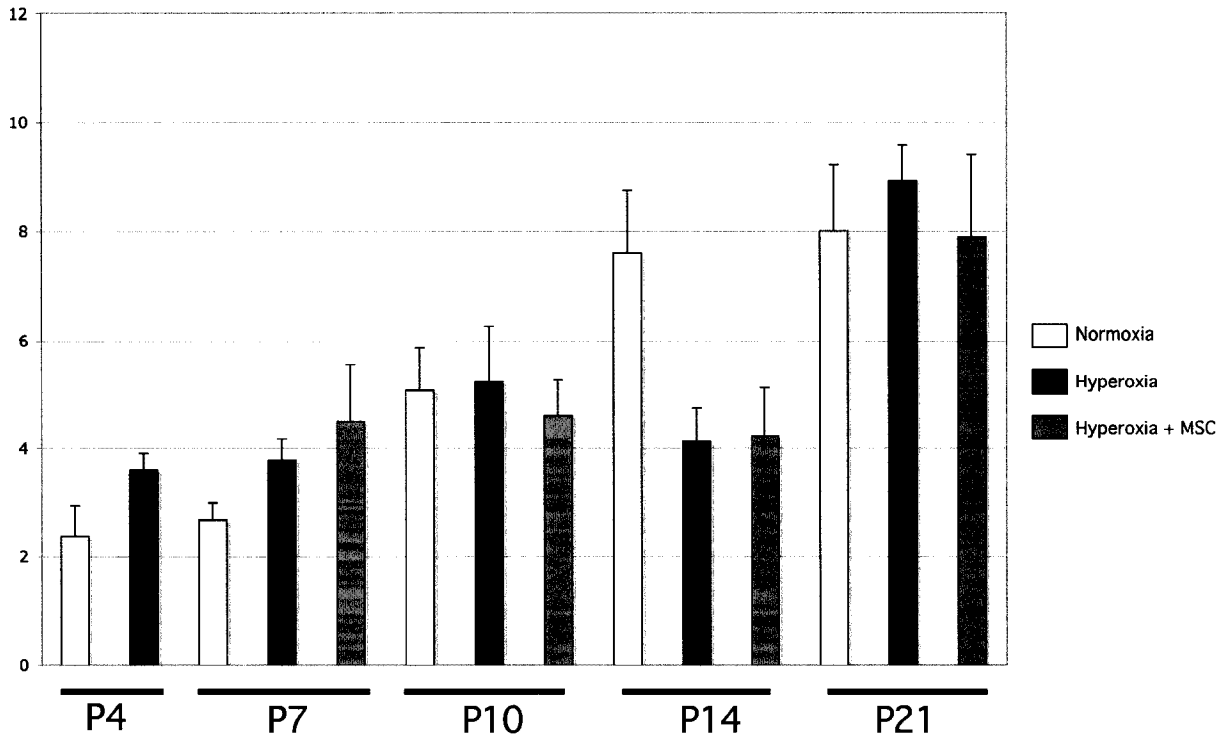


**Figure 3.17 RT-PCR Analysis of HGF mRNA Expression**
Temporal expression of HGF mRNA (normalized to 18S) did not reveal significant differences within mRNA expression between the three groups.

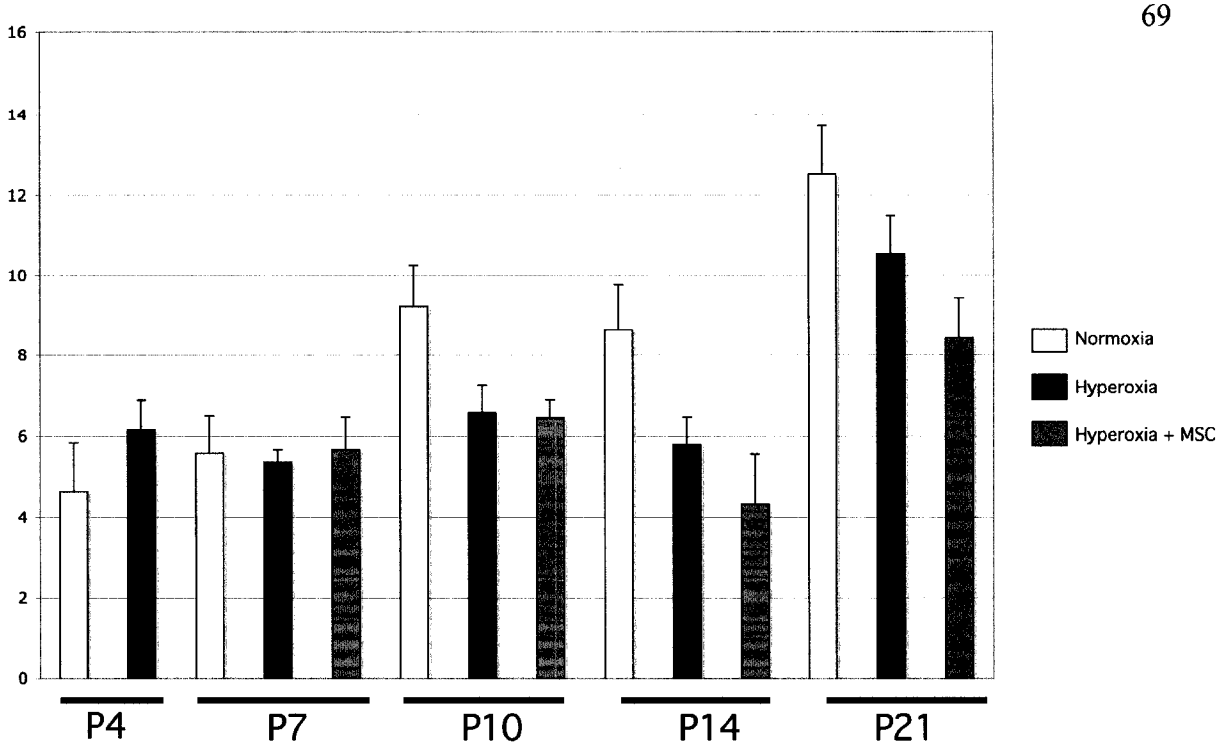


**Figure 3.18 RT-PCR Analysis of FGF7 (KGF) mRNA Expression**
Temporal expression of FGF7 (KGF) mRNA (normalized to 18S) did not reveal significant differences within mRNA expression between the three groups.

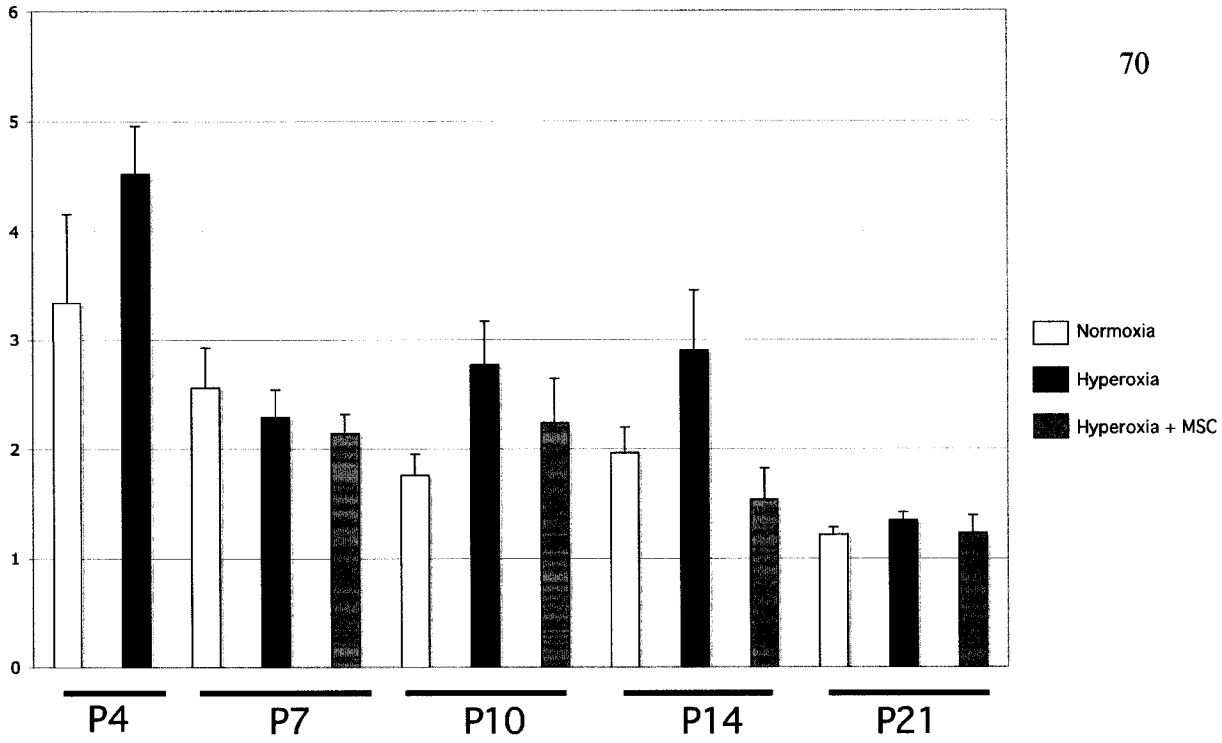


**Figure 3.19 RT-PCR Analysis of FGF10 mRNA Expression**
Temporal expression of FGF10 mRNA (normalized to 18S) did not reveal significant differences within mRNA expression between the three groups.

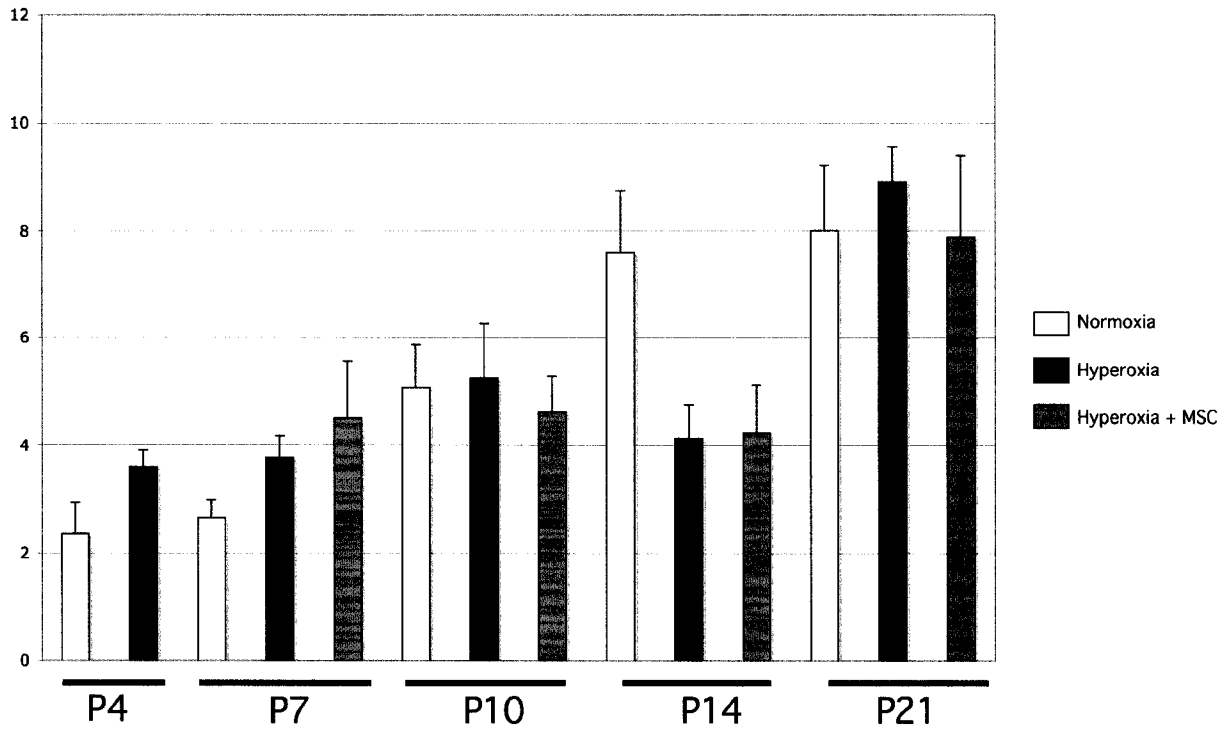


**Figure 3.20 RT-PCR Analysis of FGF18 mRNA Expression**
Temporal expression of FGF18 mRNA (normalized to 18S) did not reveal significant differences within mRNA expression between the three groups.

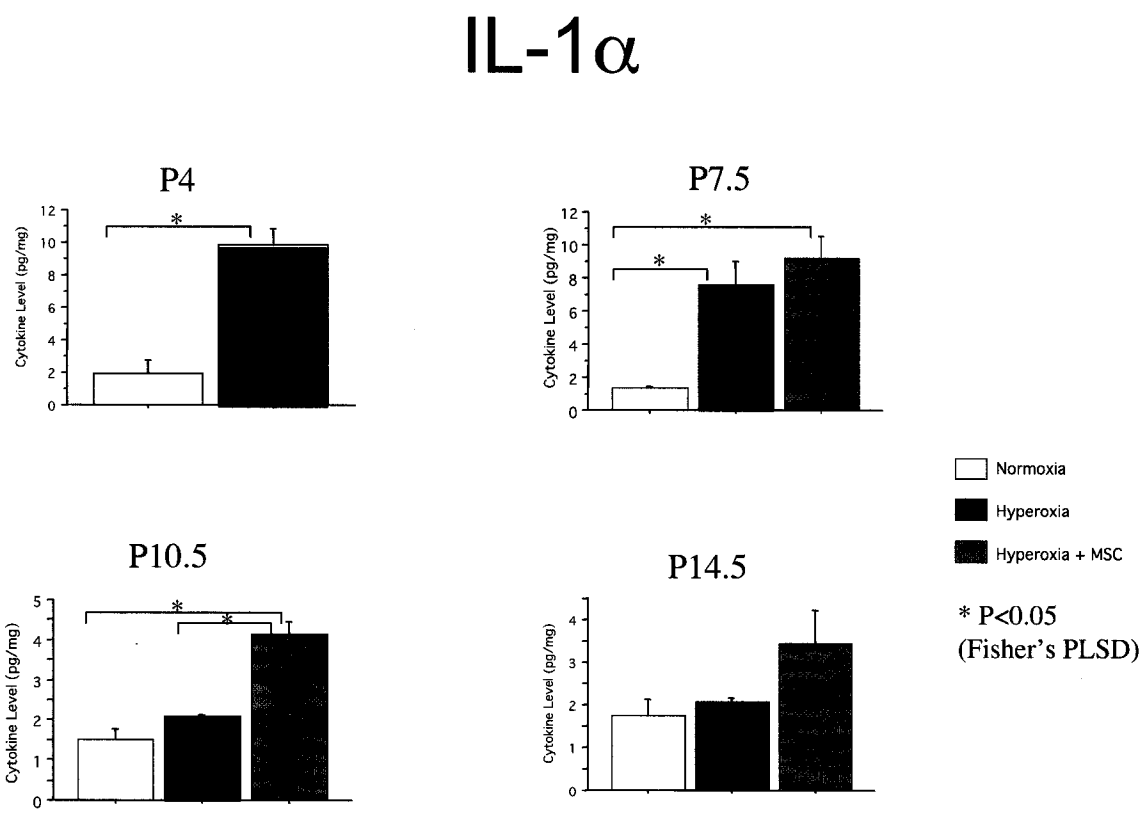


**Figure 3.21 Secreted Protein Concentrations of Il-1α**
Temporal expression of the secretion of the cytokine Il-1α reveals a significant increase in Il-1α in the hyperoxic group after only 4 days exposure to 95% oxygen. This increase was sustained through 7 days of hyperoxic exposure and returned to normal by day 10. Interestingly, Il-1α expression increased following MSC administration and this increase was maintained for the length of exposure to hyperoxia.

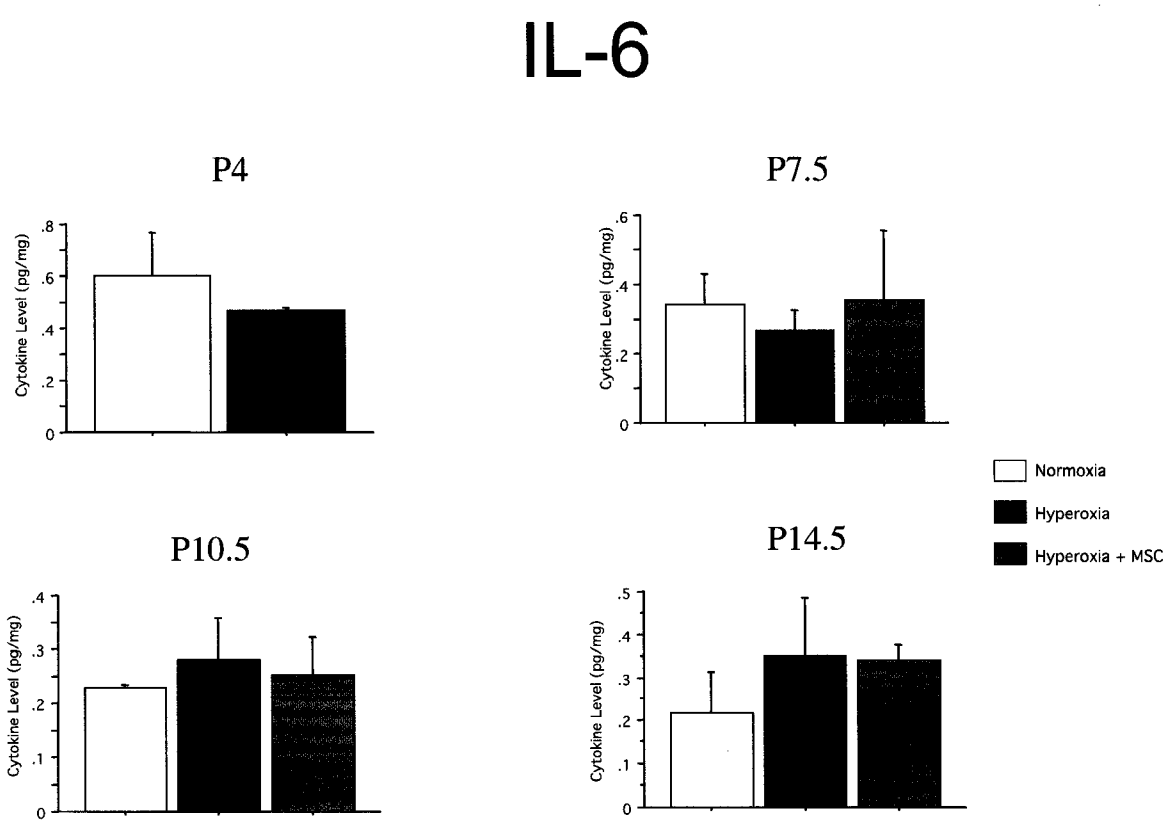


**Figure 3.22 Secreted Protein Concentrations of Il-6**
Temporal expression of secreted Il-6 protein concentrations did not reveal significant differences between the three groups.

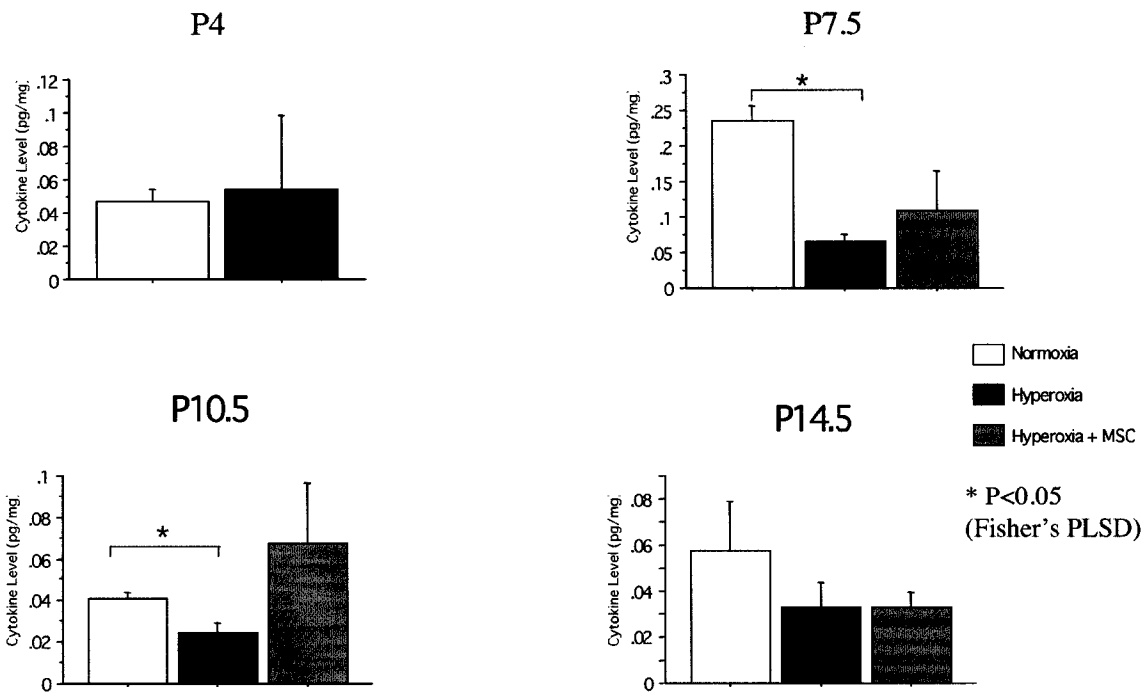


**Figure 3.23 Secreted Protein Concentrations of TNF-α**
Temporal expression of secreted TNF-α protein concentrations did not reveal significant differences between the three groups.

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# Chapter IV: Discussion

**4. Discussion**

In this report, we demonstrate that we are able to harvest bone marrow derived mesenchymal stem cells of the same phenotype and plasticity of those used by numerous other groups (Prockop 1997; Pittenger, Mackay et al. 1999; Orlic, Kajstura et al. 2001; Ortiz, Gambelli et al. 2003; Phinney and Isakova 2005; Dominici, Le Blanc et al. 2006). Also, we show that the circulating and lung resident MSC populations are significantly diminished following two weeks of 95% oxygen exposure. Furthermore, we are the first to utilize the intratracheal route of administration for MSCs to the injured lung. This therapeutic approach significantly improves survival, protects alveolar development, and ameliorates pulmonary hypertension in an experimental model of BPD. The IT administration of MSCs also improved the functional exercise capacity of our afflicted animals as compared to the diseased control animals.

Moreover, we report that IT MSCs engraft within the lung, comprising ~4% of the total cells within the distal air space when tracked by the cytoplasmic marker CFSE. Further, we demonstrate that the engrafted MSCs readily adopt the phenotype of AEC2s (75.3±24.5% of cells) *in vivo*. This *in vivo* plasticity is further supported by our work demonstrating that the MSC is able to express surfactant protein-C following exposure to the lung microenvironment in an *in vitro* modified boyden chamber. Taken together this is the first report to conclusively measure therapeutic benefit from MSC administration on lung structure, to show an improvement in exercise capacity, and to show that the MSCs are able to transdifferentiate into lung specific cells. Collectively, our findings indicate that the IT administration of MSCs is beneficial in lung pathologies characterized by alveolar destruction.

### 4.1 Selection of the Bone Marrow Mesenchymal Stem Cell

The selection of cell type for a preventative or regenerative therapy is as important as the selection of the correct drug therapy in any disease. However many stem cells lack *unique* surface markers, forcing reliance upon a panel of potential markers. Adding to the confusion was the lack of consensus on what was an acceptable panel of markers, with this only being resolved recently (Dominici, Le Blanc et al. 2006). Moreover, the nomenclature within the stem cell field is not clearly defined. Even within the bone marrow derived mesenchymal stem cells, one could argue that mesenchymal *progenitor* cell is a more accurate term.

To this end, we focused exclusively on the bone marrow MSC (instead of embryonic stem cells, side population cells, or hematopoietic stem cells) due to its relative ease of isolation (adherence on tissue culture plastic), its well defined differentiation into three different cell lineages (Pittenger, Mackay et al. 1999; Dominici, Le Blanc et al. 2006), and the promising initial reports of this cell's ability to "become" a lung cell and prevent further lung injury (Krause, Theise et al. 2001; Jiang, Jahagirdar et al. 2002; Theise, Henegariu et al. 2002; Abe, Lauby et al. 2003; Ortiz, Gambelli et al. 2003).

We were able to isolate, reproducibly, bone marrow stem cells that are inline with those previously defined in the literature. Our stem cells share the same cellular surface phenotype (CD31, CD34, CD45 negative and CD44, CD54, CD73, CD90 positive) as well as the ability to differentiate into adipocytes, chondrocytes, and osteoblasts when exposed to various culture conditions. Therefore, we are confident that the cells we harvested from the bone marrow are the same *mesenchymal stem cells* that were utilized by other groups in numerous models (Petersen, Bowen et al. 1999; Orlic, Kajstura et al. 2001; Kale, Karihaloo et al. 2003; Amado, Saliaris et al.

2005; Kajstura, Rota et al. 2005; Yoon, Wecker et al. 2005; Zhao, Courtman et al. 2005; Lee, Seo et al. 2006).

### 4.2 Hyperoxia Reduces the Number of Circulating and Lung Resident Mesenchymal Stem Cells

Previous studies have shown that chronic hypoxia can significantly alter the presence of both mesenchymal stem cells (Rochefort, Vaudin et al. 2005) and endothelial progenitor cells (Balasubramaniam, Mervis et al. 2007) in peripheral blood as well as within the lung. However, the effect of chronic hyperoxic exposure on these pools of MSCs has yet to be investigated.

Using the CFU-F assay, and subsequent confirmation of the presence of MSCs using cell surface markers and the ability for lineage differentiation, we show that these pools of MSCs are significantly blunted in both the circulating blood and within the lung itself. This finding alone raises an interesting question; what role are the circulating and lung resident MSCs playing in cellular repair following injury?

Indeed, studies that have reconstituted bone marrow following irradiation (Krause, Theise et al. 2001; Theise, Henegariu et al. 2002; Rojas, Xu et al. 2005) have shown that bone marrow cells do contribute to the production of cells within the lung. Therefore, this reduction of both circulating and lung resident MSCs may in part be responsible for the pathogenesis of BPD in this model as there are less cells available to help repair the lung following this chronic exposure to hyperoxia.

### 4.3 Mesenchymal Stem Cell Administration Unequivocally Prevents Alveolar Simplification in Experimental BPD

With this evidence that the pool of potentially repairative cells is severely diminished following two weeks of hyperoxic exposure, we set out to determine if supplementation of these MSCs directly to the damaged lung would prevent the progression of BPD.

The intratracheal route of administration of MSCs to the lung has yet to be exploited experimentally. This route is attractive for numerous reasons. Firstly, in the clinical setting, infants undergoing oxygen supplementation are also given surfactant via the trachea. Therefore, this is a clinically appealing route as one could potentially give the MSCs within a surfactant delivery system. Secondly, we are able to tightly control the dosage of cells given as well as ensure that they end up within the target organ (the lung). An intravenous strategy could potentially require a much higher dose of cells, and there is no way of controlling for the potential "wash out" effect of these cells taking residence within other organs (i.e. the liver, spleen, gut, heart).

Within this report, we unequivocally demonstrate that the IT administration of MSCs is able to preserve alveolarization. Previous reports have only demonstrated that either the MSC is contributing to the lung (Kotton, Ma et al. 2001; Krause, Theise et al. 2001) or that bone marrow stem cells were important in attenuating the features of a bleomycin induced fibrosis (Ortiz, Gambelli et al. 2003; Rojas, Xu et al. 2005). However, the effect of MSC administration, via *any* route, on alveolar health (using a technique like the mean linear intercept analysis, or the radial alveolar count) has yet to be reported. Our findings clearly show that the IT administration of MSCs preserves alveolarization during experimental BPD as quantified by the mean linear intercept.

This finding is important, as one of the current controversies within the literature is to what extent does the bone marrow MSC contribute to the lung following damage. While the

considerations already mentioned in the introduction (choice of cell type, route of administration, model of lung damage) must all be considered and have similar analyses performed, we are confident that the IT administration of MSCs in chronic hyperoxic exposure are able to significantly improve alveolarization.

Moreover, some recent studies have demonstrated the *failure* of bone marrow derived stem cells to repopulate the lung epithelium following injury (Wagers, Sherwood et al. 2002; Chang, Summer et al. 2005; Kotton, Fabian et al. 2005). However, as previously mentioned, we used a *different* cell type (the mesenchymal stem cell) than the ones utilized in these studies (the hematopoietic stem cell, unfractionated bone marrow, or the side population cell). We also used different models of injury and different routes of administration. With all of these confounding factors, it is not possible to directly compare between these studies. Each isolated cell population will have a different potential for engraftment and repair, just as each route of administration can drastically affect the dose of cells received, and how the injury model can affect cell recruitment and retention. We therefore cannot refute the claims of the failure these cells to reconstitute the lung epithelium in these studies (Wagers, Sherwood et al. 2002; Chang, Summer et al. 2005; Kotton, Fabian et al. 2005), as we focused on an entirely different cell population.

### 4.4 Mesenchymal Stem Cells Engraft into the Distal Lung

The controversies and confusion around the choice of cell type, route of administration, and injury model directly impact the reported engraftment potential of these cells. Wide ranging values, from zero engraftment to over 20% engraftment have all been reported (Kotton, Ma et al. 2001; Krause, Theise et al. 2001; Theise, Henegariu et al. 2002; Ortiz, Gambelli et al. 2003; Chang, Summer et al. 2005; Kotton, Fabian et al. 2005; Rojas, Xu et al. 2005).

Our initial results, utilizing the cytoplasmic marker CFSE, indicate that the engraftment potential of the MSCs is around 4%. This relatively modest engraftment potential cannot exclusively account for the robust affect on alveolarization that we demonstrate. From this we formed two potential explanations: 1. If the MSCs truly engrafted within the lung and formed cells within the lung that continued to divide within the lung as it matured, it is possible that we essential "bred out" the cytoplasmic staining through numerous cellular division and that our reported value does not reflect the true engraftment potential; 2. The engrafted MSCs indeed engraft at this relatively low level and have a beneficial effect on lung development through the induction of a paracrine effect.

To address the first hypothesis, we obtained transgenic rats that over-express green fluorescent protein (GFP) and administered bone marrow MSCs harvested from these animals. Our initial results indicate that indeed there may be a more robust engraftment than we originally thought, and that the injected MSCs actually contribute quite robustly to the architecture of the injured lung. Analysis of transdifferentiation and the cell types that the GFP-MSCs contribute to are on-going.

While our results with the GFP-MSCs indicate a structural improvement of the lung, we still investigated if the IT administration of MSCs induced a paracrine effect on the lung by screening for known factors important for lung growth and development. This is important as there is a growing body of literature that suggests that conditioned media from stem cells can have a similar effect on damaged organs as the injection of the stem cells themselves (Gnecchi, He et al. 2005; O'Neill, Wamhoff et al. 2005; Mazhari and Hare 2007). We focused on candidate growth factors that have been strongly implicated in lung development, with many of these already being experimentally exploited therapeutically. While our results do not clearly

demonstrate the induction of the factors we selected, there is still the possibility that a synergistic mechanism (of structural improvement along with increased growth factor production) still occurs with a potential factor that we did not investigate. The potential for modification of inflammatory cytokines, namely the sustained increase of interleukin 1-α warrants further study as well.

### 4.5 Mesenchymal Stem Cell Administration Reduces Pulmonary Hypertension, Attenuates Pulmonary Capillary Destruction, and Improves Exercise Capacity Following BPD

The use of MSCs for pulmonary hypertension is currently being investigated in depth by numerous groups; with very promising and provocative initial results (Zhao, Courtman et al. 2005; Frid, Brunetti et al. 2006; Kanki-Horimoto, Horimoto et al. 2006; Baber, Deng et al. 2007). These findings are important to our study as pulmonary hypertension is a significant complication of BPD (Abman 2001; Jobe and Bancalari 2001; Bland 2005; Coalson 2006).

Using echocardiography and directly measuring the degree of right ventricular hypertrophy (RVH; a surrogate marker of pulmonary hypertension) we conclusively demonstrate a significant improvement in the progression of pulmonary hypertension in experimental BPD following the IT MSC therapy. Moreover, utilizing barium angiograms with small animal computed tomography (CT) scanning as well as manual counting of pulmonary capillary density, we further show that the IT administration of MSCs is able to improve alveolar capillary formation (although the density does not return to normal).

Along with the improvement of alveolarization, this improvement in lung capillary density and the subsequent reduction of pulmonary hypertension, all lead to an increase in the

functional exercise capacity of our afflicted animals. This improvement is of particular interest as it demonstrates that not only do we have a structural benefit but also a functional improvement.

### 4.6 Mesenchymal Stem Cells are Able to Transdifferentiate into Type 2 Alveolar Epithelial Cells *In Vitro* and *In Vivo*

The ability of a stem cell to truly transdifferentiate into an epithelial cell is a very contentious issue. Numerous cell sources, markers of epithelial cells, and imaging modalities have all been utilized both *in vitro* and *in vivo* studies (Song and Tuan 2004; Sato, Araki et al. 2005; Kadivar, Khatami et al. 2006; Keilhoff, Goihl et al. 2006). Adding complexity to this debate is cellular fusion as a potential mechanism of action of stem cell transdifferentiation (reviewed in (Herzog and Krause 2006). Within the lung, numerous studies have shown that the administered bone marrow stem cells are able to form functional components of the alveolar epithlium *in vivo* based on the markers aquaporin-V and T1α (for type I AECs) (Kotton, Ma et al. 2001) or based on surfactant protein or morphological characteristics for type II AECs (Krause, Theise et al. 2001; Theise, Henegariu et al. 2002; Ortiz, Gambelli et al. 2003). *In vitro* the literature is even sparser, with only a single report demonstrating that MSCs, as a potential vehicle for delivery of the cystic fibrosis transmembrane regulator (CFTR) gene, can differentiate into type II AECs based on morphology and cytokeratin-18 expression (Wang, Bunnell et al. 2005).

Within our studies, we focused exclusively on surfactant protein-C (SP-C), a specific marker for the type II AEC. Our interest in the type II AEC is due to this cell being described as the putative progenitor cell within the distal lung (Mason, Williams et al. 1997; Warburton, Wuenschell et al. 1998). Therefore we speculated that we could supplement the endogenous

repair mechanisms within the lung by giving a non-committed stem cell that could "become" the tissue specific progenitor cell for the organ.

Our engraftment data with CFSE labeled MSCs indeed supports this theory. We see approximately 75% expression of SP-C co-localizing with the CFSE label suggesting that our engrafted stem cells are becoming type II AECs. However, more rigorous analysis of both the CFSE labeling (versus a genetic GFP label) as well as more detailed analysis of the other cell types within the distal lung (type I AECs, fibroblasts) is still on going. Moreover, our *in vitro* data also support the notion that our MSCs are able to transdifferentiate into type II AECs when exposed to the lung microenvironment in a modified boyden chamber. If true, this provides a very attractive and simple explanation for the benefit of MSC administration in BPD: administering a stem cell that will supplement the lung progenitor cell population and improve the health of the distal lung.

**4.7 Future Directions**

This research addresses some of the controversies in the literature, but raises numerous other questions as well. Questions that need to be immediately answered include:

1. To what extent do the GFP injected MSCs engraft into the lungs, and what lineage do the daughter cells become;
2. Do the GFP MSCs express the same plasticity in a modified boyden chamber as seen with the non-transgenic MSCs;
3. What role do the transcription factors TTF-1 and GATA-6 play in MSC transdifferentiation (Bruno, Korfhagen et al. 2000; Liu, Glasser et al. 2002);

4. What is the immediate, early engraftment and transdifferentiation of the MSCs (i.e. what happens to the MSCs 2 days post-injection?);

5. What effect on lung mechanics (compliance, elasticity, static recoil) does the MSC therapy have on the lung.

This research also provides increasing evidence for the use of MSCs as a potential vehicle to deliver gene therapy to the lungs. Furthermore, the exploration of MSCs to repair a damaged lung in an emphysema model is warranted. Moreover, the use of other potential therapeutic cells, such as the endothelial progenitor cell, are worth investigating. And lastly, it would be interesting to use a gadolidium-based marker to track the very early (5 minute, one hour) distribution of MSCs within the lung using magnetic resonance imaging (MRI).

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