University of Alberta

Epithelial and vascular progenitors in the developing lung: Newer insights and therapeutic implications

by

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Dedication

I dedicate this thesis to *my parents* for teaching me the following...

Seek, and ye shall find.

(Matthew 7:7, New Testament, *King James Version*)

…and my 18 month old *daughter Anenya* for showing me how to live that!

Abstract

Bronchopulmonary dysplasia (BPD) and congenital diaphragmatic hernia (CDH) are life-threatening lung diseases affecting newborn infants. Both diseases are characterized by impaired lung development and are currently untreatable. Dysregulation in the number or function of lung progenitor cells is one possible determinant of disrupted lung development. Stem cell augmentation is emerging as an appealing therapeutic strategy to promote lung growth. In fact, the integrated objective of the research presented in this thesis is to define the existence of resident progenitor cells in the distal lung and protect or supplement them to restore normal lung development.

This objective is addressed using two approaches. The first approach involves enhancing the survival of alveolar type 2 (AT2) pneumocytes, which are considered the 'repair cells' of the lung alveoli and believed to harbour alveolar precursor cells. Excessive AT2 cell apoptosis impairs alveolar development and results in BPD in newborn rats exposed to hyperoxia (95% O_2). Control of apoptosis in alveolar epithelial cells via overexpression of Akt (an intracellular prosurvival factor) protects hyperoxia-exposed rat pups from developing BPD. Our results constitute the framework for further therapeutic studies employing apoptosis control as a measure to prevent alveolar damage.

The next approach derives partly from existing evidence that pro-angiogenic factors promote alveolar growth. Here, we explore the correspondingly likely role of vascular progenitors in lung growth and maintenance. We hypothesized that endothelial colony forming cells (ECFCs,

recently recognized progenitors of the vascular endothelium) exist in the developing lung and impaired ECFC function underlies disrupted lung alveolar and vascular development. We found that ECFCs exist in the distal vasculature of developing human and rat lungs. Pulmonary vascular ECFCs isolated from newborn rats with hyperoxia-induced BPD or monocrotaline (MCT)-induced lung hypoplasia (simulating CDH-associated lung dysgenesis) expand less rapidly on culture, generate fewer colonies and form lesser vessel-like networks in Matrigel. Therapeutic supplementation with cord-blood ECFCs prevents BPD and restores alveolar structure in oxygen-exposed mice. ECFC treatment also attenuates MCT-induced PHT and preserves lung growth. Together, these observations suggest that ECFC supplementation represents a potential cell-based therapy for lung diseases characterized by impaired alveolar development. This research ushers in newer ideas that shall drive future research into evolving working remedies for BPD and CDH.

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List of Symbols, Abbreviations and Nomenclature

Symbol	*Definition*
Ac-LDL	Acetylated low density lipoprotein
AEC	Alveolar epithelial cell
ANOVA	Analysis of variance
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
ASC	Adult stem cell
AT1,2	Alveolar type 1, 2
BPD	Bronchopulmonary dysplasia
CDH	Congenital diaphragmatic hernia
CdM	Conditioned medium
CAC	Circulating angiogenic cell
EBM	Endothelial basal medium
cEGM2	Complete endothelial growth medium 2
CFU-EC	Colony forming unit-endothelial cell
COPD	Chronic obstructive pulmonary disease
DMEM	Dulbecco's Modified Eagle Medium
EC	Endothelial cell
ECFC	Endothelial colony forming cell
ECMO	Extra corporeal membrane oxygenation
eNOS	Endothelial NOS
ECM	Extra cellular matrix
EMAP-II	Endothelial monocyte activating polypeptide-II
EPC	Endothelial progenitor cell
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
GFP	Green fluorescence protein

hND-Fib	Human neonatal dermal fibroblasts
hUCB	Human umbilical cord blood
HUVEC	human umbilical vein endothelial cell
IGF	Insulin-like growth factor
iPSC	Induced pluripotent stem cell
i.t.	Intra tracheal
i.p.	Intra peritoneal
KGF	Keratinocyte growth factor
LSD	Least significant differences
LV-S	Left ventricle-Septum
MACS	Magnetic activated cell sorting
MCT	Monocrotaline
MLI	Mean linear intercept
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
MVD	Mean vessel density
MWT	Medial wall thickness
NICU	Neonatal intensive care unit
NOS	Nitric oxide synthase
nNOS	Neuronal NOS
PAT	Pulmonary artery acceleration time
PD	Postnatal day
PHT	Pulmonary hypertension
PPAR-γ	Peroxisome proliferator activated receptor-gamma
PPF	Pleuro peritoneal fold
PI3K	Phosphoinositol-3-kinase
PPHN	Persistent pulmonary hypertension of the newborn

PV-ECFC	Pulmonary vascular ECFC
PBS	Phosphate buffered saline
RDS	Respiratory distress syndrome
RAC	Radial alveolar count
r.p.m.	Rotations per minute
RVET	Right ventricular ejection time
RVH	Right ventricular hypertrophy
s.c.	Subcutaneous
SEM	Standard error of mean
TGF-β	Transforming growth factor-beta
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor

Chapter One: **Prolegomenon**

Explanatory Note:

This chapter was written by ARSA and edited by BT. Portions of this manuscript have already been published in the journals, Neonatology (2011; 99, 326–337), S. Karger AG, Basel, and Antioxidants & Redox Signaling (2012), Mary Ann Liebert, Inc., publishers.

1.1 Introductory remarks

Chronic lung diseases are expected to be the third leading cause of death, globally, by 2010 (http://www.who.int/healthinfo/global_burden_disease). Chornic obstructive pulmonary disease (COPD) tops this list (Gershon et al., 2011), posing a huge toll of $1.5 billion each year on hospitalizations, in Canada (Mittmann et al., 2008). But chronic lung diseases are no monopoly of adult patients! Newborn infants and children are also victims of debilitating lung diseases in increasingly large numbers. If not identified and treated early enough, these lung diseases become irrevocable and may affect life beyond childhood. Bronchopulmonary bysplasia (BPD) and congenital diaphragmatic hernia (CDH) are two major lung ailments affecting the neonatal population. Currently, there is no treatment for both conditions (Thebaud, 2011). The research presented in this thesis attempts to expand our understanding of these debilitating neonatal lung diseases and eventually suggests avenues for more effective treatment strategies.

BPD is a disruption of lung development. It primarily affects preterm neonates secondary to ventilator and O_2 therapy for acute respiratory failure (Kinsella et al., 2006b). It assumes significance in the wake of increasing preterm deliveries that occur at a rate of more than 12% of all births and account for more than 85% of all perinatal complications and death (http://iom.edu/Reports/2006/Preterm-Birth-Causes-Consequences-and-Prevention.aspx). Although surfactant therapy, antenatal steroids, and advances in perinatal care have modified the pattern of lung injury, BPD still persists as a common complication of extreme prematurity (Jobe, 2011; Thebaud and Abman,

2007a). CDH, on the other hand, is a common congenital malformation (1/2500 live births) (Colvin et al., 2005) with a very low rate of survival (~50%) (Stege et al., 2003). Even those who survive almost invariably present with pulmonary hypoplasia and varying degrees of pulmonary hypertension (PHT) (Abman, 2007). In fact, the associated pulmonary maldevelopment constitutes the key determinant of morbidity and mortality in CDH (Lally, 2002; van den Hout et al., 2009). Despite all the existing knowledge on the mechanisms contributing to lung injury (Jobe, 2011; Sluiter et al., 2011), it is unfortunate that modern medicine still lacks effective approaches to restore normal lung development in these infants.

The common denominator between BPD and CDH is impaired maturation and vascular paucity of the distal lungs (Thebaud, 2011). Promoting development of these growth-arrested lungs appears to be a logical and appealing therapeutic solution. In order to achieve this, a closer understanding of how the alveolar sacculi and the underlying capillary network develop and how these mechanisms are disrupted during disease processes, is critical. Recent advances in regenerative medicine and stem cell biology has offered us new tools and ideas for evolving such regenerative therapies for life-threatening chronic lung diseases. As set forth in the upcoming chapters, we have: (i) explored the role of an intracellular pro-survival pathway in lung alveolar regeneration (chapter 3), (ii) established the existence of endothelial colony forming cells (ECFCs), a recently discovered vascular progenitor cell phenotype, in the developing mammalian lung (Chapter

4) and (iii) investigated the potential role of these cells in lung development and repair (chapters 4 & 5).

1.2 General objectives

The unified objective of the research presented in this thesis is to understand the role and therapeutic potential of epithelial and vascular progenitor cells in the developing lung. We addressed this objective using two approaches:

i. Preventing apoptotic loss of alveolar type 2 (AT2) pneumocytes, which are believed to represent alveolar epithelial progenitors. This approach was assessed in an experimental model of oxygen-induced BPD.

ii. Establishing the presence of resident pulmonary vascular (PV)-ECFCs in the developing lung, investigating their role in disrupted alveolar growth and evaluating their potential to restore normal lung development. For these studies, experimental rodent models representing BPD and CDH-associated lung dysgenesis were employed. In BPD, the intent was to promote alveolar growth via enhancing ECFC-driven angiogenesis. In CDH, on the other hand, the primary lung abnormality is a restrictive pulmonary vascular bed that results in severe PHT. For this disease, the focus was to promote lung vascular growth and alleviate PHT, using ECFCs.

Our findings shall help evolve newer and more effective tools to combat disrupted alveolar and vascular development characterizing BPD and CDH-associated lung hypoplasia.

1.2.1 Lung regenerative potential of Akt pathway activation

Apoptosis is a fundamental feature contributing to organ morphogenesis. In the lung, cell-specific and temporally coordinated apoptosis contributes to

normal lung development. However, apoptosis occurring above physiologic levels in response to injury (hyperoxia, for example) during the critical alveolar stage of lung development may contribute to impaired alveolar architecture. In fact, several reports have implicated elevated alveolar epithelial cell apoptosis in the pathogenesis of BPD. Among the alveolar epithelial cells, alveolar type (AT)2 pneumocytes are capable of proliferating in response to injury and are believed to harbor the regenerative stem cell reservoir of the distal lung. In the light of this knowledge, we hypothesized that control of apoptotic AT2 cell death via activation of the prosurvival Akt pathway would prevent arrested alveolar development. In chapter 3, the aim was to test this hypothesis using the established newborn rat model of hyperoxia (95% O_2)-induced experimental BPD. Our results indicate that pharmacologic inhibition of Akt impairs alveolar development in newborn rats. Conversely, expression of activated Akt is decreased in the lungs of rats with O_2-induced BPD. When alveolar epithelial cells are made to overexpress a constitutively active form of Akt, they undergo decreased apoptosis under hyperoxia and this in turn protects hyperoxia-exposed rat pups from developing BPD-like changes in their lungs. Our data corroborate previous reports and further establish a framework to clarify the role of Akt-mediated regulation of cell survival in the distal lung epithelium. In addition, accommodating the current lack of dependable techniques to identify and study the actual alveolar epithelial progenitors, our findings suggest that manipulation of apoptosis in AT2 cells might have the therapeutic potential to prevent lung injury.

1.2.2 Existence and role of ECFCs in the developing lung

In both BPD and CDH-associated lung hypoplasia, stunted alveolar development is consistently associated with a marked rarefaction of distal lung vascularity. Extensive research in the last decade clearly indicates that the developing pulmonary vasculature has an important regulatory role in airway morphogenesis and alveolar maturation in mammals. Proangiogenic growth factors promote alveolar formation and preserve lung development in experimental models of alveolar growth arrest. This gave us the logical question: If angiogenic growth factors and the lung vasculature contribute to lung integrity, is it not likely for vascular progenitor cells to be involved in the same mechanisms? In order to address this question we framed our two step hypothesis. First, we suggested that a 'true' vascular progenitor cell population exists in the developing lung. Second, we assumed that the functional impairment of these cells, at least in part, underlies the pathogenesis of arrested alveolar development. ECFCs being the currently accepted 'true' vascular progenitors, we decided to address the first step of our hypothesis by examining the presence of these cells in the developing lung. The outcomes of this investigation are presented in chapters 4 and 6. Our results demonstrate that ECFCs exist in the distal vasculature of developing human and rat lungs. In the next step, we examined the functional capacity of PV-ECFCs in developmentally arrested lungs obtained from rats with experimentally induced BPD or lung hypoplasia (modeling CDH-associated pulmonary dysgenesis). These results displayed in chapters 4 and 5 indicate that

the *in vitro* survival, colony forming and vessel forming potentials of PV-ECFCs are impaired in both disease models. Our findings indicate that impaired ECFC functionality could, at least in part, underlie arrested alveolar growth in BPD and CDH-associated lung hypoplasia. They also suggest a potential treatment via therapeutic supplementation of endogenously derived ECFCs.

1.2.3 The potential of ECFC supplementation to restore normal lung development

Existing reports show that therapeutic mobilization or supplementation of angiogenesis-promoting cells (of endogenous or exogenous origin, respectively) have been successful in treating experimentally-induced lung injury. Prompted by these reports and our previous observations, we framed our hypothesis that ECFC supplementation will restore disrupted lung alveolar and vascular growth in experimental BPD and CDH-associated lung hypoplasia. Outcomes of these studies presented in chapters 4 demonstrate that therapeutic administration of human cord-blood derived (hUCB)-ECFCs or its conditioned media restores alveolar development and attenuates PHT in newborn mice exposed to hyperoxic damage. Similar therapeutic improvement is also observed in newborn rats subject to monocrotaline mediated lung hypoplasia/PHT, and the results are presented in chapter 5. Our long-term (10 month) follow-up of ECFC-treated BPD mice demonstrate persistent therapeutic benefit with no detectable adverse reactions. Together, these observations suggest that ECFC supplementation represents a potential cell based therapy for lung diseases characterized by disrupted alveolar development.

The current chapter introduces the essential elements of lung organogenesis with emphasis on lung vascular development. It also highlights how these processes are disrupted in BPD and CDH-associated lung dysgenesis. The fundamental concepts about stem cells are introduced following which the reader is provided with an overview of the existing knowledge on vascular/endothelial progenitor cells and their role in lung injury and regeneration. This shall offer the framework to further elaborate the background, hypothesis and findings of our research presented in the upcoming chapters.

1.3 Lung development- a pulmonary vascular perspective

1.3.1 Stages of lung organogenesis

A generally accepted view of how the lung develops is summarized in Figure 1-1. In the embryonic stage, the lung appears as an outpouching of the primitive foregut on day 28. This bud bifurcates into the 2 main stem bronchi. During the pseudoglandular stage, rudimentary bronchi divide by dichotomous branching and culminate in the first terminal sacs. These tubular structures are lined by columnar epithelium surrounded by mesenchymal tissue. The canalicular stage is characterized by the bifurcation of the last generations of distal bronchi. In this stage, there is also capillary invasion and differentiation of the air space epithelium into alveolar type 2 (AT2) cells (responsible for surfactant production) and alveolar type 1 (AT1) cells (which form the thin air-blood barriers). During the saccular stage the peripheral air spaces enlarge at the expense of the intervening mesenchyme, forming saccules. This is followed by the final alveolar stage when alveoli are generated via subdivision (septation) of the distal lung saccules (Bourbon et al., 2005). This process of alveologenesis begins at 36 wk gestation and extends into the first 1.5 years of post natal life.

1.3.2 Pulmonary vascular development

In the developed lung, pulmonary arteries run alongside the branching airways carrying blood to the alveolar capillary bed. The pulmonary veins, with a comparable number of tributaries, return blood from the capillary bed to the heart. The veins are separated from the airways by the alveolar region. The close

proximity of the circulation to the airway system suggests a close interaction between them both, during development and beyond (Hislop, 2005). This section highlights the key events in lung vascular development with special focus on the distal pulmonary vasculature. The next section summarizes the key regulatory steps involved in this process and briefs our current knowledge on how they are perturbed in disease state.

Pulmonary vascularization is dependent on two processes- vasculogenesis and angiogenesis. Vasculogenesis is the *de novo* formation of blood vessels from angioblasts or endothelial precursor cells that migrate and differentiate in response to local cues (growth factors, extra-cellular matrix etc.) to form vascular tubes. Angiogenesis, on the other hand, is the formation of new blood vessels from pre-existing ones (Patel-Hett and D'Amore, 2011). Initially, vasculogenesis results from the *de novo* formation of blood vessels from the blood lakes present in the mesenchyme surrounding the embryonic lung. Endothelial cell precursors have been identified without a lumen in the splanchnic mesenchyme around foregut and lung bud at 4 weeks gestation (Hall et al., 2000b). At 7 weeks, a capillary network is observed around each prospective main bronchus, connecting cranially with the aortic sac and caudally with the left atruim (Hall et al., 2002). A similar appearance occurs in mouse embryos at 10.5 days' gestation (Schachtner et al., 2000). As the airways divide towards the periphery, the capillaries coalease to form small blood vessels alongside the airways. Thus there is sustained addition of newly formed tubules to the existing vessels until the end of the pseudoglandular stage of lung development (17th week gestation in humans).

Although the developing airways appear to constitute the template for this process (Hall et al., 2000b), it is still not clear if the branching airways dictate the extension of vasculature or vice versa. In the canalicular stage, further division of the airways to form respiratrory bronchiles and sac-shaped alveolar ducts is accompanied by extensive growth of capillaries derived by angiogenesis (Hall et al., 2002). By this time, capillaries in the mesenchyme move to lie under the epithelium lined by AT1 and AT2 pneumocytes. At about 24 weeks gestation, these structures produce the alveolar-capillary gas exchange barrier with thickness comparable to that of the adult lung.

1.3.2.1 Microvascular maturation

The final and most important step of lung vascular development is the distal lung microvascular maturation, which overlaps with the alveolar stage of lung development (Burri, 1997). At the time of alveolar formation, immature inter-airspace walls are constituted by two capillary layers with a central sheet of connective tissue. During and after alveolarization, the septa with double capillary networks are restructured into the mature form, with a single network. This process gets completed in rats by the third week of post-natal life. From birth to adulthood, the alveolar and capillary surface areas expand nearly 20-fold and the capillary volume by 35 fold. Capillary expansion during this developmental stage occurs by sprouting angiogenesis and intussusceptive growth (Djonov et al., 2000). The latter involves internal division of preexisting capillary plexus via insertion of transcapillary pillars, without sprouting. However, much more needs

to be learned about intussusceptive growth of new vessels, which may potentially underlie alveolar development and remodeling throughout postnatal life.

1.3.2.2 Vessel wall development

The newly formed pulmonary arteries begin to acquire the muscular laminae in their walls as early as 38th gestational day. Initially, they are formed from smooth muscle cells that migrate from the adjacent bronchi. As the arteries increase in size, the thickness of the muscular lamina increases correspondingly with muscle cells derived from the surrounding mesenchyme. Elastic laminae and collagen are laid down between the muscle cells resulting in a mature arterial appearance (Hall et al., 2000b). Interestingly, endothelial cells have been posited as a third source of smooth muscle cells. Implying this possibility, capillary endothelial cells stain positive for α-smooth muscle actin. However, the factors controlling these processes are not very clear and their potential importance in alveolar development and remodeling remains to be resolved. Veins, on the other hand, are relatively thin walled and are believed to derive their muscle lining from mesenchymal fibroblasts and endothelial cells. The smooth muscle lining of the arteries and veins show progressive and gradual maturation that continues into early postnatal life (Hall et al., 2002).

1.3.2.3 Postnatal modification

During fetal life, the resistance to pulmonary blood flow is very high with only 10% of the right ventricular output passing through the lungs. At birth, a drastic cardiopulmonary transition occurs as the lung assumes vital gas exchange

function for the first time. This transition involves a rapid fall in pulmonary vascular resistance and pulmonary arterial pressure, and a dramatic rise in pulmonary blood flow. Cytoskeletal reorganization of the smooth muscle and endothelial cells contributes to thinner walls and wider lumen in the pulmonary vasculature undergoing transition (Haworth, 2002). In addition, a transient disassembly of actin leading to changes in the contractile myofilaments of the smooth muscle cells have been observed during the first two weeks of life (Hall et al., 2000a). However, as the cells undergo maturation, an increase in myofilament density is observed. Disrupted distal lung development may culminate in failure of the normal cardiopulmonary transition at birth. For instance, this results in a clinically challenging condition termed persistent pulmonary hypertension of the newborn (PPHN) in CDH survivors (Steinhorn, 2010).

1.3.3 Regulation of pulmonary vascular development

In contrast to the extensive information available on airway growth and development (Kimura and Deutsch, 2007; Warburton et al., 2010), we know very little about the regulation of lung vascularization. However, it is apparent that the developmental processes of epithelial, mesenchymal and vascular compartments are highly interconnected. The key regulators of lung vascular development with particular relevance to pulmonary microvasculature are summarized in this section (For a detailed review on this topic see (Hislop, 2005; Hislop and Pierce, 2000)).

During lung development, formation of new capillaries by vasculognesis occurs consistently at a similar distance from the sprouting epithelial buds, suggesting a putative control by epithelial cells (Hislop, 2005). Vascular endothelial growth factor (VEGF), a chief regulator of blood vessel growth, and its receptors are expressed in the human embryo from as early as 38 days' gestation (Acarregui et al., 1999). Interestingly, epithelial cells and myocytes express the ligand while the endothelial cells express its receptors. This spatially coordinated expression of VEGF and its receptors suggests a paracrine orchestration of vessel formation in the developing fetal tissues including the lung (Shifren et al., 1994). Assembly and stabilization of the formed vessels are dependent on factors such as tyrosine kinase receptor Tie2 and its ligand angiopoietin (Sato et al., 1995). Insulin like growth factor (IGF) and its receptors have been colocalized with endothelial markers, as early as 4 weeks gestation in human lungs explants. Blockade of IGF receptors results in abnormal growth of lung buds and diminished formation of endothelial tubules, suggesting the role of IGF in airway and blood vessel development (Warburton et al., 2000). In addition to peptide angiogenic growth factors, recent studies have revealed the importance of the gaseous transmitter nitric oxide (NO) in fetal lung vascular development and lung morphogenesis (Han and Stewart, 2006). Infact, endothelial nitric oxide synthase (eNOS), an endothelial-cell-specific isoform of NO producing enzyme is expressed in the vascular endothelium throughout development. Nevertheless, with all the ongoing research on the factors controlling lung vessel growth, a lot of questions remain unresolved. For instance, what cues distinguish and guide the

structural and anatomical differences between arteries and veins during early stages of lung vessel development? No precise knowledge exists, although it has been speculated that phenotypic differences between endothelial cells (Gale and Yancopoulos, 1999) and smooth muscle cells derived from the bronchial wall might play a role.

The distal lung microvasculature, alongside the differences in structural characteristics, also displays an apparently distinct mechanism of regulation. In the pseudoglandular stage of lung development, as early as 17 weeks gestation in humans, supernumerary arteries and veins are known to exist in addition to the pulmonary arteries alongside each developing airway. These supernumerary vessels are the ones that will be associated with the alveoli that are not developed by this time. This observation suggests a putative genetic influence on blood vessel development (Hislop and Pierce, 2000). During the canalicular stage, the capillaries come to life much close to the epithelium. Although it is not clear what attracts the capillaries to underlie the distal epithelium, it is known that they influence the epithelium to thin and promote the differentiation of AT1 and AT2 pneumocytes (Adamson and Young, 1996). Evidence also indicates that stretch activates myogenic differentiation and increased VEGF expression in vascular smooth muscle cells, allowing increased endothelial cell motility and capillary numbers (Smith et al., 2001). Moveover, the interaction of arteries with airways continues into alveolar development. For example, secondary crest formation is always accompanied by a capillary loop derived from the primary septae (Zeltner and Burri, 1987). Increased Flk-1 (VEGF receptor) expression in the lungs of

mice during perinatal life is another example (Bhatt et al., 2000). Flk-1 inhibition results in reduced arterial and alveolar numbers in rats (Jakkula et al., 2000). Reversal of VEGF-inhibitor induced impaired lung growth and PHT by inhaled NO is one more instance demonstrating the interconnectedness between the developmental processes of distal lung epithelial and endothelial compartments (Tang et al., 2004).

Much more needs to be learned about the molecular and genetic events underlying lung vascular development and time-specific mechanisms that regulate growth and function at each stage. Likewise, in the advent of recent knowledge on the role of endothelial precursor cells such as endothelial progenitor cells (EPCs) in lung development (Borghesi et al., 2010), more needs to be known about the role of these cells and their lineal relationships in embryonic and postnatal lung development.

1.4 Abnormal pulmonary vascular development

Abnormalities in lung vasculature may be a result of abnormal development of the vascular tree in utero, the branching pattern or the structure of the vessel wall. In BPD, extreme prematurity surmounted by perinatal injury results in reduction of alveolar formation with abnormal structure and decreased vascularity (Jobe, 1999). In CDH, on the other hand, there is equal reduction in arteries and airway numbers and consequenty a reduction in alveolar number (H and Tibboel, 1998). The following section defines and introduces the commonly suggested mechanisms contributing to the pathogenesis of BPD and CDH. Existing knowledge on the various factors potentially underlying abnormal vascular development in these conditions are emphasized.

1.4.1 Bronchopulmonary dysplasia

1.4.1.1 Definition:

According to the earlier descriptions, BPD was defined as a disorder occurring in relatively larger preterm infants (around 34 wks gestation, 2200 g birth wt) (Northway et al., 1967) ventilated for neonatal respiratory distress. It included mucosal metaplasia of airways, emphysema and interstitial fibrosis ("old BPD") (Cerny et al., 2008; Northway et al., 1967). Fortunately, surfactant therapy, antenatal steroids, and improved perinatal care have almost eradicated the incidence of BPD in these larger preemies. However, thanks to incremental advances in neonatal intensive care, premature babies become viable as early as 24 wks of gestation (500-1500 g) (Bourbon et al., 2005). Lungs of these

extremely premature infants are in their late canalicular or early saccular stage of development where inadequate capillary development and alveologenesis preclude sufficient gas-exchange to support independent survival. BPD in these infants (termed “new BPD”) primarily involves fewer and poorly vascularized alveoli, with a reduced frequency of airway injury (Coalson, 2003; Rojas et al., 1995).

1.4.1.2 Overview of pathogenesis:

BPD is a multifactorial disease with several pre and postnatal factors contributing to its pathogenesis. Postnatal oxygen exposure in the setting of decreased host antioxidant defenses, barotrauma/volutrauma on a very immature lung, chorioamnionitis and preeclampsia contribute to BPD (For more extensive coverage on this toipc, see (Bourbon et al., 2005; Gien and Kinsella, 2011; Hansen et al., 2010; Jobe, 2003). Briefly, the pathophysiological processes underlying the development and progression of BPD can be broadly compartmentalized into three interdependent mechanisms (Figure 1-2):

i. *Defective elastogenesis and extracellular matrix remodeling:* Elastin deposition in the saccular walls of the developing alveoli plays a spatially instructive role in the budding of secondary septa and formation of mature alveoli. Lungs of mice devoid of the elastin gene fail to progress beyond the saccular stage and usually die soon after birth from cardiorespiratory failure resulting from fewer, dilated distal air sacs with attenuated septae (Wendel et al., 2000) and an overgrowth of smooth muscle cells in the pulmonary arteries (Li et al., 1998). Exposure of newborn mice to

mechanical ventilation in conjunction with hyperoxia, mimicking intensive care interventions in the NICU, increases lung elastase activity, causing elastin degradation and redistribution of elastic fibers from septal tips to alveolar walls (Bland et al., 2008). These "BPD like" changes are significantly attenuated by blocking lung protease activity with intrapulmonary injections of recombinant human elafin (Hilgendorff et al., 2011). Alveolar septation also involves the deposition of other ECM components, including collagens and proteoglycans. Marked distortion of collagen architecture is observed in infants with BPD, with thickened, tortuous, and disorganized fibers (Thibeault et al., 2003). Disrupted ECM remodeling attributable to dysregulation in the levels and activity of gelatinases such as matrix metalloproteinase (MMP)-2 and -9 is another suggested mechanism of impaired lung development (Buckley and Warburton, 2002; Danan et al., 2002; Ekekezie et al., 2004).

ii. *Altered alveolar epithelial growth and epithelial-mesodermal interactions:* AT2 cells are critical candidates in alveolar growth and maintenance (Dobbs et al., 2010; Mason, 2006). During distal lung development, spatio-temporally coordinated cell-cell interactions of the alveolar epithelium with the mesoderm are crucial for the formation of a functional alveolar architecture (Bourbon et al., 2005). The importance of signals originating from differentiated epithelial cells in alveolar septation is evidenced by the need for optimal expression of transcription factors Foxa2 (Wan et al., 2004), GATA6 (Liu et al., 2002) and the AT1 cell

differentiation marker, T1α (Ramirez et al., 2003). Keratinocyte growth factor (KGF), one of the key regulators of alveolar maturation, is released by alveolar fibroblasts (Ulich et al., 1994). Paucity in KGF levels has been directly correlated with susceptibility for BPD (Bourbon et al., 2005). Stretch-mediated surfactant synthesis in the developing lung is also regulated by a parathyroid hormone related peptide-dependent paracrine loop between AT2 cells and alveolar lipofibroblasts (Schultz et al., 2002). Sustaining this paracrine pathway using peroxisome proliferator activated receptor-gamma (PPAR-γ) agonists has been effective in preventing oxygen, volutrauma or infection mediated injury in the developing lung (Torday and Rehan, 2007).

iii. *Impaired development of lung microvasculature:* In addition to defective alveolar formation, BPD is characterized by a pronounced paucity of distal lung microvasculature (Bhatt et al., 2001; Gien and Kinsella, 2011). It is highly likely for a vascular component to underlie, at least in part, the pathobiology of BPD (Stenmark and Abman, 2005). Being the primary focus of the research constituting this thesis, this mechanism is elaborated in greater detail in the upcoming section.

1.4.1.3 Pulmonary vascular abnormalities in BPD:

The interdependence between alveolar formation and angiogenesis is being increasingly recognized thanks to several observations and studies done over the past decade (Thebaud and Abman, 2007b). It is also clear that secondary abnormalities almost invariably occur in one process when the other is primarily

affected (Jakkula et al., 2000; McGrath-Morrow et al., 2005). Disrupted interactions between several pro and antiangiogenic mediators could potentially be responsible for the observed vascular alterations in BPD (Bourbon et al., 2005).

When exposed to hyperoxia or prolonged mechanical ventilation, the developing lung almost invariably suffers varying degrees of microvascular disruption in conjunction with alveolar damage. It is very likely that alterations in angiogenic growth factors might underlie this process (D'Angio and Maniscalco, 2002). Development of lung vasculature is chiefly controlled by a well-coordinated interaction of angiogenic growth factors and their receptors in concert with the ECM. Expression of proangiogenic growth factors such as VEGF and angiopoietin (Ang)-1 & 2 are observable very early during lung organogenesis (Han and Stewart, 2006; Hato et al., 2009). It is very clear that VEGF signaling plays a crucial role in the development of lung microvasculature and in the postnatal growth and maintenance of alveolar structure (Thebaud and Abman, 2007b). Even brief treatment with genetic or pharmacologic VEGF inhibitors during the critical period of lung development in newborn rodents results in fewer and larger alveoli and capillary rarefaction in distal lungs (Galambos et al., 2002; Jakkula et al., 2000; McGrath-Morrow et al., 2005; Thebaud et al., 2005). Conversely, strategies that enhance microvascular integrity via mitigating hyperoxia-induced alterations in lung VEGF levels significantly improve the outcome of alveolar injury (Kunig et al., 2005; Thebaud et al., 2005). Angiopoietins, on the other hand, enhance the response of endothelial cells to

VEGF, promoting vascular stabilization and maturation (Thomas and Augustin, 2009). It is believed that the regulation of Tie2 signaling through a balance in the expression of Ang1 and Ang2 is crucial for normal lung vascular development (Hato et al., 2009; van der Heijden et al., 2009). Studies also indicate that Ang-1 contributes to lung protection via attenuating inflammation (Hegeman et al., 2010; McCarter et al., 2007) and supporting pulmonary vascular homeostasis (Kugathasan et al., 2009). In addition, transcription factors such as hypoxia inducible factor (HIF) 1/2, activation protein-1, and Sp-1 play a role in regulating angiogenic growth factor expression (Josko and Mazurek, 2004). Animals deficient in HIF-2α exhibited defective lung morphogenesis accompanied by neonatal respiratory distress due a selective defect in pneumocyte maturation and surfactant production (Compernolle et al., 2002).

A decade of research on pulmonary vascular development has shed new light on the critical role of NO and NO synthases (NOS) in this process as well as their contribution to the genesis of BPD (Han and Stewart, 2006). Constitutive expression of endothelial and neuronal NOSs (eNOS & nNOS, respectively) at strategic locations in the developing lung suggests a contributory role of these enzymes in airway branching morphogenesis (Young et al., 2002). Genetically modified mice deficient in eNOS demonstrate severely impaired compensatory lung growth, indicating that eNOS is probably critical for alveolar regeneration and maintenance (Leuwerke et al., 2002). Even mild exposure to hypoxia during the early postnatal lung development impairs alveolarization and reduces vessel density in these mice (Balasubramaniam et al., 2003). The primary function of

NOS enzymes is the time-dependant synthesis and release of NO, a critical downstream mediator of several potent angiogenic growth factors (Han and Stewart, 2006; Sessa, 2009). It is also believed that NO might act upstream of angiogenic growth factors via transcriptional control of their expression (Dulak and Jozkowicz, 2003). Preterm baboons with ventilation-induced BPD demonstrate marked decline in pulmonary NOS expression indicative of an attenuated capacity for endogenous NO production in this disease state (Afshar et al., 2003). On the other hand, evidence exists that exogenous supplementation of NO preserves alveolar structure and lung vascular remodeling to varying extents in experimental models of hyperoxia (Lin et al., 2005), bleomycin (Tourneux et al., 2009) and mechanical ventilation-induced BPD (Bland et al., 2005). Given that VEGF-induced angiogenesis is in part mediated by NO (Papapetropoulos et al., 1997), alveolar arrest and pulmonary hypertension induced in neonatal rats by VEGF receptor blockade are effectively reversed by inhaled NO treatment (Tang et al., 2004). Potentiating the effect of endogenous NO via prolonging the half-life of its downstream mediator cyclic guanosine monophosphate also promotes lung angiogenesis and attenuates oxygen-induced lung injury (Ladha et al., 2005). Collectively, these findings explain the beneficial effects of early and prolonged, low-dose, inhaled NO observed in three recent, randomized controlled trials to prevent BPD (Ballard et al., 2006; Kinsella et al., 2006a; Schreiber et al., 2003). Nonetheless, further analyses on the role of endogenous NOSs, VEGF, the interplay of NO and superoxide, and protein nitration with concurrent exogenous

NO administration is required to obtain a closer understanding of effects of inhaled NO in the newborn lung (Rieger-Fackeldey and Hentschel, 2008).

Contrastingly, evidence from the developing lung indicates that proangiogenic pathways and mediators are opposed and held in a temporally orchestrated balance by factors that negatively influence new blood vessel formation (Quintos-Alagheband et al., 2004). These anti-angiogenic factors are candidates likely to play a contributory role in BPD evolution (Quintos-Alagheband et al., 2004). Endothelial monocyte activating polypeptide-II (EMAP-II) is one such negative regulator of vessel formation(Berger et al., 2000), the expression of which is negatively correlated with vascularization in the developing lung (Schwarz et al., 1999). EMAP II expression appears elevated in the lung tissue of infants with BPD as well as in the premature baboon model (Quintos-Alagheband et al., 2004), suggesting that this protein may contribute to the interruption of vascular development in BPD. Endostatin is another endogenous anti-angiogenic protein known to inhibit endothelial proliferation and migration in tumour tissue, in addition to promoting apoptosis and predisposing G1 arrest of endothelial cells. Endostatin also antagonizes VEGF receptor 2 activation and interferes with cell-matrix interactions that are necessary during angiogenesis (Ribatti, 2009). Interestingly, a study measuring endostatin levels in the cord plasma of preterm neonates weighing less than 1500 g reports a positive correlation between high concentrations of endostatin in cord plasma and the development of BPD (Janer et al., 2009). More interestingly, ventilated preterm infants who developed BPD or died, had a decreased angiopoietin-1/endostatin

ratio on the 1st, 3rd and 15th days of life, suggesting an imbalance toward inhibition of pulmonary angiogenesis (Thomas et al., 2009).

In brief, it appears convincing that the arrested and dysmorphic lung vascular growth in BPD results at least partly from altered signaling of angiogenic factors, their receptors, and the impaired funtioning of NO synthases. With the expanding knowledge on the biology and role of EPCs in new vessel formation, it seems equally convincing that these cells might also contribute to the disease process. Existing evidence for the potential role and therapeutic usefulness of EPCs in BPD has been elaborated in later sections of this chapter.

1.4.2 Congenital diaphragmatic hernia

1.4.2.1 Definition:

Congenital diaphragmatic hernia (CDH) is a complex congenital malformation of the thoracic cavity that includes two basic components- a diaphragmatic defect and developmental dysgenesis of the lungs. The defect in diaphragm development allows intra-abdominal viscera to enter the fetal chest cavity during the early stages of lung development. The resultant encroachment by the abdominal viscera on the developing lung buds is believed to cause altered lung growth and maturation. As a result, infants with CDH are afflicted with smaller-than-normal lungs (lung hypoplasia) and an associated decrease in the cross-sectional area of the pulmonary vasculature (pulmonary vascular hypoplasia). The observed lung dysgenesis and its ensuing clinical manifestations

constitute the prime determinants of CDH related morbidity and mortality (Lally, 2002; van den Hout et al., 2009).

1.4.2.2 Overview of pathogenesis:

The diaphragm starts to develop at 4 weeks of gestation in humans and its basic structure becomes defined during the process of embryonic folding and separation of the body cavities. An infolding in the ventral body wall gives rise to the septum tranversum which fuses with similar infoldings from the posterolateral sides, the structures surrounding the esophagus and the esophageal mesentery to close the pleuroperitoneal canals at apporximately 8 weeks gestation. Using rodent models, Greer and his group demonstrated that the formation of transient structures termed pleuro-peritoneal folds (PPFs) is a key event in mammalian diaphragm development (Allan and Greer, 1997; Clugston et al., 2006). PPFs are formed at the union of the pleuro-pericardial folds and the septum transversum and are colonized by myogenic cells and phrenic axons. Subsequent expansion and distribution of these cells in the PPFs gives rise to the neuromuscular component of the developing diaphragm (Babiuk et al., 2003). Improper formation of the septum transversum and the PPFs are likely causes of defective diaphragm development in CDH. Indeed, abnormalities in the PPFs have been identified in distinct models of CDH induced by methods such as nitrofen-exposure, vitamin-A deficiency or Wt1 null-mutation (Allan and Greer, 1997; Clugston et al., 2006).

Depending on the location and nature of the diaphragmatic defect, CDH can be phenotypically characterized into several subtypes. A defect in the

posterolateral diaphragm (Bochdalek type) accounts for greater than 95% of cases and is almost synonymous with clinically diagnosed CDH. Other less common types of CDH include eventration of the diaphragm, defects of the central tendon, and retrosternal (Morgagni) hernias (Clugston and Greer, 2007). Nevertheless, in all CDH variants, the integrity of the diaphragm as a barrier between the abdomen and thoracic cavity is diminished. The ensuing herniation of abdominal viscera into the thoracic cavity interferes with the formation and maturation of the tracheobronchial tree and lungs in the successive weeks of gestation ("compression" hypothesis) (Grisaru-Granovsky et al., 2009). However, this rather simplistic view of CDH associated lung dysgenesis became challenged by data from various in utero therapeutic trials and experimental models (Jesudason, 2002). For instance, studies indicate that fetal diaphragmatic repair and hernia reduction have not enhanced survival in CDH (Harrison et al., 1997). Even the temporary fetal tracheal occlusion technique (Kitano and Adzick, 1999), an in utero surgical procedure intended to secure normal lung growth, failed to yield reliable clinical benefits in CDH survivors (Done et al., 2008). Several transgenic and toxin-induced models of CDH also appear to suggest that the lung malformation could be an independent part of the emerging CDH phenotype and may even precede diaphragmatic hernia (Keijzer and Puri, 2010) (Rottier and Tibboel, 2005). Although these studies draw new light into the deeper ramifications CDH, none of the studied surgical, transgenic and toxicological models have succeeded in completely explaining the developmental and biological basis of the lung anomaly. Even more unclear is our knowledge on the

determinants of the lung vascular abnormalities associated with CDH. Probably, a well-orchestrated interplay of genetic, epigenetic and environmental factors together with an 'embryologic timing' of the injury (a "multiple-hit" hypothesis) shall more adequately explain the complexities underlying the origin of CDH and its associated lung malformation.

1.4.2.3 Pulmonary vascular abnormalities in CDH:

CDH associated pulmonary maldevelopment comprises striking dysgenesis of the vascular network in the distal lung. Human and experimental CDH lungs display decreased numbers of pulmonary vascular generations and enhanced smooth muscle laminae in the arterial walls (Chinoy, 2002; Kinane, 2007). Consequent to the abnormalities in the lung vascular bed, most cases of CDH present with PHT.

Persistent pulmonary hypertension of the newborn, commonly termed PPHN, is the earliest presentation of PHT in CDH neonates (Kinsella et al., 2005). At birth, a drastic cardiopulmonary transition occurs as the lung assumes vital gas exchange function for the first time. This transition involves a rapid fall in pulmonary vascular resistance and pulmonary arterial pressure, and a dramatic rise in pulmonary blood flow. The hypoplastic vasculature seen in CDH lungs results in the failure of normal cardiopulmonary transition, resulting in PPHN (Steinhorn, 2010). The hemodynamic complications that follow result in severe and clinically unresponsive hypoxemia. Indeed, one of the greatest strides in the management of neonates with CDH has been the strict attention and priority provided to tackling PPHN associated respiratory failure and hypoxemia, rather

than the immediate need to close the diaphragmatic defect (van den Hout et al., 2009). Inhaled NO and extracorporeal membrane oxygenation (ECMO) have tremendously simplified PPHN management (Kinsella et al., 2005; Sluiter et al., 2011). However, 50% of newborns with PPHN are refractory and fail to respond to inhaled NO (Steinhorn, 2008), implying a fundamental abnormality in the structure of the pulmonary arterial walls. Even though a partial structural improvement with respect to decreased medial smooth muscle mass and adventitial connective tissue is observable after ECMO administration (Shehata et al., 1999b), in the long-term, the structural defect tends to persist. Moreover, owing to significant reduction in the pulmonary vascular bed, PHT in CDH infants endures beyond the neonatal period in the form of late and chronic variants (Kinsella et al., 2005). Long-term consequences of these protracted PHT variants are yet to be documented (Peetsold et al., 2009).

The precise mechanisms contributing to refractory PHT in CDH are presently not very clear. However, they may primarily relate to (i) a hypoplastic pulmonary vascular bed and (ii) altered pulmonary vasoreactivity (Chinoy, 2002; Geggel and Reid, 1984; Meyrick and Reid, 1983). Consequently, researchers have extensively investigated these factors and their potential contribution to PPHN in CDH. A brief overview of those studied factors is as follows:

The VEGF pathway: The VEGF/NO pathway, along with its upstream and downstream mediators, plays a central role in orchestrating normal vessel development and vascular reactivity. In fact, this is the most extensively studied pathway of mediators in the context of PHT in CDH (Abman, 2007; Mathew,

2011). During lung organogenesis, capillary networks surround the terminal lung buds and expand by formation of new capillaries from preexisting vessels as the lung bud grows into the surrounding mesenchyme. This angiogenesis process in the embryo is mainly driven by hypoxia, which serves as a stimulus for the induction of angiogenic growth factors. Of these factors, VEGF appears to be the most potent. VEGF action is primarily mediated through the VEGF receptor 2 (also termed Flk-1), which is one of the earliest markers expressed on endothelial cells (Yancopoulos et al., 2000). As might be expected, VEGF expression is decreased in the lungs of nitrofen-induced CDH rats (Okazaki et al., 1997). Nitrofen, particularly downregulates the production of VEGF during gestation and attenuates the VEGF peak normally observed at the onset of the canalicular stage. This effect seems most pronounced in the peripheral lung tissue (Chang et al., 2004). Contrastingly, increased levels of VEGF is detected in the small, pressure regulating pulmonary arteries of necroscopic lung specimens from CDH patients (Shehata et al., 1999a). However, as the authors suggest, this may reflect an unsuccessful attempt by the developing fetus to increase the pulmonary vascular bed in the hypoplastic lungs, in order to alleviate PHT (Boucherat et al., 2010). It may also relate to the regional differences in VEGF expression or defective downstream mediators of VEGF, and their significance in regulating vascular development.

Upstream regulation of VEGF: VEGF mRNA expression is regulated by hypoxia through hypoxia inducible factor 1 (Hif-1) (Patel-Hett and D'Amore, 2011), which in turn is controlled at the protein level (via controlling its

degradation) by the tumor suppressor, von Hippel-Lindau protein (Vhl) (Krek, 2000). Hypoxia prevents this association of Vhl with Hif-1, resulting in stabilization of Hif-1 protein and leading to the activation of the wide range of its target genes, including VEGF. Interestingly, HIF-1α (a HIF-1 subtype) expression is downregulated in CDH lungs (van der Horst et al., 2011), particularly in the endothelium of arteries, veins, and capillaries (de Rooij et al., 2004). An apparently increased (de Rooij et al., 2004) or unaltered (van der Horst et al., 2011) Vhl expression is observed in these lungs. These findings imply that aberrant expression of HIF-1 dependent genes may hamper the development or functional adaptability of the pulmonary vasculature (Miniati, 2007).

Nitric Oxide: VEGF activates nitric oxide synthases (NOS) such as eNOS and inducible NOS (iNOS) in the vessel wall, which in turn produce NO. NO is a critical mediator in many physiological and pathological processes, including vascular development. In fact, it acts both as an upstream and downstream mediator of VEGF mediated angiogenesis. Importantly, in nitrofen-induced CDH lungs, both expression and activity of NOS are decreased (Karamanoukian et al., 1996). In addition to justifying the extensive clinical use of NO to combat PPHN in CDH infants, these findings also add credence to the efforts by researchers exploring therapeutic possibilities with downstream mediators of NO. Indeed, NO-cGMP augmentation, via sildenafil mediated phosphodiesterase inhibition (Luong et al., 2011; van der Horst et al., 2010) or direct guanylyl cyclase activation (Deruelle et al., 2005), offers encouraging control of PHT in CDH lungs.

Factors mediating vasoreactivity: Researchers have also investigated several other mediators of pulmonary vasoreactivity in the context of CDH associated PHT. For instance, the vascoconstrictor endothelin-1 and its receptors deserve being mentioned due to their consistent association across human and experimental CDH. Reports show that endothelin-1 and the endothelin-A receptor proteins (de Lagausie et al., 2005; Keller et al., 2010) and their mRNA are upregulated in human and nitrofen-induced CDH lungs. Studies have also indicated that an imbalance of vasoconstrictive and vasodilatory eicosanoids may be involved in PHT, in experimental and human CDH (H and Tibboel, 1998). It is true that differences in expression of these mediators in themselves does not necessarily indicate functional involvement. Nonetheless, these studies suggest that the imbalance in expression of these regulators may contribute to or aggravate preexistent altered vasoreactivity in CDH patients. For a more detailed study, the reader is referred to extensive reviews by Abman (Abman, 2007) and Mathew (Mathew, 2011) on this topic.

In summary, the points that stand out clearly are: (i) CDH is more a disease of lung development, rather than a sheer surgical defect in the diaphragm; (ii) lung dysgenesis and its associated clinical manifestations constitute the key determinants of morbidity and mortality attributable to CDH; (iii) the origin of lung malformation is most likely an independent part of the emerging CDH phenotype and may even precede the diaphragmatic hernia; (iv) hypoplastic vasculature, structurally as well as functionally, is the primary element of CDH-associated lung dysgenesis. Very similar to BPD, promoting lung vascular growth

may be the promising approach to attain reliable structural and functional improvement in BPD. With the advent of novel stem cell-based therapeutic tools, this shall be an achievable target and the following sections elaborate in greater detail on the state of this art.

1.5 Stem cells in lung biology and lung diseases

Regenerative medicine is a promising new domain of medicine that has ushered in new hopes of more effective management of several long-standing ailments in several different organ systems in the body (Daley, 2010). The complicated three-dimensional structure of the lungs and the involvement of many cell types in lung function make research on lung regeneration challenging. For a long time, attempts have been made to regenerate the lungs by the administration of small molecules to stimulate the endogenous regenerative ability of lung cells (Warburton and Olver, 1997). However, the recent discoveries of lung tissue stem cells are now opening new doors in lung regenerative medicine, offering new perspectives for understanding and treating lung diseases. As indicated in the above sections, conditions of impaired lung development such as BPD and CDH indispensably require a regenerative arm in their treatment strategies. This section attempts to introduce some fundamental concepts about stem cells followed by a comprehensive summary of what we currently know about lung stem cells. This is followed a brief overview of the efforts initiated to harness the therapeutic potential of stem cells for lung regeneration.

1.5.1 Stem Cells: Concepts and Applications

Stem cells are primitive cells capable of extensive self-renewal and have the potential to give rise to multiple differentiated cellular phenotypes. Progenitor cells, on the other hand, are cells that can proliferate and differentiate into restricted cellular phenotypes (Stripp, 2008). The stem/progenitor cells are not

only critical for organogenesis and growth during early stages of development, but also contribute to organ repair and regeneration throughout postnatal life.

1.5.1.1 Developmental potency of stem cells.

Fundamental to understanding the function and therapeutic potential of different types of stem cells is the concept of developmental potency, which refers to the range of possible fates open to cells during differentiation. Stem cells exhibit varying differentiation potencies and are typically categorized into embryonic and somatic stem cells. Even though fertilized eggs are totipotent (differentiate into all cell types that constitute the entire organism), they do not self-renew by simple cell division. This makes embryonic stem cells (ESC), derived from the early blastocyst, the most potent of stem cells. These cells are pluripotent (differentiate into cell types derived from all three germinal lineages of the developing embryo- endoderm, mesoderm and ectoderm) and capable of indefinite self-renewal. In contrast, somatic stem cells (also termed adult stem cells, ASC) are cells that have assumed increasing degrees of fate restriction as the embryo develops and are either multipotent (differentiate into a limited range of cell types) or unipotent (generate only one cell type) (Stevenson et al., 2009). Residual pools of such multi or unipotent stem cells are hypothesized to reside in almost all adult organs, contributing to their ability to repair and regenerate following injury.

1.5.1.2 Classical vs non-classical stem cell hierarchies.

While stem cells are held critical for growth and development throughout childhood, residual pools of ASC are considered important for tissue repair and

maintenance through adulthood. Highly proliferative tissues such as the intestinal epithelium or the hematopoietic compartment of the bone marrow depend on a pool of ASC that are organized in a 'classical hierarchy', to maintain homeostasis (Stripp, 2008). In marked contrast, anatomically complex tissues that turn over more slowly (brain, heart, lung and kidney) do not appear to support a classical stem cell hierarchy. Such tissues are believed to be maintained by stem/progenitor cell populations that are organized in a 'non-classical hierarchy' and recruited facultatively for regeneration following injury. For example, in the liver, mature hepatocytes are responsible for post-resection tissue reconstitution. However, under certain conditions, cells from a facultative pool of ASC (termed 'oval' cells), are also called into action (Zaret and Grompe, 2008). Very similarly, in the lung, several local epithelial cell types can function both as differentiated functional cells and as transit-amplifying progenitors, that proliferate in response to airway or alveolar injuries (Rawlins and Hogan, 2006). Nevertheless, recent research suggests that the adult lung also harbors rare populations of multipotent epithelial stem cells that are regulated by specific micro-environmental cellular niches and are putatively recruited to repopulate the damaged epithelium (Giangreco et al., 2002; Kim et al., 2005; McQualter et al., 2010; Rawlins et al., 2009a).

1.5.1.3 Regulation of stem cell function.

There are a lot of unanswered questions about the role and regulation of stem cells in tissue formation, organogenesis and tissue repair following injury. However, it is becoming more and more evident that the capacity to behave as a

stem cell ("stemness") is, to a significant extent, a functional state rather than an intrinsic cellular characteristic. The decisions about cell fate and maintenance of stem cell populations are controlled by a multitude of variables that include genetic, epigenetic and cellular micro-environmental factors (Blau et al., 2001; Watt and Hogan, 2000). One classical example highlighting the importance of these factors in stem cell fate determination is the ability of ASC residing in the bone marrow to cross lineage barriers and adopt the phenotype of target organs such as liver, lung, GI tract and skin epithelia (Krause et al., 2001). Another example is the engineering of pluripotency into somatic cells by ectopic expression of transcription factors linked to pluripotency (Takahashi and Yamanaka, 2006). The resulting induced pluripotent stem cells (iPSC) are functional equivalents of ESC and represent a major milestone in our understanding of stem cell behavior and function.

1.5.1.4 Concept of stem cell-replacement.

One reason for studying stem cells is their usefulness in cell-replacement therapy. The self-renewal and regenerative capabilities of tissue-resident stem cell populations are naturally limited due to damage that accumulates with advancing age or disease. This may occur via simple exhaustion of the residual stem cell pools or as a consequence of genetic or micro-environmental changes that impede proper stem cell function. These changes can potentially be reversed via stimulation of the endogenous stem cell pools or their therapeutic replacement with stem cells derived from exogenous sources. Such cell-replacement therapies exist already for hematological disorders using bone marrow-derived stem cells

(Gahrton and Björkstrand, 2000) and similar approaches have started showing promise in other debilitating childhood and adult disorders (Helmy et al., 2010; Horwitz et al., 1999; Vats et al., 2005). Another potential approach in cell therapy is to administer functionally mature cells of required tissue specificity, generated by targeted differentiation of ESC. However, isolation of human ESC for research or therapeutic applications is entangled in biological and ethical constraints. This is where iPSC offer an ethically sound alternative to model specific diseases *in vitro* as well as for autologous cell therapy (Li et al., 2010). In addition to their use in tissue replacement, certain stem cell types may have utility as adjuvant therapies by virtue of their increasingly recognized trophic and immunomodulatory effects (Figliuzzi et al., 2009; Le Blanc et al., 2004; Tögel et al., 2005). Evidence shows that stem cells could also find application as delivery vehicles for targeted gene therapy (Myers et al., 2010).

Along these lines, the quest for a deeper understanding of lung stem/progenitor cell biology and its therapeutic translation has encountered several advances and challenges in the recent years. These will be discussed below with emphasis on the role of stem cells in lung alveolar growth and maintenance.

1.5.2 Lung Stem Cells

Lungs are complex organs constituted by 40 or more cell types derived from all three germ layers (Kim, 2007). Normal lung morphogenesis involves spatiotemporally coordinated interactions between the stem/progenitor cells of

different cellular compartments, which are later recapitulated during lung regeneration and repair following injury (Shi et al., 2009).

1.5.2.1 Lung epithelial stem/progenitor cells.

The epithelial lining of the lung, generated almost exclusively from the foregut endodermal cells, is a crucial player in maintaining organ function and homeostasis (Griffiths et al., 2005). This cellular compartment is subdivided into at least four distinct anatomical zones along the proximodistal axis (Table 1-1) with each characterized by unique cellular organization and repair mechanisms (Blaisdell et al., 2009). Organizational complexity coupled with prolonged turnover times of the adult lung epithelial cells has hindered the identification of true lung epithelial stem cells. However, it has been observed that relatively differentiated airway and alveolar epithelial cell types are capable of proliferating in response to epithelial injury (Rawlins and Hogan, 2006). This observation drew the focus of lung stem cell research into identifying and defining those epithelial cell subpopulations that appear to contribute to post-injury regeneration. Basal cells (Hong et al., 2004; Rock et al., 2010), clara cells (Stripp, 2008) bronchoalveolar stem cells (Kim et al., 2005) and ATII cells (Brody and Williams, 1992) have all been shown to exhibit stem cell properties. A representative list of lung cell populations with putative progenitor characteristics is summarized in Table 1-1.

Progenitor cells of the alveolar epithelium: Cuboidal ATII cells have long been considered as the progenitors of the alveolar epithelium based on their capacity to replenish themselves and to generate terminally differentiated ATI

cells (Adamson and Bowden, 1974; Brody and Williams, 1992). Since then, ATII cells have been speculated to contain a subpopulation of progenitors or cells that can undergo reactivation into a progenitor-like state in response to injury cues. Using an acute model of oxygen-induced injury, Driscoll et al demonstrated the existence of a telomerase-positive subpopulation within the general ATII cell population, during the recovery phase (Driscoll et al., 2000). This outlook was further strengthened by a later study where Reddy et al classified ATII cells into E-cadherin-positive and -negative fractions and showed heightened telomerase-activity and injury resistance in the latter subset (Reddy et al., 2004).

Recent research, however, suggests the existence of multipotent stem cells in the distal lung that are capable of differentiating into epithelial cells specific to the airway and the alveoli. Kim et al demonstrated the existence of dual-lineage bronchioalveolar stem cells at the airway-alveolar junction that express both airway (CC10) and alveoar (SP-C) markers (Kim et al., 2005) and proliferate in response to airway and alveolar injury (Kim et al., 2005; Nolen-Walston et al., 2008). However, based on the employed techniques, there has been some ambiguity about the lineage potential (Bertoncello and McQualter, 2010; Snyder et al., 2009) and contribution of these cells to alveolar repair (Rawlins et al., 2009b). Another population of Oct-4+ stem/progenitor cells have been described by Ling and colleagues to specifically exist at the bronchoalveolar junction. These cells co-express other stem cell markers such as SSEA-1, Sca-1 and cytokeratin 7+ and do not express c-kit, CD34 or p63. They require mesenchymal stroma for growth and maintenance and have the potential to differentiate into ATI & II

cells. Interestingly, these Oct-4+ cell subpopulation are particularly susceptible to SARS-coronavirus infection resulting in the loss of lung repair capability(Ling et al., 2006b).

More recently a population of self-renewing $EpCAM^{hi}CD49f^{pos}CD104^{pos}CD24^{low}$ epithelial colony forming units (cfus) has been identified. These epithelial cfus form colonies when co-cultured with EpCAMneg Sca-1pos lung mesenchymal stem cells showing that epithelial-mesenchymal interaction is essential for the formation of colonies by the epithelial colony forming units. In addition, epithelial growth factors, HGF and FGF-10 support the generation of epithelial colonies and could replace the mesenchymal support (McQualter et al., 2010). Another very recent study has identified a novel population of c-kit positive, self-renewing, multipotent, clonogenic cells in the distal airways of the human lung. These undifferentiated human lung cells form bronchioles, alveoli and pulmonary vessels, integrated structurally and functionally with the cryoinjured lung (Kajstura et al., 2011). Although several questions still remain unanswered, the above findings suggest that the distal human lung might contain undifferentiated, multipotent stem cells that potentially play a role in lung homeostasis.

1.5.2.2 Lung mesenchymal progenitors.

Additional lung cell types, including airway smooth muscle, fibroblasts, and the vasculature, are derived from the mesoderm. Interaction between the epithelial cells, mesenchymal microenvironment (including extracellular matrix proteins and growth factors) and the adjacent pulmonary vasculature regulates the

structural and functional maturation of the developing lung (Shi et al., 2009). Our knowledge on lung mesenchymal precursors is very limited. Evidence however indicates the existence of small populations of resident lung cells expressing certain phenotypic characteristics of mesenchymal cells with progenitor capacity. For example, resident lung "side population" (SP) cells, which appear to have both mesenchymal and epithelial potential, have been isolated based on their capacity to efflux Hoescht dye (Majka et al., 2005; Reynolds et al., 2007). Their role in endogenous lung repair is not fully understood. Following the discovery of the plasticity characteristics of ASC that allow them to cross lineage barriers and adopt functional phenotypes of other tissues, a lot of interest has been diverted to understanding their role in repair and maintenance of the lungs (Herzog et al., 2003). Experimental evidence indicates that the injured lung stimulates the release and preferential homing of mesenchymal stem cells (MSC), a population of ASC derived from the bone marrow (Liebler et al., 2008; Rojas et al., 2005). The mechanism by which exogenous progenitors such as bone marrow MSC assume lung phenotype and its clinical significance, however, remains unclear (Neuringer and Randell, 2004). In addition, by virtue of their ubiquitous presence and unquestionable importance in organogenesis and tissue repair, endothelial progenitor cells (EPC), a population of circulating and resident vascular precursor cells, have also recently received widespread attention in the context of lung development and regeneration (Kovacic and Boehm, 2009). Interesting findings indicating the therapeutic potential of these mesenchymal progenitor populations

and the need for further investigations in elucidating more precisely their role in lung regeneration are highlighted in the next sections.

1.5.3 Stem cells and lung regeneration

The recent surge in our knowledge of stem cell biology and the availability of advanced research tools in this field has motivated researchers into exploring the role of lung stem cells in the pathogenesis of chronic lung diseases that lack effective therapies. Indeed, several major lung diseases are believed to involve dysregulation in the numbers and/or the function of resident lung stem/progenitor cells (Neuringer and Randell, 2004). For instance, depletion or functional impairment of epithelial or endothelial progenitor cells might underlie the pathogenesis of alveolar growth arrest (BPD) or destruction (emphysema). Similarly, a defect in vascular progenitor cells could potentially underlie intractable PHT in CDH lungs. Augmentation of endogenous stem cells may minimize lung injury, promote repair, and possibly regenerate lost tissue, in these diseases (Alphonse and Thebaud, 2011; Toshner and Morrell, 2010).

Numerous preclinical studies have provided compelling evidence for the beneficial effects of cell therapy using exogenous stem cells for a large variety of lung diseases (Table-2). Animal models mimicking major lung diseases such as acute lung injury/acute respiratory distress syndrome (ALI/ARDS), idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease (COPD), cystic fibrosis, asthma, pulmonary hypertension and BPD have been employed in these studies (Agostini, 2010; Blaisdell et al., 2009; Weiss et al., 2008) (Table 1-2). As

evident from the existing literature, MSCs are by far the most extensively studied ASC type. Their remarkable differentiation plasticity, extraordinary immunomodulatory properties, as well as their ability to be recruited to sites of injury (Schipani and Kronenberg, 2008) have made them the most widely investigated stem cells in preclinical therapeutic studies. EPCs, by virtue of their ubiquitous presence and importance in organogenesis and tissue repair have also recently received widespread attention (nearly the second most commonly studied ASCs) in the context of lung development and regeneration (Kovacic and Boehm, 2009). In addition to ASCs, attempts have been made to therapeutically use ESCs via targeted differentiation into alveolar epithelial progenitors (Wang et al., 2010). With extensive research on ESCs being hindered by ethical controversies (Agostini, 2010; Daley, 2010), the dawn of iPS cells has ushered in new promises of patient-specific pluripotent stem cell-based therapies (Madonna, 2012). From the therapeutic perspective, it is noteworthy that a major part of the benefits of exogenous stem cells have been attributed to cell derived paracrine factors, rather than direct cell engraftment (Abreu et al., 2011). In fact, the observed lung engraftment of stem cells is not more than 5% in most of the studies cited in Table. 2. Identification of these paracrine factors may lead to more efficient and safe therapies in the future.

While the therapeutic benefit of exogenous stem cells for lung diseases has been widely explored, very few studies have sought to tap into the therapeutic potential of endogenous lung progenitors. For instance, Hegab et al have isolated a population of Sca-1+CD45-CD31- endogenous lung multi-lineage progenitors

capable of self-renewal and differentiation into pulmonary epithelial cells. These cells could attenuate alveolar damage and improve survival in a mouse model of elastase-induced emphysema (Hegab et al., 2010). The study by Kajstura and colleagues is another example where the authors show the existence of multipotent, clonogenic human stem cells in distal airway niches that could regenerate airway and vascular components of cyroinjured mouse lungs *in vivo*. However, this study did not demonstrate the regenerative ability of the newly identified c-kit positive stem cells in a lung disease model (Kajstura et al., 2011). Incomplete knowledge about the existence, defining characteristics and role of distal lung stem cells and the difficulty in isolating and maintianing them in culture are the major road blocks to realizing their true therapeutic potential. In this backdrop, treatment approaches targeting improved survival of resident lung stem cells gain relevance. In fact, the study presented in chapter 3 of this thesis describes one such approach. A possible reason for the demonstrated therapeutic benefit of Akt pathway activation, in this study, could be the improved survival and regenerative capability of putative progenitor cell populations posited to exist in the alveolar niches (Reddy et al., 2004).

Finally, it should be realized that stem cell "deficiency" is not always the cause for lung diseases involving stem cells. Abnormally elevated proliferation of certain stem cell types can also be an equally important cause of disease. For instance, fibrotic reactions and scarring is a common consequence of several disease processes in the lung involving epithelial injury. Such diseases can be viewed as diseases of fibroblast stem cell "hyperproliferation" (Pierro and

Thebaud, 2010), where inhibiting excessive growth of stem cells might be a valid therapeutic goal.

1.6 EPCs, ECFCs and pulmonary vascular homeostasis

The previous sections explained our basic understanding of lung vascular development and its indispensable role in normal lung growth and function. Vascular dysgenesis was also underlined as an important contributory mechanism to the pathogenesis of BPD and CDH-associated lung hypoplasia. The section that followed introduced stem cells and elaborated how these cells could potentially underlie normal and impaired lung development. The upcoming section offers the reader insights into our current understanding of vascular precursor cells (ie. "vessel-forming" stem cells) and their possible role in lung development, injury and repair. It introduces some basic definitions and ideas that are relevant through chapters 4 and 5, which carry the major scientific content of this thesis.

1.6.1 EPCs and postnatal vasculogenesis

Almost until the end of the previous century, the accepted paradigm for new vessel formation in postnatal life was by endothelial sprouting from pre-existing vessels, via angiogenesis (Heil et al., 2006). Vasculogenesis, which involves *de novo* formation of blood vessels from uncommitted endothelial precursors (angioblasts), was thought to occur only during fetal life (Risau, 1997). However, in 1997, Asahara et al. (Asahara et al., 1997) demonstrated the existence of a population of circulating cells which displayed the properties of endothelial cells in addition to exhibiting stem/progenitor cell characteristics. In a mouse model of hind-limb ischaemia, this new population of 'endothelial progenitor cells (EPCs)' appeared to generate new blood vessels. This seminal

discovery ushered in the concept of 'postnatal vasculogenesis', where EPCs were purported to give rise to mature endothelial cells and form blood vessels *de novo*.

This novel paradigm of postnatal vasculogenesis spawned a new field of investigation in defining the basic and translational properties of EPCs. In addition to extensive efforts in developing *in vitro* cell culture methods for isolating and purifying these cell populations, intensive research has been dedicated to identifying how these cells may be mobilized and used therapeutically to promote vascular repair (Asahara, 2007; Khoo et al., 2008). Several animal studies have tested therapeutic targeting of circulating EPC numbers to restore organs such as the heart (Kocher et al., 2001; Schuster et al., 2004), brain (Zhang et al., 2002), retina (Otani et al., 2002), and ischaemic hindlimb (Kalka et al., 2000; Sasaki et al., 2006), after experimental injury. Similar strategies have also been successfully explored in models mimicking newborn and adult lung diseases (Ishizawa et al., 2004b; Takahashi et al., 2004a; Zhao et al., 2005b). Even more interestingly, clinical data correlated higher circulating EPC numbers with a reduced cardiovascular risk (Dzau et al., 2005) and improved outcomes in pulmonary ailments (Fadini et al., 2007; Huertas and Palange, 2011) and sepsis (Cribbs et al., 2008).

1.6.2 ECFCs, the 'true' vascular progenitors?

Despite a profusion of published work since 1997, there are still considerable controversies. The outcomes of a few clinical trials using bone marrow-derived EPCs are inconclusive and not as robust as predicted by

experiments with animal models (Dimmeler and Zeiher, 2009; Mund et al., 2009). One possible explanation for this discrepancy could be the lack of a proper definition for human EPCs and the resulting heterogeneity in cell populations used in the therapeutic trials (Zampetaki et al., 2008). In fact, further work on the cells isolated by Asahara's group has demonstrated that these cells lack proliferative capacity and express the leucocyte (CD45) and monocyte (CD14) markers (Rehman et al., 2003). Even more intriguingly, studies have challenged the basic validity of the contribution of these cells to neovasculogenesis (Ohle et al., 2012; Purhonen et al., 2008; Ziegelhoeffer et al., 2004). Thanks to the recent advances in our understanding, it has become clear that these circulating cells are fundamentally different from being endothelial precursors and should therefore be better termed as circulating 'angiogenic' cells (and not 'EPCs') (Yoder, 2010).

A primary limitation holding back the rapid progression of EPC research is the lack of a defined set of markers that can unambiguously identify and allow the clean isolation of this cell type (Timmermans et al., 2009). However, in the continued quest to delineate 'true' EPCs, a cell type displaying endothelial phenotype and features of a real progenitor was recently identified by Ingram et al in 2004 (Ingram et al., 2004). These cells were termed endothelial colony forming cells (ECFCs) or 'late outgrowth' EPCs (owing to 'late' appearance of their colonies in culture compared to 'early' appearing circulating angiogenic cells) (Hur et al., 2004). ECFCs exist as resident progenitors in the vascular endothelial lining and a fraction of them are also found circulating in the peripheral blood (Ingram et al., 2005; Ingram et al., 2004). ECFCs include an entire hierarchy of

clonally expanded cells with proliferation potentials ranging from high to low. They demonstrate a robust proliferative potential when maintained in culture. In addition, these cells express almost all makers of mature endothelial cells, form capillary-like structures *in vitro* and generate new blood vessels *in vivo* (Yoder, 2010; Yoder et al., 2007). More importantly, ECFCs do not express hematopietic/myeloid markers such as CD14, CD45 and CD115 nor do they display phagocytic activity (Yoder et al., 2007), suggesting their primary endothelial nature.

In summary, currently, the term 'EPC' is vaguely applied to denote at least two distinct cell populations: (i) the minimally proliferative hematopoietic or macrophage-like phenotype (circulating angiogenic cells and colony forming unit-Hill colonies) and (ii) the highly proliferative non-hematopietic phenotype (ECFCs) (Kirton and Xu, 2010; Timmermans et al., 2009; Yoder et al., 2007). Based on their timing of emergence in culture, these populations have also been described as 'early' and 'late' outgrowth EPCs, respectively (Hur et al., 2004; Yoon et al., 2005) (Figure 1-3). ECFCs, which are phenotypically indistinguishable from cultured endothelial cells, demonstrate a very high potential for proliferation, the capacity for self-renewal and the ability to form blood vessels, *de novo* (Ingram et al., 2004; Yoder et al., 2007). This suggests that ECFCs could be the primary progenitor population participating in neovasculogenesis and thus might be considered ‘true EPCs’ (Kirton and Xu, 2010; Yoder et al., 2007). Nevertheless, 'early-outgrowth' angiogenic cells are also important for new vessel formation, as indicated by their contribution to vascular homeostasis in several

tested animal models (Kirton and Xu, 2010). One suggested model (Figure 1-4) of postnatal vasculogenesis is that ECFCs may act as unipotent precursors generating the vascular endothelial lining, while the other angiogenic cells support vessel formation through secretion of appropriate cytokines and modification of the ECM (Yoder, 2010). However, more detailed studies are necessary to validate this model and to eventually modulate it therapeutically.

1.6.3 Vascular progenitors in lung injury and repair

The aforementioned sections highlighted the key advances in our understanding of EPC-dependent new blood vessel formation and its implications for tissue repair and regeneration. Considering the importance of EPCs in vessel formation and maintenance throughout postnatal life, their contribution to preserving pulmonary vascular homeostasis should be equally critical. Indeed, recent experimental and clinical observations suggest that EPCs contribute to lung repair. Lipopolysaccharide (LPS)-induced murine lung injury is associated with a rapid release of EPCs into the circulation that contribute, together with other bone marrow-derived progenitor cells, to lung repair (Yamada et al., 2005). In elastase-induced emphysema, bone marrow-derived cells develop characteristics of endothelial cells and contribute to repair the alveolar capillary wall (Ishizawa et al., 2004a; Ishizawa et al., 2004b). Patients with acute lung injury have twofold higher numbers of circulating EPCs than healthy control subjects (Burnham et al., 2005), suggesting a possible biological role for the mobilization of these cells in lung disease. More interestingly and similar to the prognostic role of EPCs in

ischemic diseases, improved survival in acute lung injury correlates with increased circulating EPCs (Yamada et al., 2005) and severity of illness (Burnham et al., 2005). Likewise, the number of circulating EPCs is significantly increased in patients with pneumonia and patients with low EPC counts tend to have persistent fibrotic changes in their lungs even after recovery from pneumonia. EPCs are also decreased in patients with restrictive and chronic lung disease (Fadini et al., 2006), where EPC counts correlate with disease-severity.

In addition to their role in the injury-repair processes of the adult lung, ECFCs also seem to be crucial for normal lung development and maturation. This is evident from the therapeutic potential they exercise in experimental models of impaired lung development. Since hyperoxia-mediated alveolar growth arrest (representing BPD) and PHT (commonly associated with CDH) constitute the major target diseases of the research presented in this thesis, ECFC studies with relevance to these two conditions have been grouped and described in the following sub-sections for convenience.

1.6.3.1 EPCs and hyperoxia-induced BPD

Oxygen challenge is one of the most extensively studied injury mechanisms in BPD. Animal and human observations in recent years offer interesting clues about the behavior of vascular progenitor cells during oxidative stress. Baker et al observed that cord blood of preterm infants yields higher numbers of ECFCs compared to term infants (Baker et al., 2009). However, the authors report that the preterm ECFCs are more sensitive to oxygen *in vitro*, compared to term ECFCs. Contrastingly, Borghesi et al report that ECFC are

lower in numbers in the cord blood of preterm infants who subsequently developed BPD (Borghesi et al., 2009). Interestingly, earlier animal studies have shown depletion of circulating and resident populations of progenitors with endothelial potential. One study demonstrates decreased numbers of CD45-/Sca-1+/CD133+/VEGFR-2+ EPC in the peripheral blood, bone marrow and lungs of oxygen challenged neonatal mice (Balasubramaniam et al., 2007). Using the same animal model, another study shows a decrease in the number and endothelial differentiation potential of Hoechst33342 dye-effluxing multipotent lung 'side population' cells (Irwin et al., 2007). More interestingly, observations in our studies indicate a compromise in the proliferative, clonogenic and *in vitro* vessel forming potential of PV-ECFC derived from the lungs of newborn rats with experimental BPD (chapter 4). These observations suggest an oxygen-induced depletion of vessel-forming progenitors and imply this as a possible mechanism underlying arrested lung vascular growth in BPD. However, sparing the endothelial progenitors, our knowledge on the fate of other stem cell populations within the BPD lung is still rudimentary and more extensive research may offer the scope for newer therapeutic approaches.

Importantly, the observed depletion of circulating and lung resident EPC in BPD (Borghesi et al., 2009) suggests a potential rescue from arrested alveolar and vascular growth via therapeutic supplementation with EPC of exogenous origin. In fact, oxygen-exposed newborn mice treated intravenously with bone marrow-derived angiogenic cells (a population of bone marrow derived early outgrowth EPC) demonstrate nearly complete restoration of lung structure that is

indistinguishable from room air controls (Balasubramaniam et al., 2010). Administration of hUCB-ECFCs also restores arrested alveolar growth and alleviates pulmonary hypertension in hyperoxia-exposed newborn mice (data presented in chapter 4).

1.6.3.2 EPCs and PHT

To date, most existing studies investigating the role of EPCs in lung vascular dysgenesis and PHT have examined the number and functional state of circulating early-outgrowth EPCs, particularly of bone-marrow origin (Toshner and Morrell, 2010). Circulating EPCs derived from patients with PHT due to various causes showed decreased capacity to migrate and incorporate into tube-like structures when cultured *in vitro* (Diller et al., 2008; Junhui et al., 2008), indicating a compromise in their functional state. Counter-intuitively, evidence also indicates an apparent contributory role for EPCs in the pulmonary vascular remodeling observed in PHT. Bone-marrow derived circulating progenitor-like cells appear to be recruited into the remodeled vessel walls of pulmonary arteries (Davie et al., 2004; Toshner et al., 2009). Blocking stromal derived factor-1/CXCR4 axis, the main bone marrow progenitor homing pathway, reversed pulmonary vascular remodeling and PHT (Young et al., 2009).

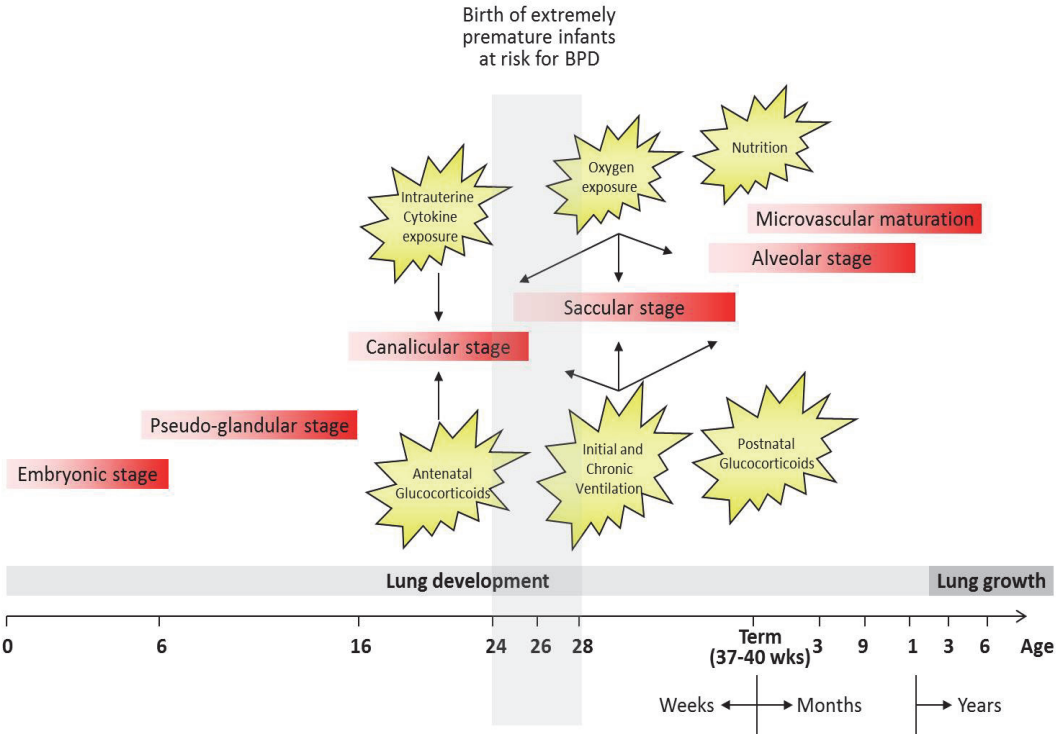
Interestingly, the earliest studies on EPCs in PHT centered around their therapeutic potential. Ex vivo expanded early-outgrowth EPCs demonstrate therapeutic benefit in short term animal models of PHT (Nagaya et al., 2003; Raoul et al., 2007b; Takahashi et al., 2004a; Zhao et al., 2005b). Subsequently, there were two small randomised trials in humans demonstrating an improved

hemodynamic response and exercise capacity following a single infusion of the same cells (Wang et al., 2007; Zhu et al., 2008). Another very recent study by Smadja and colleagues suggests that ECFCs might mediate the clinical benefits of prostanoids in PHT (Smadja et al., 2011). Pertinently, the studies presented in chapter 5 examines the functional state of resident lung ECFCs in neonatal rats with PHT and proceeds to evaluate the therapeutic potential of cord-blood ECFCs in the same disease model (chapter 5).

1.7 General Summary

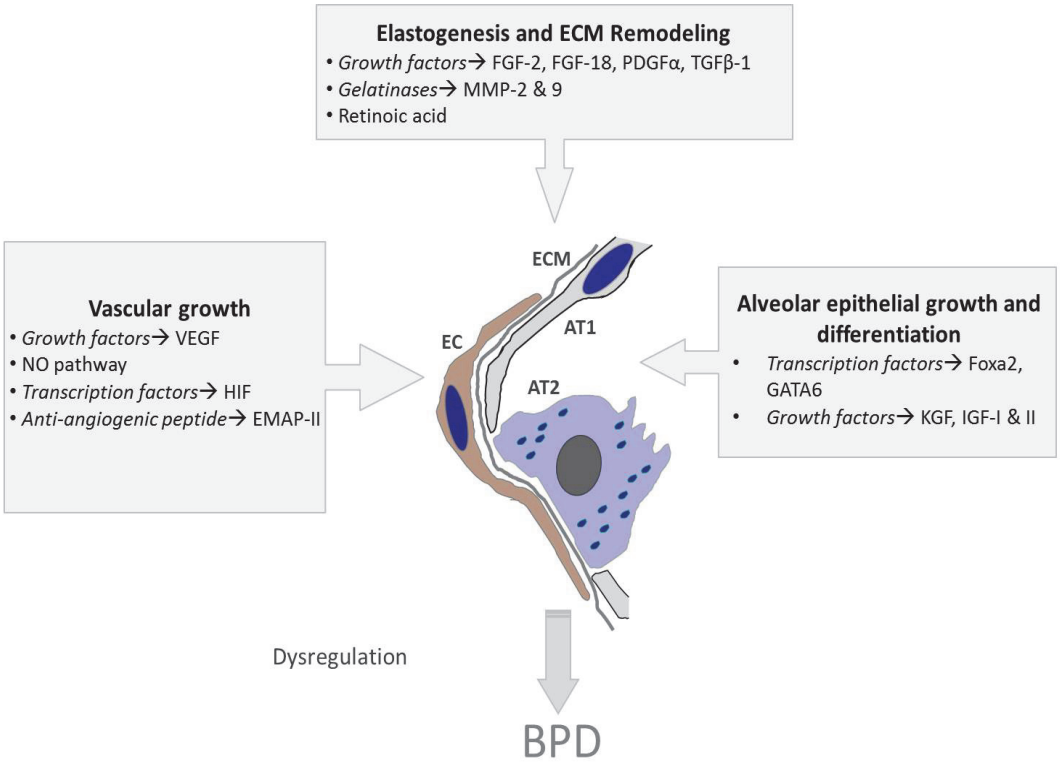
The purpose of the preceding sections was to provide a basic understanding of the mechanisms contributing to the pathogenesis of two major neonatal ailments, BPD and CDH, both characterized by disrupted lung development. In order to obtain an in depth comprehension of the molecular, pathophysiological and clinical underpinnings of BPD and CDH, the reader is referred to more extensive reviews elsewhere (see the following reviews: (Abman, 2007; Bourbon et al., 2005; Kinane, 2007; Thebaud and Abman, 2007b)). Nonetheless, it should be apparent that impaired vascular development is one of the critical determinants of arrested alveolar growth, warranting detailed research. With the evolving knowledge on vascular progenitor cells and their contribution to postnatal vasculognesis, the need to establish their existence (chapter 4) and examine their functional involvement in lung injury (chapters 4 and 5) assumes paramount importance. In this era of amazing advancements in stem cell biology and regenerative medicine, it is extremely timely to recognize and harness the potential that ECFC-based treatment strategies represent for serious lung diseases still lacking effective remedies. The upcoming chapters will address these issues, systematically.

Figure 1-1 Normal lung development and pathophysiological events contributing to BPD and decreased alveolarization in preterm infants.



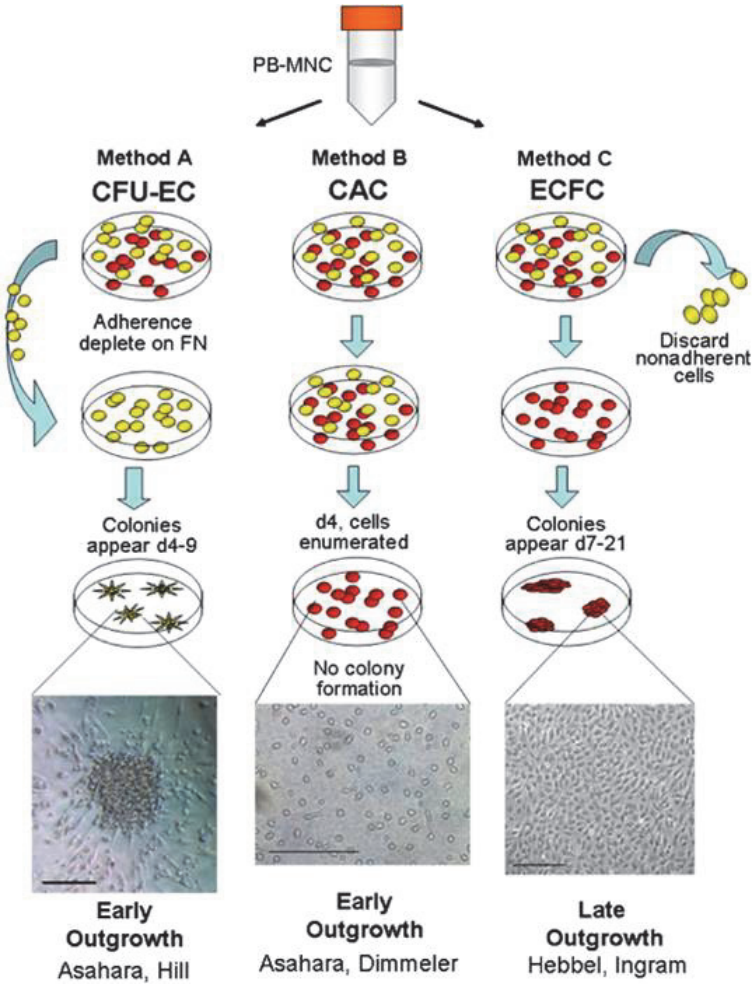
Schematic depicting the classical stages of histological lung development. It shows that preterm infants at risk of developing BPD are born at the late canalicular or early saccular phase of lung development. Pre-, peri- and postnatal adverse events contribute to interrupt the normal sequence of lung development leading to the arrest in lung growth observed in these infants

Figure 1-2 Outline of the multifactorial pathogenesis of BPD.



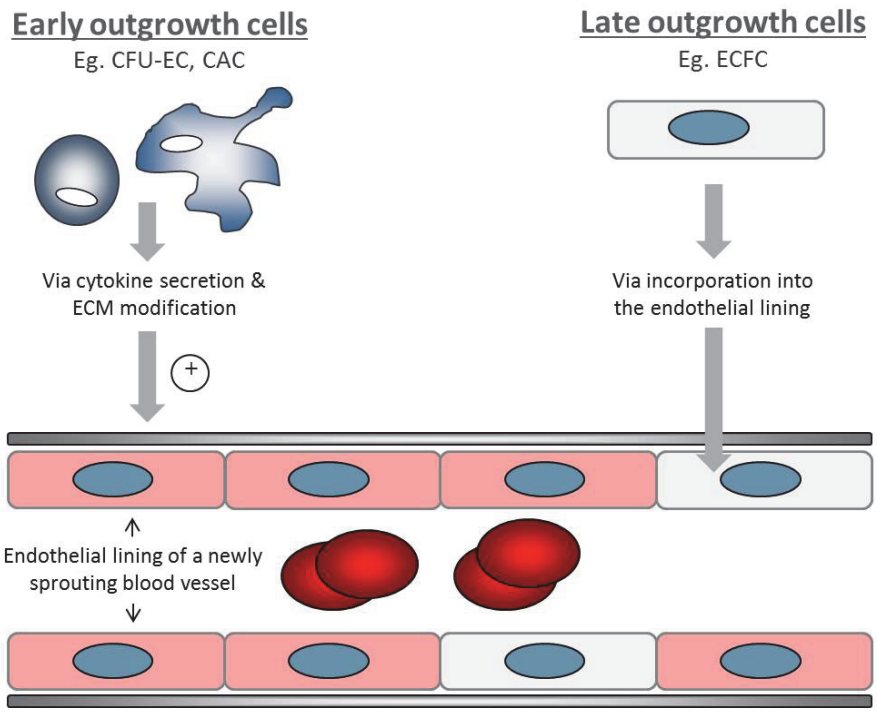
This simplified schematic outlines three major mechanistic compartments that underlie the pathogenesis of BPD. ECM- extra cellular matrix, FGF- fibroblast growth factor, PDGFα- platelet derived growth factor-α, TGF-β1- transforming growth factor β1, MMP- matrix metalloproteinase, NO- nitric oxide, HIF-2α- hypoxia inducible factor 2α, EMAP-II- endothelial-monocyte activating polypeptide II, KGF- keratinocyte growth factor, VEGF- vascular endothelial growth factor, IGF- insulin like growth factor. See text for description and references

Figure 1-3 Common methods of EPC culture.



Culture of colony-forming unit-endothelial cells (CFU-EC, Method A, scale bar =100 μm) includes a 5-day process wherein non-adherent MNCs give rise to the EPC colony. Circulating angiogenic cells (CAC, Method B, scale bar=200 μm) are the adherent mononuclear cells of a 4- to 7-day culture procedure. CAC cultures typically do not display colony formation. Endothelial colony-forming cells (ECFC, Method C, scale bar=400 μm) are derived from adherent MNCs cultured for 7–21 days in endothelial conditions and colonies display a cobblestone morphology. ECFC emerge much later in culture as compared to both CFU-EC and CAC. Hence, ECFC have been called 'late outgrowth' EPCs, while CFU-EC and CAC have been called 'early outgrowth' EPC. *Adapted with permission from Prater et al, 2007.*

Figure 1-4 Early and late outgrowth EPC in blood vessel formation.



This figure depicts that ECFC (late-outgrowth EPC) get incorporated into and generate the endothelial lining of newly sprouting blood vessels. The circulating early-outgrowth EPC support new vessel formation via cytokine secretion and ECM modification.

Table 1-1 A representative (not exhaustive) list of candidate endogenous lung stem/progenitor cells described in human and rodent lungs.

Location	Species	Candidate stem cell	Attributed phenotype	Niche	Defining characteristics	
Proximal trachea	Mouse	Unknown cell type	Tracheal epithelial cells	Submucosal glands	BrdU labeling-retained cells following i.t. detergent or SO_2 mediated epithelial injury	(Borthwick et al., 2001)
Distal trachea and bronchi	Mouse	Basal cells	Tracheo bronchial epithelial cells	Inter-cartilaginous zones	Cytokeratin 14-expressing multipotent progenitor cells capable of restoring differentiated tracheal epithelium following naphthalene injury; associated with innervated NEBs	(Hong et al., 2004)
Bronchioles	Mouse	Variant Clara cells	Distal airway epithelium	NEBs	Express CCSP; survive and repoulate distal airway epithelium following naphthalene injury; divide infrequently during steady state	(Hong et al., 2001)
Bronchioles and alveoli	Mouse	Bronchio alveolar stem cells (BASC)	Bronchio alveolar epithelial cells	Bonchio alveolar duct junction	Resistant to naphthalene injury and proliferate in reponse; co-express CCSP and SP-C	(Kim et al., 2005)
	Mouse	Pulmonary Oct-4+ stem/ progenitor cells	Alveolar type-I and -II pneumocytes	Bonchio alveolar duct junction	Oct4+, SSEA-1+, Sca-1+, cytokeratin-7+ cells; serially passaged, differentiate terminally into type-II and -I pneumocytes; susceptible to SARS-CoV infection	(Ling et al., 2006a)

Location	Species	Candidate stem cell	Attributed phenotype	Niche	Defining characteristics	
	Mouse	Multipotent lung epithelial progenitors	Ariway and alveolar epithelium	Intrapulmonary aiways and alveoli (not localized)	EpCAMhi, CD49f+, CD104+, CD24lo, Sca-1-, CD45-, CD31- lung epithelial cfus, form colonies in Matrigel, serially passaged and retain multipotent potential	(McQualter et al., 2010)
	Human	Self-renewing, multipotent, clonogenic cells	Ariway and alveolar epithelium and vascular endothelium	Distal airways	C-kit+ population	(Kajstura et al., 2011)
Alveoli	Mouse	Alveolar type-II pneumocytes	Alveolar type-I pneumocytes	Alveolar surface	All alveolar type-II pneumocytes	(Adamson and Bowden, 1974)
	Rat	A subset of alveolar type-II pneumocytes	Alveolar type-I and mature type-II pneumocytes	Alveolar surface	E-cadherin negative subset of alveolar type-II cells, proliferative, high telomerase activity, resistant to oxygen-induced injury	(Reddy et al., 2004)

Acronyms: BrdU: 5-bromo-2-deoxyuridine; CCSP: Clara cell secretory protein; NEB: neuroendocrine bodies; SARS-Co V: severe acute respiratory syndrome coronavirus; SP-C: surfactant protein C; SSEA-1: stage specific embryonic antigen-1

Table 1-2 Studies testing the therapeutic effect of stem cells in various experimental lung disease models, classified by the stem/progenitor cell type and disease model.

Disease Model	Applied Cell Type	Outcomes	Suggested Mechanisms	References
Embryonic Stem Cells				
Bleomycin (i.t.)- induced acute LI	Human ESC-derived cells with AT2 epithelial phenotype (i.t.)	Improved body weight and survival Improved arterial oxygen saturation Decreased collagen deposition	Structural engraftment & AT1 differentiation Paracrine-mediated mechanisms	(Wang et al., 2010)
Adult Stem Cells				
Exogenous Stem or Progenitor Cells				
Bleomycin (i.t.)-induced LI/fibrosis	Bone marrow-derived MSC	Reduced fibrosis Reduced inflammation	IL-1 receptor antagonism Decrease of NO metabolites, proinflammatory & angiogenic cytokines	(Kumamoto et al., 2009; Ortiz et al., 2007; Rojas et al., 2005)
	Wharton's jelly-derived MSC	Reduced fibrosis	Decreased TGF-β and TIMP activity Increased MMP-2 activity	(Moodley et al., 2009)
	Bone marrow-derived HSC with or without KGF overexpression (i.v.)	Reduced fibrosis	KGF-induced endogenous AT2 cell proliferation	(Aguilar et al., 2009)
Asbestos-induced LI/fibrosis	Allogenic whole bone marrow (i.v.)	Abrogation of inflammation and fibrosis		(Levis et al., 2008)
E. coli endotoxin (i.p.)-induced systemic inflammation and LI	Bone marrow-derived MSC (i.v. or i.t.)	Decreased systemic and local inflammation Improved survival	Cell-cell interactions Paracrine mechanisms Decreased proinflammatory and increased antiinflammatory cytokines Antioxidant mechanisms	(Xu et al., 2007) (Gupta et al., 2007) (Iyer et al., 2010)
E. coli pneumonia	Bone marrow-derived MSC (i.t.) and MSC CdM	Reduced bacterial in the lung homogenates and in the bronchoalveolar lavage fluid	Secretion of human cathelicidin hCAP-18/ LL-37	(Krasnodembskaya et al., 2010)

Disease Model	Applied Cell Type	Outcomes	Suggested Mechanisms	References
E. coli endotoxin (i.t.)-induced LI	Bone marrow-derived MSC overexpressing Ang-1 (i.v. or i.t.)	Decreased inflammation Decreased alveolar permeability	Decreasing inflammatory cytokines Ang-1 mediated effects	(Mei et al., 2007) (Xu et al., 2008)
Burn injury-mediated acute LI	Bone marrow-derived MSC (i.m)	Decreased inflammation and apoptosis	Decreased activation of epithelial NF-kB	(Yagi et al., 2010a; Yagi et al., 2010b)
CLP-induced sepsis and acute LI	Bone marrow-derived MSC (i.v.)	Control of sepsis Improved survival	Prostaglandin E2 mediated macrophage IL-10 secretion	(Németh et al., 2009)
Hyperoxia-induced lung injury	Bone marrow-derived MSC (i.t.)	Prevention of alveolar and vascular growth arrest	Paracrine mechanisms Immunomodulatory factors	(van Haaften et al., 2009) (Aslam et al., 2009)
	hUCB-derived MSC (i.t.)	Decreased inflammation Improved alveolarization	Epithelial differentiation Decreased pro-inflammatory and fibrotic cytokines	(Chang et al., 2009)
Lung-specific Fas ligand overexpression	hUCB-derived CD34+ hematopoietic progenitor cells (i.n.)	Pulmonary epithelial reconstitution	Long-term engraftment, functional differentiation, replication and clonal expansion; no cell fusion observed	(De Paepe et al., 2011)
Papain-mediated emphysema	Bone marrow-derived MSC (i.v.)	Emphysema attenuation	Engraftment and AT2 differentiation of MSC Decreased alveolar epithelial apoptosis	(Zhen et al., 2008)
Elastase-induced emphysema	Bone marrow-derived MSC (i.t.)	Reduced alveolar destruction	Paracrine effects-HGF, EGF and secretory leukocyte protease inhibitor secretion	(Katsha et al., 2011)
PPE-induced emphysema	Adipose tissue-derived MSC (i.v. or cultured on PGA scaffolds and transplanted after LVRS)	Gas exchange and exercise tolerance restored	Growth factor release (HGF, VEGF)	(Shigemura et al., 2006a; Shigemura et al., 2006b)
Ragweed-mediated asthma	Bone marrow-derived MSC (i.v.)	Decreased asthma-specific allergic response	TGF-β production Regulatory T-cell recruitment	(Nemeth et al., 2010)
CFTR-KO mice with airway injury	Bone marrow-derived MSC (i.v.)	Detection of lung CFTR expression and activity	Cell engraftment and induction of CFTR expression	(Loi et al., 2006)

Disease Model	Applied Cell Type	Outcomes	Suggested Mechanisms	References
Monocrotaline-mediated PHT	Bone marrow-derived MSC with or without eNOS overexpression (i.v. or i.t.)	Improved RV pressure overload and function Improved lung structure Improved survival	eNOS mediated vasodilatation VEGF mediated enhanced microvasculature Paracrine effects	(Baber et al., 2007; Kanki-Horimoto et al., 2006; Raoul et al., 2007a; Umar et al., 2009)
	Bone marrow-derived EPC (i.v.)	Restored pulmonary hemodynamics Increased microvascular perfusion	eNOS-controlled vascular growth	(Zhao et al., 2005a)
	Peripheral blood-derived EPC (i.t.)	Improved cardiac funtion Improvement in small vessel medial thickness and lung neovascularization		(Takahashi et al., 2004b)
Ovalbumin-mediated asthma/allergic airway inflammation	Adipose tissue-derived MSC (i.v.)	Decreased local and systemic allergic response	Decreased Th2 activity	(Cho et al., 2009) (Park et al., 2010)
	Bone marrow-derived MSC CdM	Reduced airway hyperresponsiveness and remodeling	Paracrine mechanisms	(Ionescu et al., 2011)
Endogenous Stem or Progenitor Cells				
Elastase-induced emphysema	Lung resident multilineage progenitors $Sca1^{+}CD45^{-}$ $CD31^{-}$ (i.t.)	Attenuated elastase-induced alveolar damage Longer survival	Immunomodulation via paracrine mechanisms	(Hegab et al., 2010)

Acronyms: Ang-1, angiopoietin-1; AT1, alveolar epithelial type 1; AT2, alveolar epithelial type 2; CdM, conditioned media; CFTR-KO, cystic fibrosis transmembrane regulator-knock out; CLP, caecal ligation and puncture; EGF, epidermal growth factor; eNOS: endothelial nitric oxide synthase; EPC, endothelial progenitor cell; HGF, hepatocyte growth factor; HSC, hematopoietic stem cell; hUCB, human umbilical cord blood; i.t., intra-tracheal; i.p., intra-peritoneal; i.v., intra-venous; i.n., intra-nasal; IL, interleukin; KGF, keratinocyte growth factor; LI, lung injury; LVRS, lung volume reduction surgery; MMP, matrix metalloproteinases; MSC- mesenchymal stem cell; PHT, pulmonary hypertension; PPE, porcine pancreatic elastase; RV, right ventricle; TGF-β, Transforming growth factor-β; Th2, helper T-cell type 2; TIMP, tissue inhibitor of metalloproteinases; VEGF, vascular endothelial growth factor.

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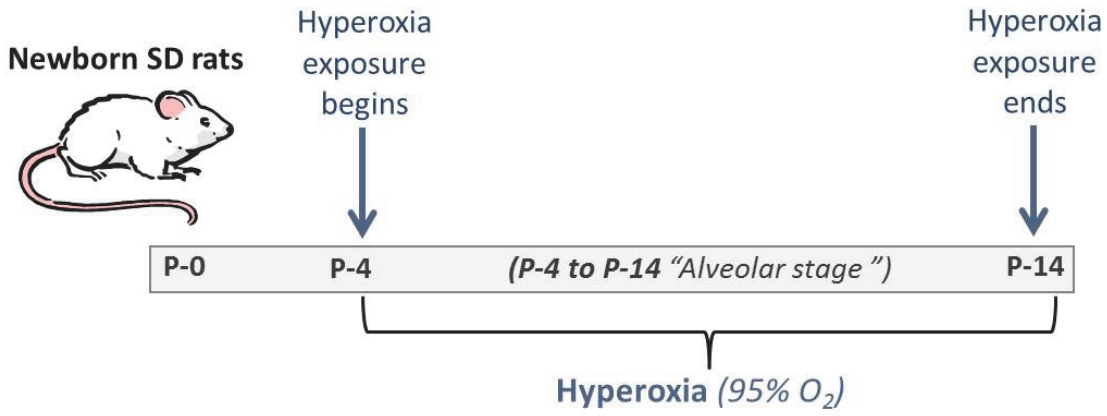
Chapter Two: **General Methods**

Explanatory note: The purpose of this chapter is to describe some of the basic methodologies employed during the course of the research presented in this thesis. These methods were commonly used in later chapters and are entered here in order to prevent repetition. Subsequent chapters still include a methods section to describe techniques predominantly unique to that concerned project.

2.1 Oxygen-Induced BPD Model

In this study, we used two rodent models of oxygen-induced BPD. For the rat BPD model, newborn rat pups were exposed to normoxia (room air; control group) or hyperoxia (95% oxygen; BPD group) from birth to post-natal day (PD)-14 (Figure 2-1) in sealed Plexiglas chambers (OxyCycler; BioSpherix, Lacona, NY) with continuous oxygen monitoring, as previously described (Thebaud et al., 2005). For the mouse model of BPD, newborn rag-/- mice of the hyperoxia group were exposed to 85% oxygen from PD-5 to PD-14 in a similar set-up. Dams were switched every 48 hours between the hyperoxic and normoxic chambers, to prevent damage to their lungs (Frank et al., 1978) and provide equal nutrition to each litter. Litter size was adjusted to 8-12 pups to control for effects of litter size on nutrition and growth. Lungs were harvested on PD-14 in rats and PD-28 in mice and processed as required by our experiments.

Figure 2-1. Schematic of oxygen-induced rat BPD model



Newborn Sprague-Dawley rat pups were housed in 95% O_2 from PD-4 to PD-14. Exposure to this high oxygen environment during the crucial alveolar stage of lung development results in alveolar lesions and pulmonary hemodynamic changes that very closely mimic human BPD. The changes observed in these oxygen-damaged lungs include extensive alveolar simplification and striking rarefaction of distal lung vascularity.

2.2 Lung tissue collection and preparation for basic histology

Routinely, the right lungs were cryopreserved for future analysis and the left lungs were instilled with and fixed in formalin for histology. Briefly, after complete ligation of the right lung at its hilum, the left lung was inflated with 10% zinc formalin solution at a constant pressure of 20 cm of H_2O for 5 minutes (Thebaud et al., 2005). After tracheal ligation, the uninflated right lung lobes were carefully removed distal to the ligation, snap frozen in liquid nitrogen and stored immediately at -80^{o}C for subsequent protein studies. The inflated left lungs were placed in formalin, overnight.

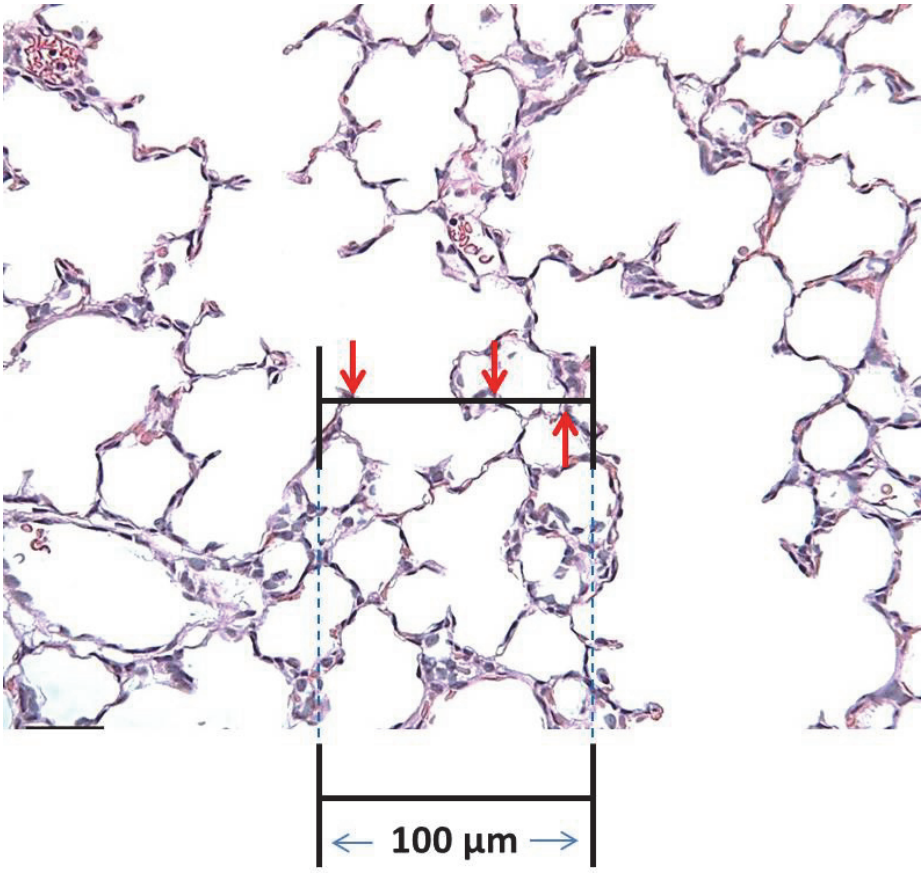
After volume measurements via water displacement (Scherle, 1970), the fixed lung tissue was dehydrated in a graded series of ethanol (50%, 75%, 90%, 95% and absolute alcohol) and cleared with xylene before being embedded in molten paraffin wax. Serial 4 mm thick sections of the paraffin embedded tissue was cut using a rotary microtome (Leica RM2135; Leica Microsystems, Wetzlar, Germany) and mounted on pre-subbed glass slides. For basic histology, the slides were rehydrated and counterstained with haematoxylin and eosin. Following tissue staining, slides were dehydrated using a generated series of ethanol and cover-slipped with mounting medium.

2.3 Lung Morphometry

After processing and embedding the lungs in paraffin, serial step sections, 4 μm in thickness, were taken along the longitudinal axis of the lobe. The fixed distance between the sections was calculated so as to allow systematic sampling of 10 sections across the whole lobe. Lungs were stained with hematoxylin and eosin (H&E) and alveolar structure was examined using a computer controlled motorized microscope stage (Leica Microsystems Canada, Richmond Hill, ON) with Openlab imaging software (Improvision, Coventry, UK). The mean linear intercept (MLI) parameter was calculated as previously described by Thebaud and colleagues (Thebaud et al., 2005) (Figure 2-2). Briefly, a scale bar of 100 μm was placed over several random non-overlapping locations on each lung section and the number of intercepts with the respiratory tissue was counted. MLI (L_m, measured in μm) was calculated from the total number of intercepts (m), the length of the scale bar (100 μm), and the number of times the scale bar was placed on the sections (N), using the following formula (Dunnill, 1964):

$$\mathbf{L_m = (N * 100)/m}$$

Figure 2-2 Diagrammatic representation of MLI measurement technique



Shown is the diagrammatic representation of the 100 µm scale bar that is randomly placed over different non-overlapping locations on the lung sections. The number of intercepts with the respiratory tissue (indicated by red arrows) is counted and applied to calculate MLI.

2.4 Barium Angiogram

For barium instillation, the animals were anesthetized with a dose of pentobarbital. Once in surgical plane, with the heart still beating, the chest was opened and 5 ml 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 (van Haaften et al., 2009). The lungs were then fixed with formalin instillation and prepared for histology as described in the previous section. Barium-filled pulmonary arteries were counted per 10X high-powered field. Lungs of four animals/group, five sections/lung, and 10 high-power fields/section were counted.

2.5 Right Ventricular Hypertrophy

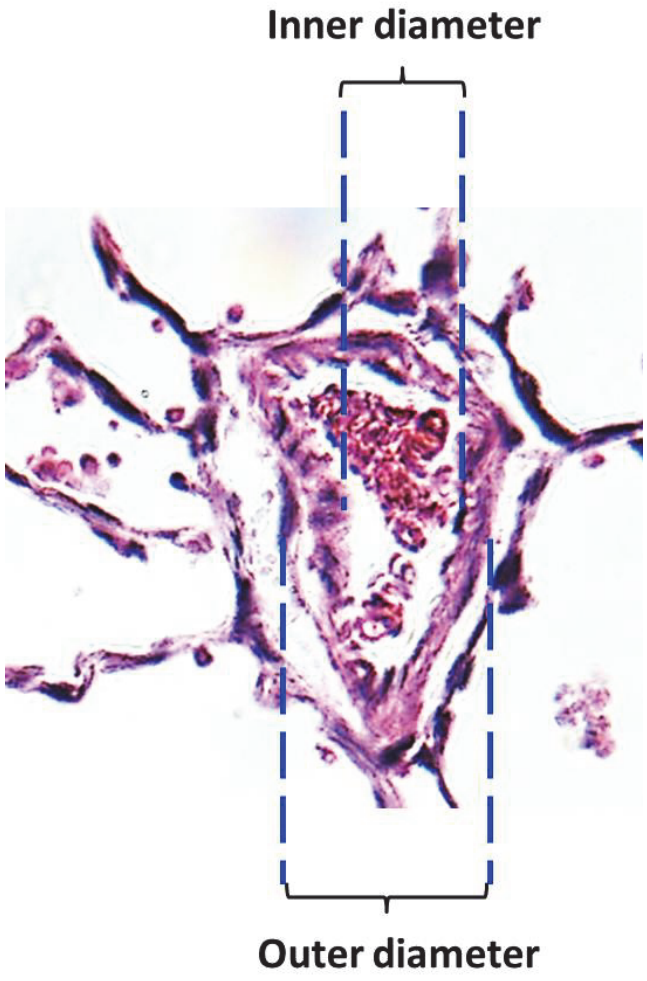
Right ventricle and left ventricle with septum were carefully dissected out and allowed to dry overnight. Their dry weights were measured separately to determine the right ventricle to left ventricle-septum ratio (RV/LV-S) as an index of right ventricular hypertrophy (RVH), as originally described by Fulton and colleagues (Fulton et al., 1952).

2.6 Vascular medial wall thickness measurement

To assess pulmonary vascular remodeling, H&E stained lung sections were examined under 40x magnification and random images of blood vessels between 30-100 μm were captured. 5 rats per group were studied and from each rat at least 3 separate lung sections were examined. The percent medial wall thickness (% MWT) was calculated as follows (Ladha et al., 2005) (Figure 2-3):

% MWT = [(2 x wall thickness/external diameter) x 100]

Figure 2-3 Sample representation of vessel wall thickness measurement



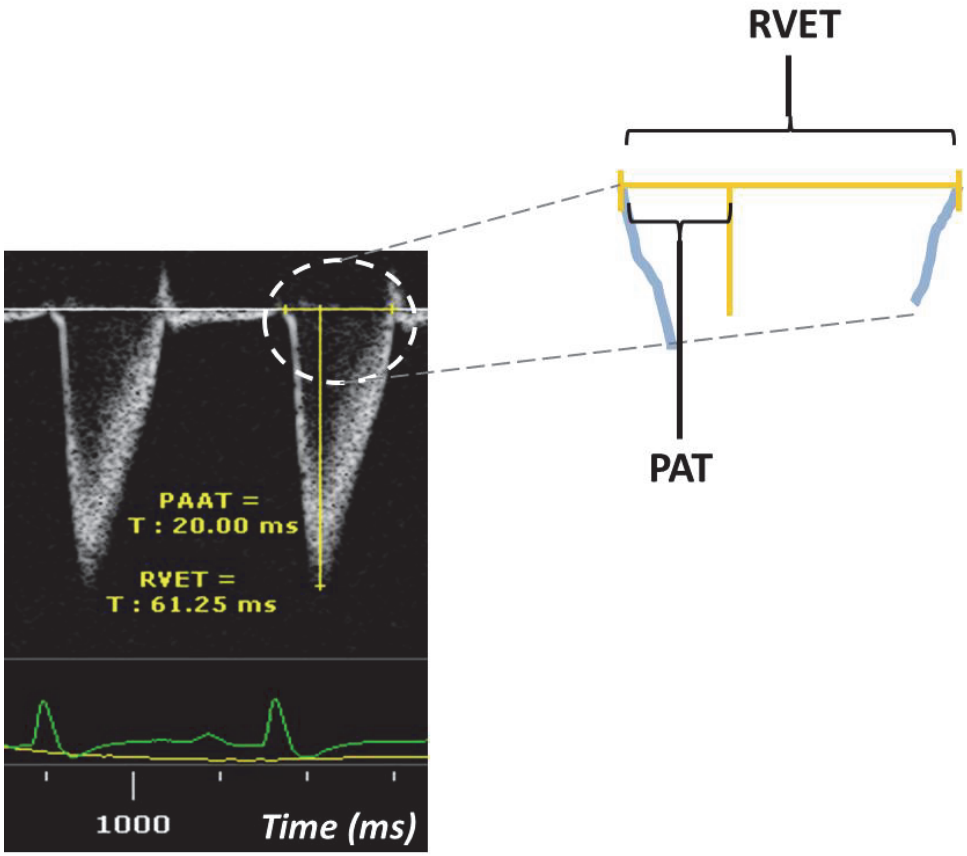
Shown is a representative cross section of a pulmonary blood vessel. The external and internal diameters of the vessel wall are measured as indicated. Their difference yields wall thickness, which is then used in the formula described earlier to deduce percent MWT.

2.7 Pulmonary artery echocardiography

Pulse-wave Doppler of pulmonary outflow was recorded in the parasternal view at the level of the aortic valve. The sample volume was placed proximal (3 mm) to the pulmonary valve leaflets and aligned to maximize laminar flow. Pulsed-wave Doppler of pulmonary outflow was recorded in the parasternal view at the pulmonary valve level. All evaluations were performed with a (maximal) sweep speed of 200 mm/s (McMurtry et al., 2004).

After recording the pulmonary outflow Doppler profile, the velocity-time integral was derived by tracing the outer edge of the profile. Pulmonary arterial acceleration time (PAT) was measured from the time of onset of systolic flow to peak pulmonary outflow velocity. Right ventricular ejection time (RVET) was measured as the time from onset to completion of systolic pulmonary flow (Figure 2-4). The PAT measurements were normalized with RVET or heart rate for comparisons (Jones et al., 2002).

Figure 2-4 Representation of pulmonary outflow Doppler profile with measurements.



Shown is a sample recording of the pulmonary artery outflow in a 14 day old rat pup, using pulsed-wave Doppler. Pulmonary artery acceleration time (PAT) was measured from the time of onset of systolic flow to peak pulmonary outflow velocity. Right ventricular ejection time (RVET) was measured as the time from onset to completion of systolic pulmonary flow.

2.8 Lung function testing by forced oscillation technique (FlexiVent^{TM})

Animals were anesthetized using ketamine (10 mg/kg) and xylazine (5 mg/kg) mixture and spontaneous breathing was abolished by an intraperitoneal injection of the muscle relaxant, pancuronium bromide (1 mg/kg). An 18-gauge cannula inserted securely into the trachea was used to connect the mice to a computer-controlled small animal ventilator (FlexiVent, Scireq, Montreal, QC). The mice were quasi-sinusoidally ventilated with a tidal volume of 10 ml/kg at a frequency of 150 breaths/minute and a positive end-expiratory pressure (PEEP) of 3 cm of H2O. Measurements of static and dynamic compliance were obtained using the following perturbations- total lung capacity (TLC) maneuver, single compartment (snapshot) and pressure-volume loops with constant increasing "pressure" (PVr-P) (Vanoirbeek et al., 2010; Yee et al., 2009). The mentioned parameters were measured at least thrice and the data with a coefficient of determination (COD) of 0.9 or above were selected for analysis.

2.9 Intra-jugular cell administration

This technique was used to administer intra-jugular injections in rag-/- mice or nude rats. After halothane anesthesia, the neck was dissected with a midline incision and the internal jugular vein was located and prepared. Using a 30-gauge needle (Becton-Dickinson, Mississauga, ON, Canada) 100 μL of DMEM containing 100,000 cells was slowly and directly injected into the jugular vein. After closing the incision with biological glue (3 mol/L, Vetbond, St. Paul, MN), the mice or rats were allowed to recover.

2.10 MTT assay to assess *in vitro* cell viability

The assay is based on a mitochondrial-dependent reduction of the yellow tetrazole 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT; Invitrogen, Eugene, OR) to purple colored formazan. The measured absorbance after dissolution of this colored product is proportional to the number and metabolic viability of the cells contained in the tested sample.

The cells to be examined were plated in equal numbers in 24 well plates and maintained in uniform culture conditions. During assessment, the monolayers of the tested cell samples were washed with phosphate buffered saline (PBS) and topped with fresh, serum-free Dulbecco's Modified Eagle Medium (DMEM). MTT was dissolved in PBS (5 mg/mL), filtered, warmed and added to each well to give an effective MTT concentration of 0.5 mg/mL, in the medium. After 2 hrs of incubation in regular culture conditions (5% CO_2 at 37^{o}C in a humidified incubator), the medium was removed and the cell monolayers were washed with PBS (x1). The formed formazan crystals were dissolved in dimethyl sulfoxide, and the absorbance of each sample was spectrophotometrically measured at 550 nm with a Spectra Max 190 microplate reader (Molecular Devices, Downingtown, PA).

2.11 Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Three types of statistical analysis were used in this thesis as appropriate; (i) Paired t-test, when comparing the population mean of two matched groups obtained from paired observations from the same sample set, (ii) Student's unpaired t-test, when comparing the means of two independent populations and (iii) One way analysis of variance (ANOVA) to test for differences amongst three or more independent groups. Fisher's least significant differences (LSD) or Bonferroni's post-hoc analyses were used to determine significant differences in values. For all analyses, a p-value <0.05 was considered as being statistically significant. Data were compiled using Excel (Microsoft Office Excel; Microsoft Corporation, Redmond WA) and imported into SPSS Statistics v17.0 (SPSS Inc., IBM Canada Ltd., Markham, ON) for statistical analysis and to generate graphs for publication.

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Chapter Three: **Activation of Akt Protects Alveoli from Neonatal Oxygen-induced Lung Injury**

Explanatory Note:

Majority of the experimental work described in this paper was performed by ARSA, with technical assistance from Vadivel A, Eaton F, Barr AJ and Boisvenue J. The manuscript was written by ARSA and edited by BT. Data presented in this chapter has been published in the journal "American Journal of Respiratory Cell and Molecular Biology", American Thoracic Society (Alphonse, R.S., Vadivel, A., Coltan, L., Eaton, F., Barr, A.J., Dyck, J.R.B., and Thébaud, B. (2011). Activation of Akt protects alveoli from neonatal oxygen-induced lung injury. Am. J. Respir. Cell Mol. Biol. 44, 146–154).

3.1 Introduction

Premature births represent 10% of all births, but account for 85% of all perinatal morbidity and death (Goldenberg et al., 2008). Extremely premature neonates (born at less than 28 weeks of gestation) are at an increased risk for bronchopulmonary dysplasia (BPD), a chronic lung disease of infancy, associated with significant mortality and long term morbidity (Kinsella et al., 2006). The hallmark of BPD is simplification of the lung alveoli and absence of vascularized alveolar ridges (Jobe and Bancalari, 2001). Multiple risk factors including oxygen toxicity and barotrauma contribute to the pathogenesis of BPD (Jobe and Bancalari, 2001). Prolonged exposure to supraphysiological oxygen concentrations results in increased production of cytotoxic oxygen free radicals that can overwhelm the host antioxidant defense mechanisms (Auten and Davis, 2009) and cause lung injury, characterized by death of alveolar epithelial cells (AEC) and endothelial cells (Crapo et al., 1980).

Hyperoxia induced cell death is multimodal and may involve necrosis and apoptosis, two distinct mechanisms of cell death that have specific biochemical, morphological and functional characteristics (Barazzone et al., 1998a). In necrosis, injury leads to extensive disruption of plasma membrane and cell lysis. In contrast, apoptosis is a more regulated form of cell death that involves the activation of proteases and nucleases within an intact plasma membrane (Majno and Joris, 1995). Of these two primary modes of cell death, apoptosis has been described to predominate in hyperoxia-mediated lung injury in various animal models *in vivo* (Mantell et al., 1999; McGrath-Morrow and Stahl, 2001a;

Otterbein et al., 1998) and was observed occurring primarily along the alveolar surface of the peripheral airways (Barazzone et al., 1998b; Chowdhury et al., 2006a).

Apoptosis is a fundamental feature contributing to organ morphogenesis. Apoptosis regulates cell number by removing the superfluous cells during their rapid developmental proliferation. Controlled by intercellular signaling, apoptosis also plays a pivotal role in tissue remodeling and homeostasis (Chowdhury et al., 2006a; Kerr et al., 1972). In the lung, cell-specific and temporally coordinated apoptosis contributes to normal lung development (Del Riccio et al., 2004). Yet, the role of apoptosis during the canalicular and alveolar stage of normal lung development is incompletely understood. Likewise, apoptosis occurring above the physiologic levels in response to injury (hyperoxia for example) during the critical alveolar stage of lung development may contribute to impaired alveolar architecture. Several reports have documented elevated epithelial cell apoptosis in lungs of preterm infants with respiratory distress syndrome (RDS) or early BPD (Hargitai et al., 2001; Lukkarinen et al., 2003; May et al., 2004). Dysregulated TGF-β pathway along with Fas-mediated epithelial cell apoptosis have also been implicated in the pathogenesis of BPD (Del Riccio et al., 2004).

The biological pathways controlling apoptosis are exquisitely organized at the molecular level and several pro- or anti-apoptotic regulatory molecules have been investigated thus far (Chittenden et al., 1995; Williams and Smith, 1993). Akt/protein kinase B, the downstream target of phosphotidylinositol-3-kinase (PI3K), is one such vital regulatory molecule responsible for maintaining cell

viability (Kandel and Hay, 1999). Its activation has been shown to improve survival in a wide range of cell types.

We hypothesized that exaggerated apoptosis would disrupt normal alveolar development and conversely, protection of AEC death via activation of the pro-survival Akt pathway would prevent arrested alveolar development in experimental oxygen-induced BPD. We show that pharmacological inhibition of Akt impairs alveolar development in newborn rats. We also demonstrate that activated Akt is decreased in lungs of rats with oxygen-induced BPD. In addition, we show that adenoviral mediated over-expression of activated Akt protects oxygen-induced alveolar epithelial cell death *in vitro* and preserves alveolar architecture in experimental oxygen-induced BPD *in vivo*.

3.2 Materials and Methods

All procedures were approved by the Animal Health Care Committee of the University of Alberta.

Some of the methodologies used in this paper have already been described in chapter 2 and are not repeated here. The following sections concern methods specific to this study and not described elsewhere in this thesis.

3.2.1 Pharmacological PI3K/Akt inhibition

Wortmannin (1.4 mg/kg/day, Sigma, St. Louis, MO.) was administered subcutaneously to rat pups from postpartum days 4-14 (P4-14) during the alveolar period of lung development. The dose was based on a previous publication (Singh et al., 2001). Body weight was assessed at P14 and lungs were harvested the same day for immunoblot analysis and lung morphometry.

3.2.2 Immunoblot Analysis

Lysed cell or lung homogenates were subjected to SDS-PAGE in gels containing 5% or 8% acrylamide and transferred to nitrocellulose. Membranes were blocked in 5% milk/1x TBS/0.1% Tween 20 and then immunoblotted at 1:500 dilution (unless otherwise specified) with either rabbit anti-phospho-Akt (Ser-473), rabbit anti-Akt, rabbit anti-tubulin, or rabbit anti-phospho-S6 ribosomal protein (Ser-240/244) (1:1000 dilution) (Cell Signaling Technology, Danvers, MA) in 5% bovine serum albumin/1x TBS/0.1% Tween 20 overnight at 4 °C as we previously described (Kovacic et al., 2003). After being washed extensively, the membranes were incubated with peroxidase-conjugated goat anti-rabbit secondary antibody in 5% milk/1x TBS/0.1% Tween 20. After further washing, the antibodies were visualized using the Amersham Biosciences-enhanced chemiluminescence Western blotting detection system.

3.2.3 Cell Line and Culture Conditions

RLE-6TN, a rat lung epithelial cell line (ATCC Culture Collection, Rockville, MD) was maintained in F-12 medium in 75-cm^2 tissue culture flasks at 37^{o}C in 5% CO_2-95% air. The medium was supplemented with 10% (vol/vol) fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. RLE cell monolayers were lifted from the flasks by addition of 2 mL of 0.05% trypsin and were subcultured on 75-cm^2 tissue culture flasks or seeded on 24-well tissue culture plates (BD Labware, Franklin Lakes, NJ) or 4-well chamber slides (Costar Life Sciences, Wikes Barre, PA) at a density of (12.5 - 20.0) x103

cells/well for the various experiments. Cells were allowed to attach overnight, transduced with Ad.Akt or Ad.GFP and were cultured in normoxic control conditions (5% CO_2-95% air) or exposed to hyperoxia (95% oxygen/5% CO_2, Xvivo, Biospherix, Lacona, NY) for periods from 24-48 hrs with media change every 2 days.. All cell culture media, supplements and chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

3.2.4 Preparation of Recombinant Adenoviral Vector

Replication-deficient adenovirus expressing green fluorescent protein (Ad.GFP) or the constitutively active mutant of Akt1 (Ad.Akt) were constructed as we have previously described (Chan et al., 2004; Kovacic et al., 2003).

3.2.5 In vitro Akt Transduction

Based on preliminary studies an MOI (molecules of infection) of 25 pfu/cell (particle forming units) was used for the *in vitro* transduction studies. RLE cells seeded on culture plates or T75 flasks were incubated in complete tissue culture medium for ~18 h, as described above. At this time, the medium was removed and replaced by serum-free medium containing the adenoviral particles (Ad.Akt or Ad.GFP) for 8-10 hours. The medium was then aspirated and replaced with 10% FBS containing medium. The cells were then either cultured in normoxia or hyperoxia for 48 hrs before the assays were done.

3.2.6 In Vivo Ad.Akt Intratracheal Administration

Gene delivery into the airways of newborn rats was performed through intratracheal puncture at P4 as previously described (Thebaud et al., 2005). After halothane anesthesia, the trachea was exposed through a neck incision. The gene of interest (25 μL) was delivered through a tracheal puncture with a short, 30-gauge needle (Becton-Dickinson). After closure of the incision with biological glue (Vetbond, 3 mol/L), rats were allowed to recover (Vadivel et al., 2009).

3.2.7 Analysis of Apoptosis

Protein expression for active caspase 3 (ab13847, Abcam, Cambridge, MA, USA) in whole lungs or RLE cell lysates was measured with immunoblotting using available antibodies, as previously described (Vadivel et al., 2009). The intensity of the bands was normalized to the intensity of a reporter protein (actin) using the Kodak Gel-doc system (Kodak, Rochester, NY).

3.3 Results

3.3.1 Pharmacological inhibition of Akt with wortmannin impairs alveolar development in newborn rats

In vivo, subcutaneous injection of wortmannin in rat pups between P4-14 during the alveolar period of lung development reduced body weight and decreased lung weight to body weight ratio (Figure 3-1A). Wortmannin efficiently decreased lung phospho-Akt (P-Akt) levels at serine 473 (Figure 3-1B), which has been shown to be highly indicative of Akt activation (Kovacic et al., 2003).

Wortmannin treatment had no effect on FRAP/mTOR kinase activity measured by phosphorylated S6 ribosomal protein expression (Figure 3-1B). This decrease in P-Akt was associated with enlarged air spaces reminiscent of BPD as reflected by a 35% decrease in the RAC in Akt-inhibited lungs compared to controls assessed at P14 (Figure 3-1C).

3.3.2 Activated lung Akt expression is decreased in hyperoxia-induced BPD in newborn rats

Rat pups exposed to hyperoxia from P4-14 displayed the characteristic features of BPD including alveolar simplification, with larger and fewer alveolar airspaces and decreased septation as compared with room air controls (Figure 3-2A) decreasing the RAC significantly by 33% in the lungs of hyperoxia exposed pups (Figure 3-2B). Immunoblotting of lung homogenates revealed a marked decrease in the expression of activated Akt in the hyperoxic rat pups in comparison with room air controls (Figure 3-2C).

3.3.3 Akt activation improves survival of RLE cells exposed to hyperoxia

RLE cells of all treatment groups were exposed to 95% oxygen for 48 h and control cells were exposed to room air. Efficient adenovirus-mediated gene transfer was confirmed by green fluorescence of RLE (Figure 3-3A) and increased P-Akt levels (Figure 3-3B). The viability of RLE cells, assessed by a colorimetric MTT assay, was significantly decreased in hyperoxia as compared with room air housed cells (Figure 3-4A). Ad.Akt significantly increased survival in comparison with hyperoxia-exposed untreated and Ad.GFP infected groups.

3.3.4 Akt activation decreases apoptosis of RLE cells exposed to hyperoxia

RLE cells cultured in 95% O_2 for 48 hrs showed an increase in activated caspase-3 expression (Figure 3-4B) compared to normoxic control cells, suggestive of apoptosis. Ad.Akt gene transfer significantly protected RLE-cells from O_2-induced apoptosis. Ad.GFP gene transfer had no protective effect and showed demonstrable apoptotic activity (Figure 3-4B).

3.3.5 Adenoviral mediated Akt gene transfer preserves alveolar architecture in hyperoxia-induced BPD in newborn rats

Based on these *in vitro* data and since Akt inhibition decreases alveolarization and because oxygen-induced arrested alveolarization is associated with decreased P-Akt levels, we investigated the therapeutic potential of Akt gene therapy to restore normal alveolarization in the rat BPD model. *In vivo* intratracheal adenovirus mediated Akt gene therapy increased lung P-Akt levels (Figure 3-5A). Newborn rats exposed from P4-14 to hyperoxia displayed the characteristic features of arrested alveolar development with larger and fewer alveolar airspaces and decreased septation as compared with normoxic animals. *In vivo* Akt gene therapy preserved alveolar growth in hyperoxic rats as assessed by the RAC (Figure 3-5B). Hyperoxic lungs showed increased active caspase 3 expression compared to room air control lungs (Figure 3-6). Akt gene therapy significantly attenuated lung apoptosis (Figure 3-6).

3.3.6 Akt activation reduces pulmonary hypertension in hyperoxia-induced BPD in newborn rats.

Chronic exposure to hyperoxia was associated with a significant increase in RVH and MWT of small pulmonary arteries (Figure 3-7). Akt activation attenuated these structural features of pulmonary hypertension as indicated by a reduction in RV/LV+S and MWT.

3.4 Discussion

Here, we show that activation of the prosurvival factor Akt is important for normal lung development and demonstrate the therapeutic potential of postnatal, intratracheal Akt gene transfer in experimental oxygen-induced neonatal lung injury. As proof of concept, we showed that inhibition of Akt phosphorylation with wortmannin, a pharmacological upstream inhibitor of Akt, impaired alveolarization, mimicking BPD. Conversely, irreversible oxygen-induced hypoalveolarization was associated with decreased activation of Akt. Furthermore, Akt overexpression through intratracheal Akt gene therapy improved survival and was able to preserve and restore normal alveolarization in the experimental model of oxygen-induced BPD.

Apoptosis is a distinct form of programmed cell death characterized by loss of cell function and rapid morphologic changes, culminating in cell death without inflammation (Chowdhury et al., 2006a; Kerr et al., 1972). It is a fundamental feature in embryogenesis, where it is necessary for fashioning of the body and molding of various organs (Vaux and Korsmeyer, 1999). In addition to

its role in developmental biology, apoptosis is also important for remodeling of tissues and the progression of diseases (Chowdhury et al., 2006b). In the lung, apoptosis occurs in a cell-specific, spatio-temporally coordinated (Kresch et al., 1998a) manner during all stages of gestational as well as postnatal development (Kresch et al., 1998b; Schittny et al., 1998). Epithelial apoptosis has been implicated as a major contributor of lung remodeling during late gestational development. Factors affecting the rate of apoptosis during lung development influence the branching pattern of the developing lung. Prosurvival factors such as epidermal growth factor (Henson and Gibson, 2006; Warburton et al., 1992) and bombesin (Kresch et al., 1999) have been observed to enhance branching morphogenesis of the distal lung. On the other hand, proapoptotic agents such as TGF-β and mullerian inhibiting substance, inhibit branching morphogenesis (Catlin et al., 1997; Serra et al., 1994).

A significant portion of the mammalian lung development takes place after birth, where apoptosis emerges as an important process in the transformation of the air-filled distal lung saccules into functional alveoli. Vascular endothelial growth factor, known to exhibit anti-apoptotic effects on AEC prevents alveolar damage in hyperoxic lung injury in neonatal rats (Kunig et al., 2005; Thebaud et al., 2005). More interestingly, through a series of elegant studies, de Paepe and colleagues implicate the Fas/FasL system as a critical regulator of type 2 AEC apoptosis in physiological alveolar remodeling (De Paepe et al., 2004; De Paepe et al., 2000). Recently, using a transgenic mouse model, they further show that FasL overexpression targeted to postcanalicular stages of lung development is

sufficient to induce AEC apoptosis and arrested alveolar development, mimicking BPD (De Paepe et al., 2008). By analogy, we hypothesized that promoting or inhibiting the prosurvival Akt pathway will affect lung alveolar development. Similar to FasL overexpression (De Paepe et al., 2008), pharmacological Akt inhibition, impaired alveolarization and produced histological changes reminiscent of BPD in newborn rats.

Above physiological levels, apoptosis can lead to ultrastructural abnormalities and may be a potential culprit in reducing the number of alveoli, characteristic of BPD. Interventions necessary to support respiratory function in premature infants (including mechanical ventilation and oxygen therapy) may compromise the fine balance between growth and apoptosis in the developing lung, leading to arrested alveolar development. Extensive alveolar epithelial apoptosis has been widely reported in adult rodent lungs (Buckley et al., 1998; Kazzaz et al., 1996; McGrath-Morrow and Stahl, 2001a) following hyperoxic injury. In neonatal mice exposed to hyperoxia for only 3.5-6.5 days, caused apoptosis in the alveolar epithelium (McGrath-Morrow and Stahl, 2001b). The extent of apoptosis was proportional to the duration of hyperoxic exposure. In the above instances, failure of prosurvival mechanisms in the oxygen-challenged AEC could have made them more susceptible to apoptotic elimination. To confirm the possible involvement of PI3K/Akt in this apoptotic response, we proceeded to determine the level of lung Akt expression in newborn rats exposed to 95% oxygen, from postnatal day 4 to 14. The lungs of these animals showed alveolar simplification, the characteristic hallmark of BPD. Interestingly,

expression of activated Akt was decreased in the lungs of hyperoxia-exposed animals. This observation indicates the potential role of the prosurvival PI3K/Akt pathway in determining the ability of AEC to resist the hyperoxic challenge.

Following this observation, we were interested in the next logical step of determining the possible therapeutic benefits of activating the PI3K/Akt pathway in the lung. Exciting insights into the pro-survival role of Akt have been revealed by studies in other organ systems (Franke et al., 1997). Overexpression of activated Akt prevented apoptosis in primary cultures of cerebellar neurons that were injured by survival factor withdrawal or inhibition of PI3K. Expression of dominant-negative forms of Akt interfered with growth factor mediated survival in these cells, indicating that Akt is necessary and sufficient for neuronal survival (Dudek et al., 1997). Similar observations have been made *in vitro* on fibroblasts, lymphoid and epithelial cell lines. Interestingly, activation of PI3K/Akt signaling improved survival of lung AEC-like A549 cells (Bao et al., 2005) exposed to Fas-mediated apoptotic stimuli. Extending this observation, we hypothesized that Akt activation would inhibit hyperoxia-mediated AEC apoptosis and preserve lung integrity. As a first step, we determined the protective effect of Akt activation on RLE cells exposed to hyperoxia. RLE cells exposed to 95% O_2 underwent apoptosis measured by cleaved caspase-3 expression. Activated caspase-3 expression was significantly decreased by transfection of the activated Akt gene. Genetic activation of Akt also significantly improved cell survival in RLE cells cultured in hyperoxia. Finally, we show that *in vivo* intratracheal Akt gene therapy protects lung architecture and prevents alveolar growth arrest in newborn rats

exposed to hyperoxia from postnatal day 4 to 14. These observations reaffirm the importance of PI3K/Akt mediated signals in protecting the lung epithelium from apoptotic stimuli that mediate oxidant-induced lung damage.

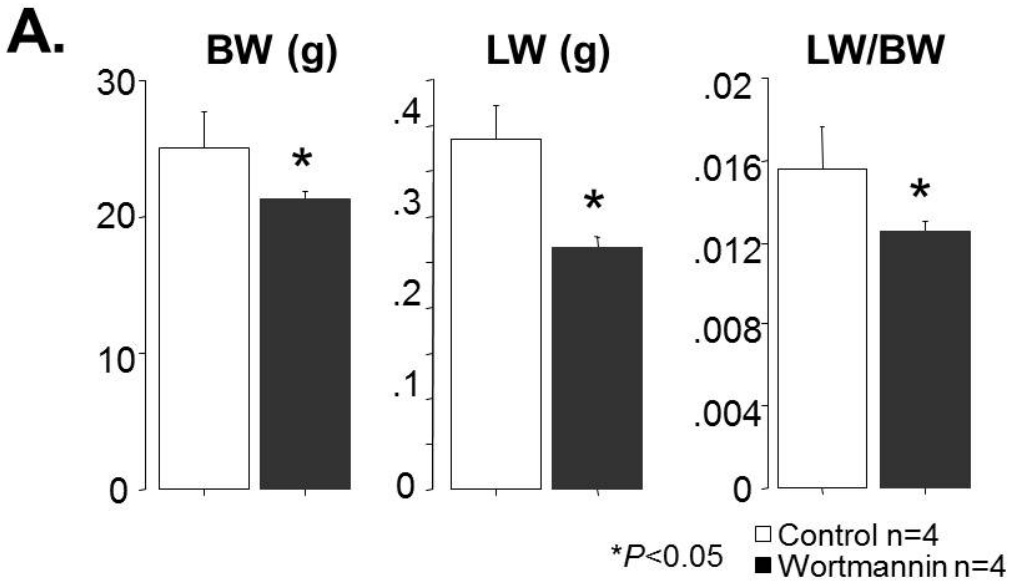
Akt activation has already been shown to improve survival and delay the onset of acute lung injury in hyperoxia exposed adult mice (Singh et al., 2001). But those experiments were performed using adult mice; our observation addressed the question of the effect of Akt overexpression on septation and alveolarization of the neonatal lung during the late canalicular through alveolar stage of lung development. Since lungs of adult animals exposed to hyperoxia respond very differently from that of developing lungs of premature newborns (Frank et al., 1978), our studies bring in a new insight on the role of PI3K/Akt prosurvival pathway on distal lung development and hyperoxia defense and complement the recent observations by de Paepe et al (De Paepe et al., 2008; De Paepe et al., 2004; De Paepe et al., 2000). However, a treatment strategy based on Akt activation does carry the potential harm of inhibiting physiological apoptosis and inducing proliferation in cells other than alveolar epithelial cells- fibroblasts in particular (Wu et al., 2009). This can result in enhanced fibrosis and inefficient alveolar repair and recovery. A possible way to circumvent this adverse effect is to develop targeted gene delivery or pharmacological approaches that specifically protect the AEC2.

In summary, we demonstrate that activation of the prosurvival factor Akt prevents alveolar epithelial damage and preserves alveolar development in newborn rats subject to oxygen-induced lung injury. Our results corroborate

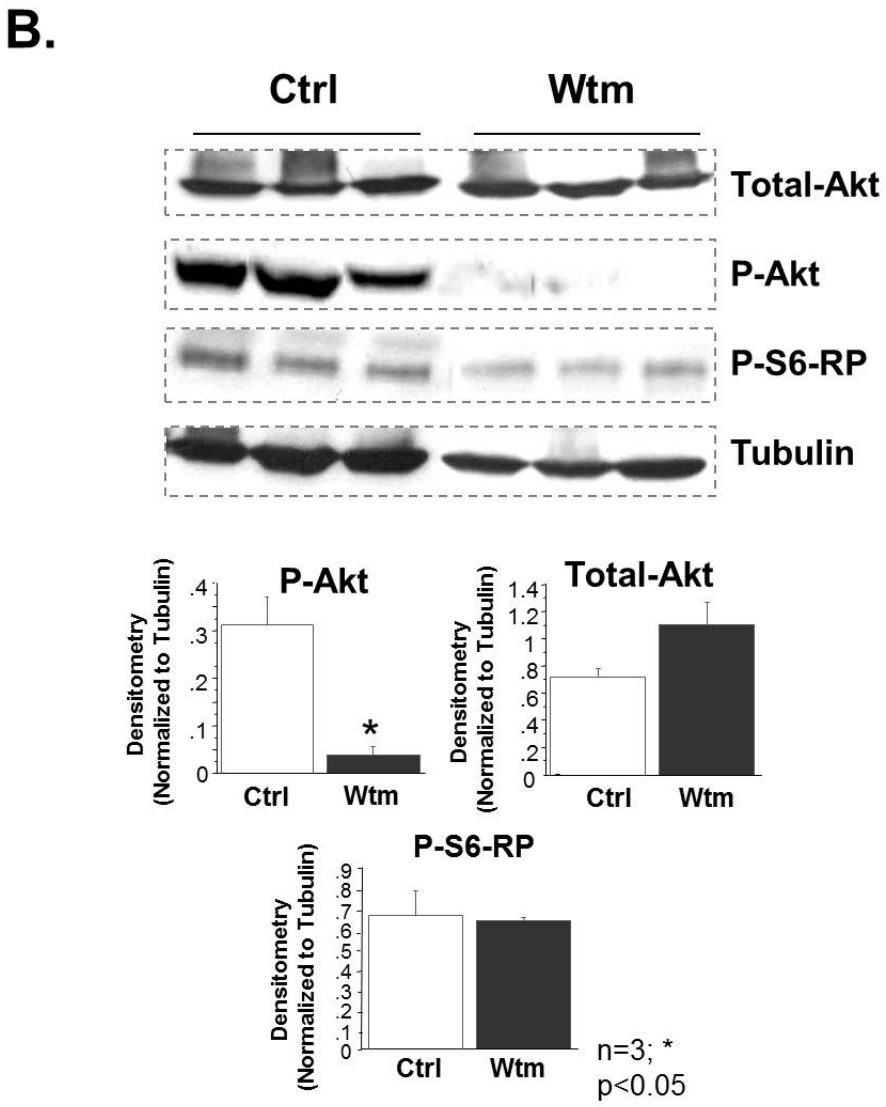
previous investigations and further establish a framework to dissect the role of PI3K/Akt mediated inhibition of apoptosis in the human lung epithelium. We speculate that manipulation of apoptotic pathways may have therapeutic potential to prevent lung injury.

Figures

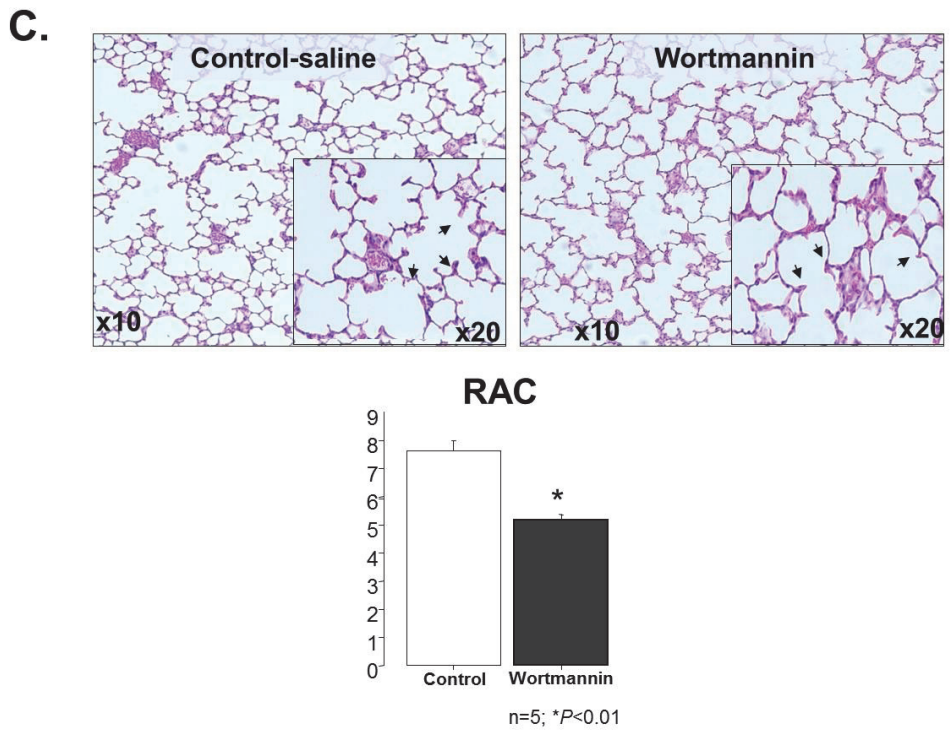
Figure 3-1 Pharmacological inhibition of lung PI3K/Akt impairs alveolar development.



Pharmacological inhibition of lung PI3K/Akt with wortmannin decreases body weight (BW), lung weight (LW), LW/BW ratio (A).

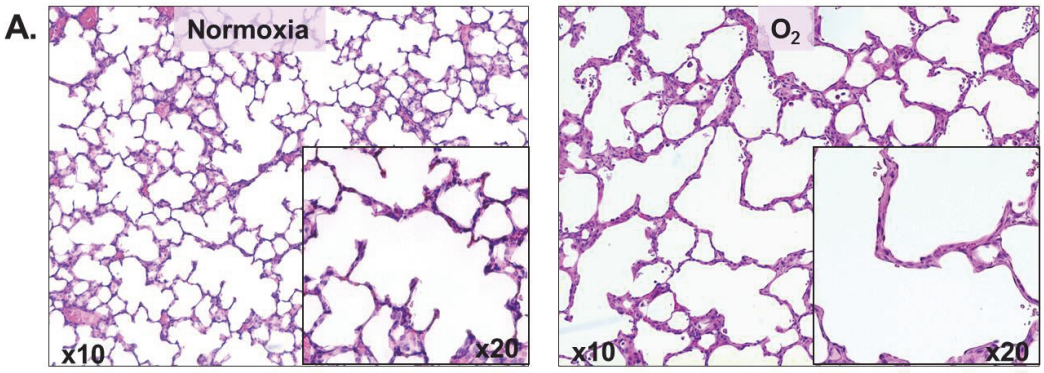


Immunoblot of frozen lung homogenates show decreased phospho-Akt (P-Akt) levels in wortmannin treated groups compared to untreated controls. No significant difference in the levels of phosphorylated S6-ribosomal protein (downstream indicator of mTOR activity) is observed in the wortmannin treated vs. control groups (n=3/group, *P<0.05).

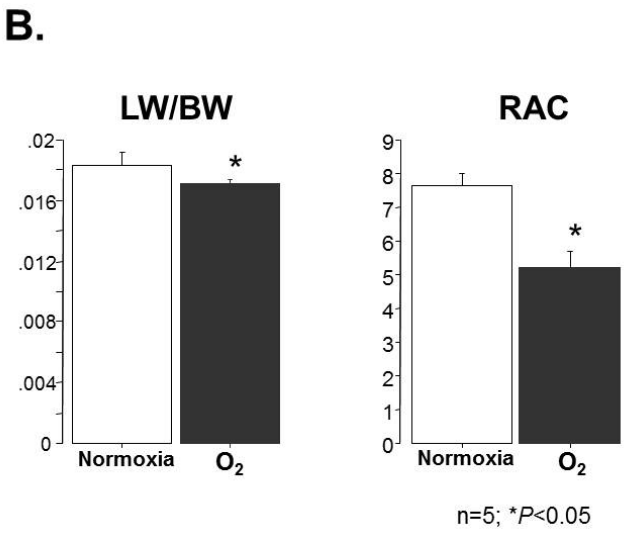


Representative H&E stained lung sections at P14 of wortmannin treated rat pups show alveolar simplification reminiscent of the histological changes seen in BPD and significantly decreased the Radial Alveolar Count (RAC) as compared to control, vehicle (saline) treated animals (n=4/group, *P<0.05).

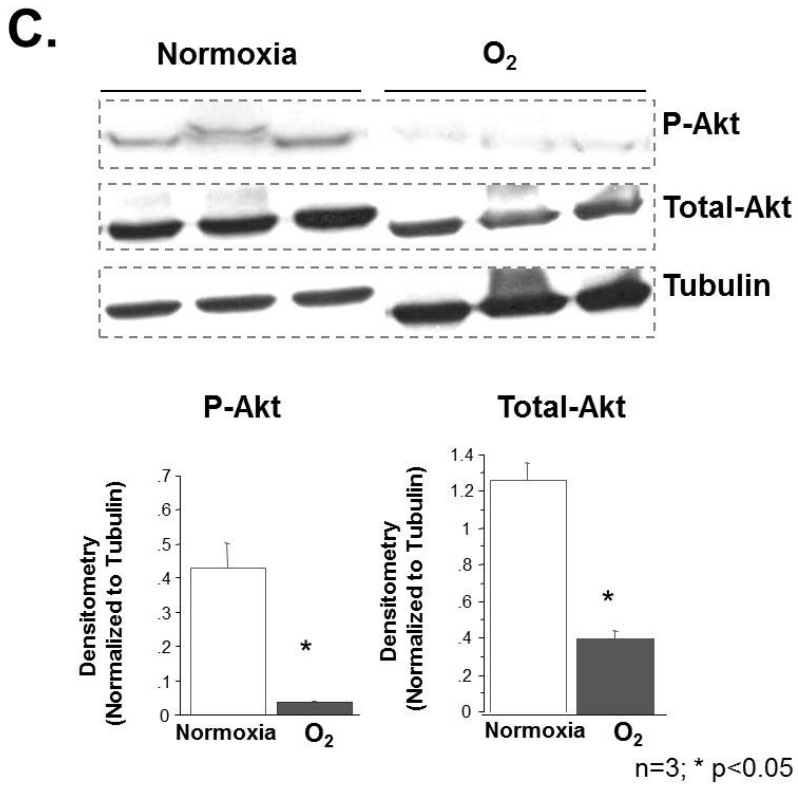
Figure 3-2 Impaired alveolar development in hyperoxia is associated with decreased phospho-Akt expression



Representative H&E stained lung sections of rat pups housed in room air and hyperoxia.

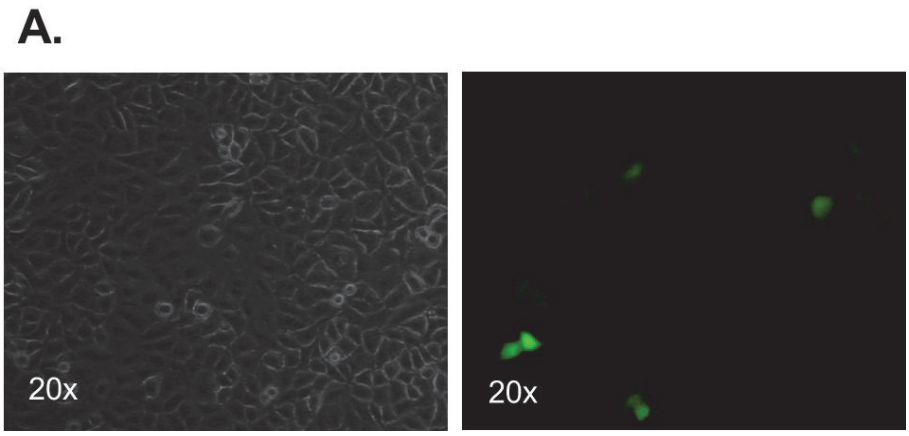


LW/BW ratio and RAC are significantly lower in the hyperoxic group compared to controls (n=5/group *p<0.05).

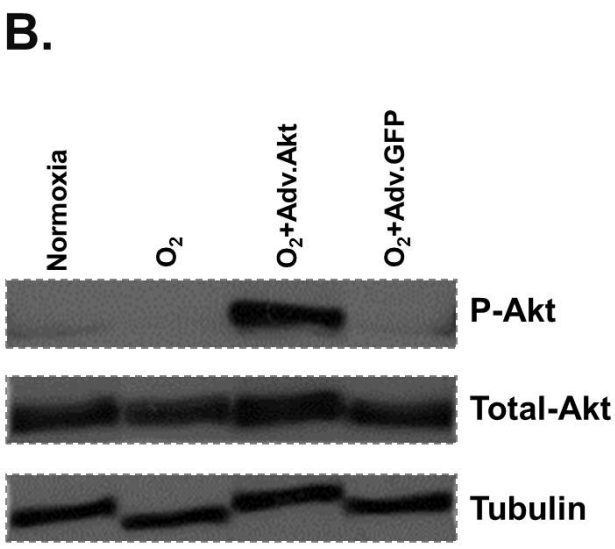


Immunoblot of frozen lung samples shows decreased phospho-Akt (P-Akt) and total Akt levels in hyperoxic group compared to control-room air housed animals (n=3/group *p<0.05).

Figure 3-3 Demonstration of efficient gene transfer *in vitro.*

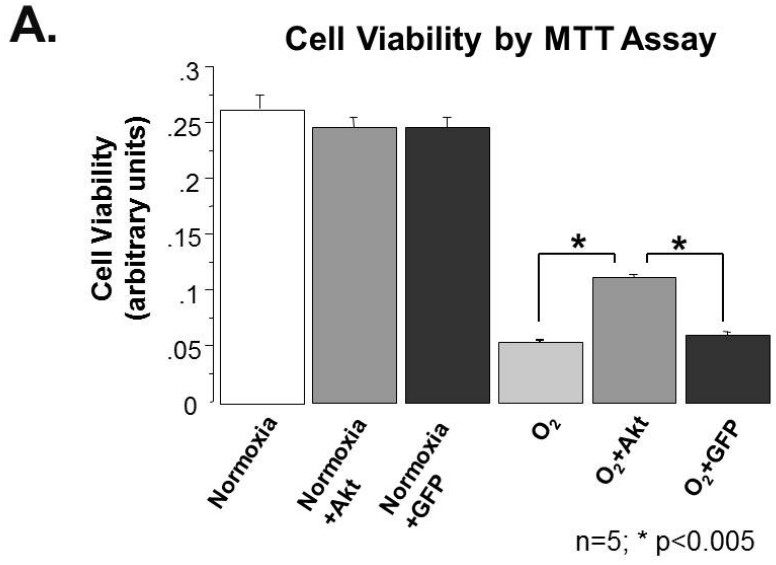


Representative fluorescent microscope pictures showing GFP fluorescence in Ad.GFP transduced RLE cells.

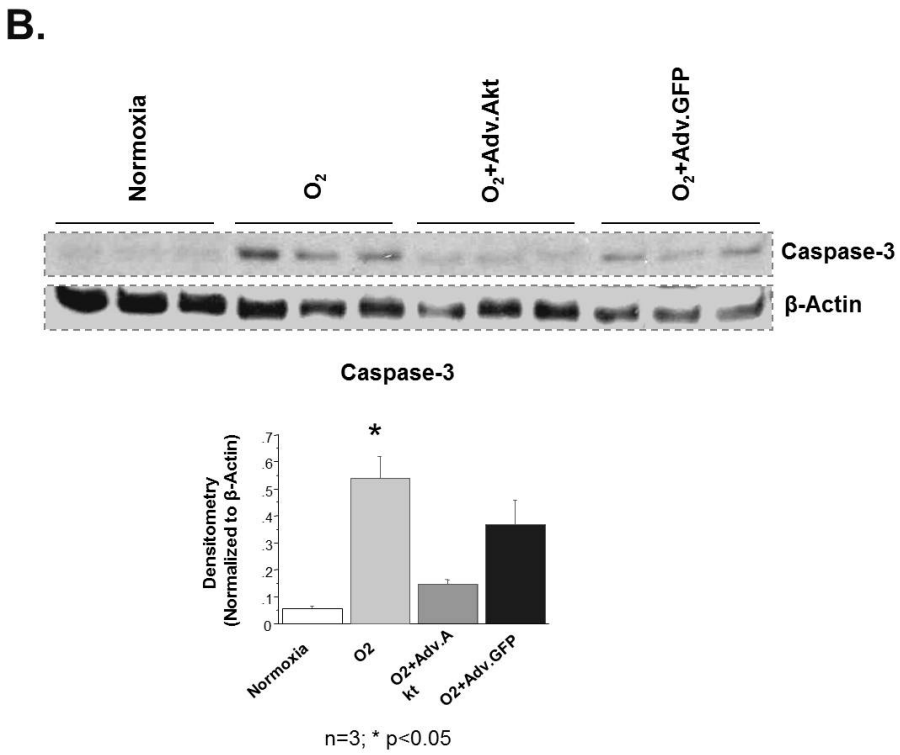


Immunoblots showing increased expression of phospho-Akt (P-Akt) in Ad.Akt transfected RLE cells.

Figure 3-4 Akt activation significantly improves alveolar cell viability and decreases apoptosis *in vitro.*

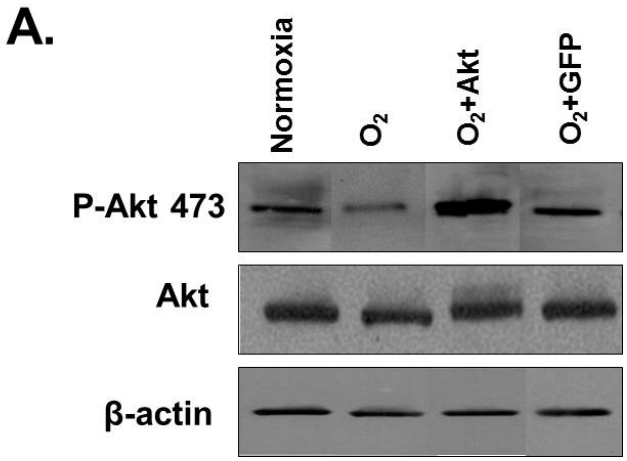


RLE cells were cultured for 48 hrs in room air (normoxic control) or 95% hyperoxia.. Mean data of cell viability as assessed by the MTT assay shows that hyperoxia significantly decreases cell viability as compared with room air exposed cells. Expression of activated Akt significantly improves cell viability in hyperoxia (n=5, *p<0.05)

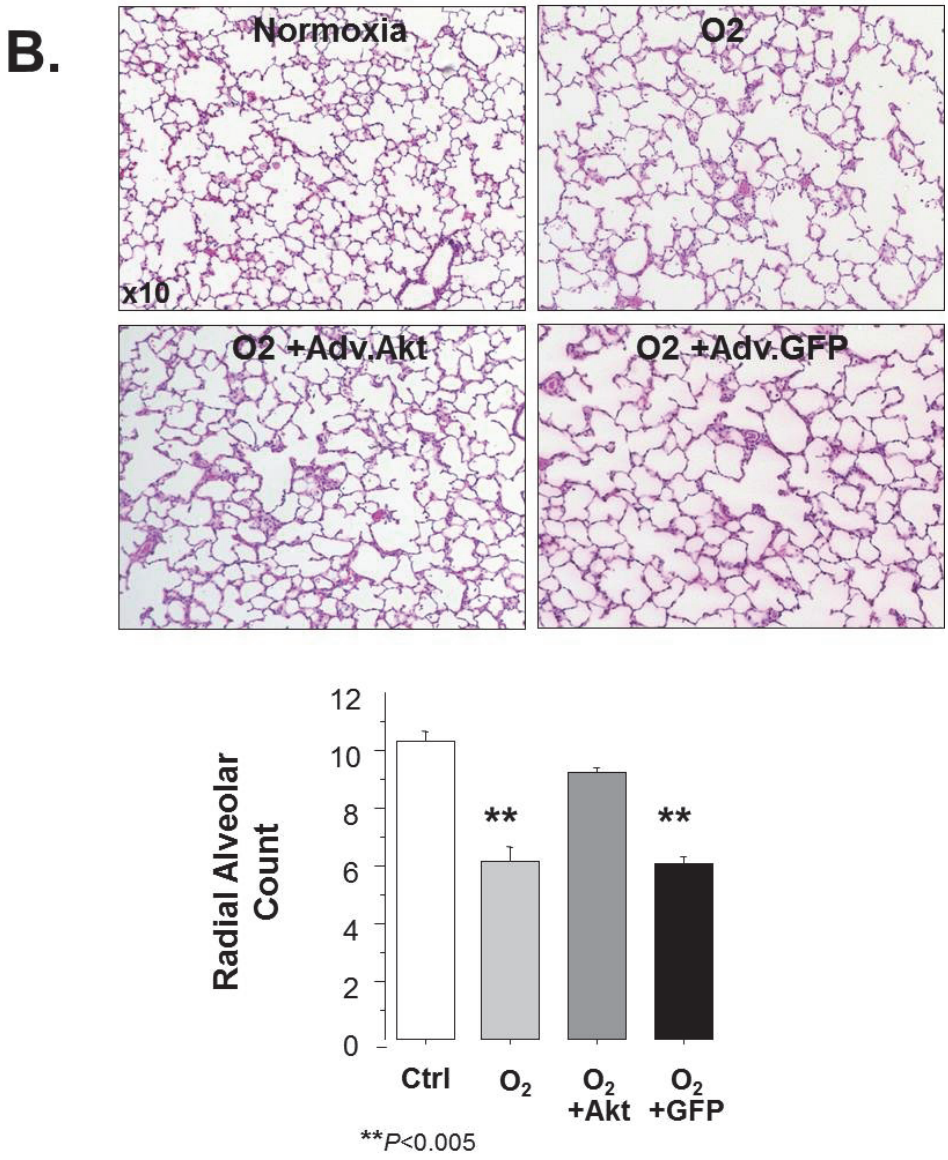


RLE cells were cultured in room air or 95% O_2 for 48 hrs. Mean data of cleaved caspase-3 activity assessed by immunoblotting shows that hyperoxia exposed cells undergo demonstrable apoptosis in comparison with cells cultured in normoxic conditions (B). Cleaved caspase-3 expression was significantly smaller and comparable to normoxic controls in Ad.Akt transfected O_2-exposed RLE cells. RLE cells transfected with Ad.GFP, however, showed apoptotic activity.

Figure 3-5 *In vivo* Adenovirus-mediated Akt gene transfer preserves alveolar growth in oxygen-induced BPD.

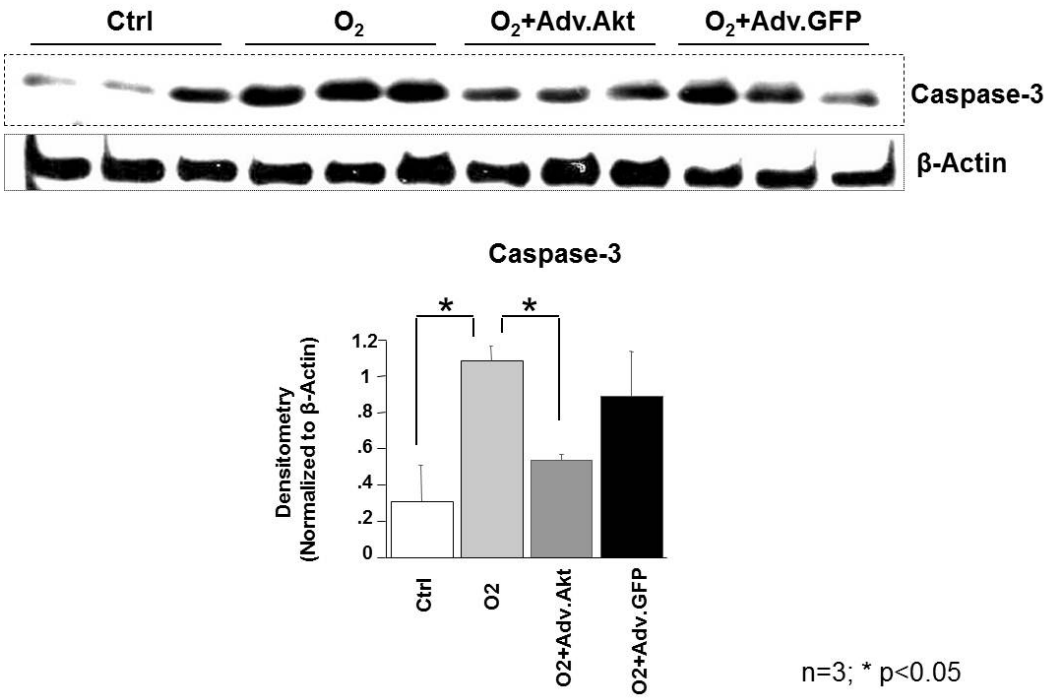


Immunoblots showing decreased phospho-Akt (P-Akt) expression in hyperoxic exposed lungs. Intratracheal Ad-mediated gene therapy, but not Ad.GFP, increased expression of P-Akt in hyperoxic lungs.



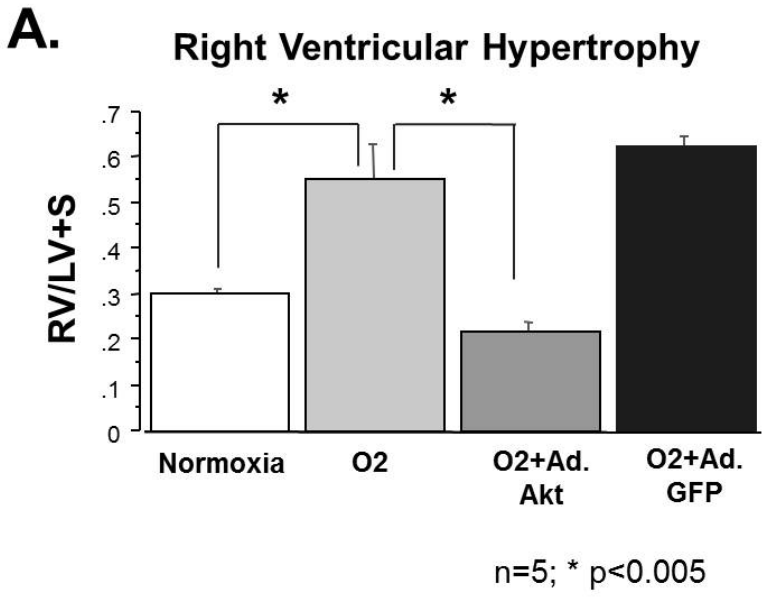
Representative H&E stained lung sections of the 4 experimental groups at P21. Oxygen-exposed lungs display the characteristic features of alveolar simplification (larger and fewer air sacs with decreased septation). Lungs exposed to oxygen and injected with Ad.GFP show the same histological characteristics. Conversely, *in vivo* intratracheal Ad.Akt gene therapy resulted in smaller and more numerous alveoli. Quantification of alveoli structures using the RAC confirms improved alveolarization in animals expressing activated Akt as compared to the other oxygen-exposed groups.

Figure 3-6 *In vivo* Adenovirus-mediated Akt gene transfer decreases apoptosis in oxygen exposed BPD lungs.

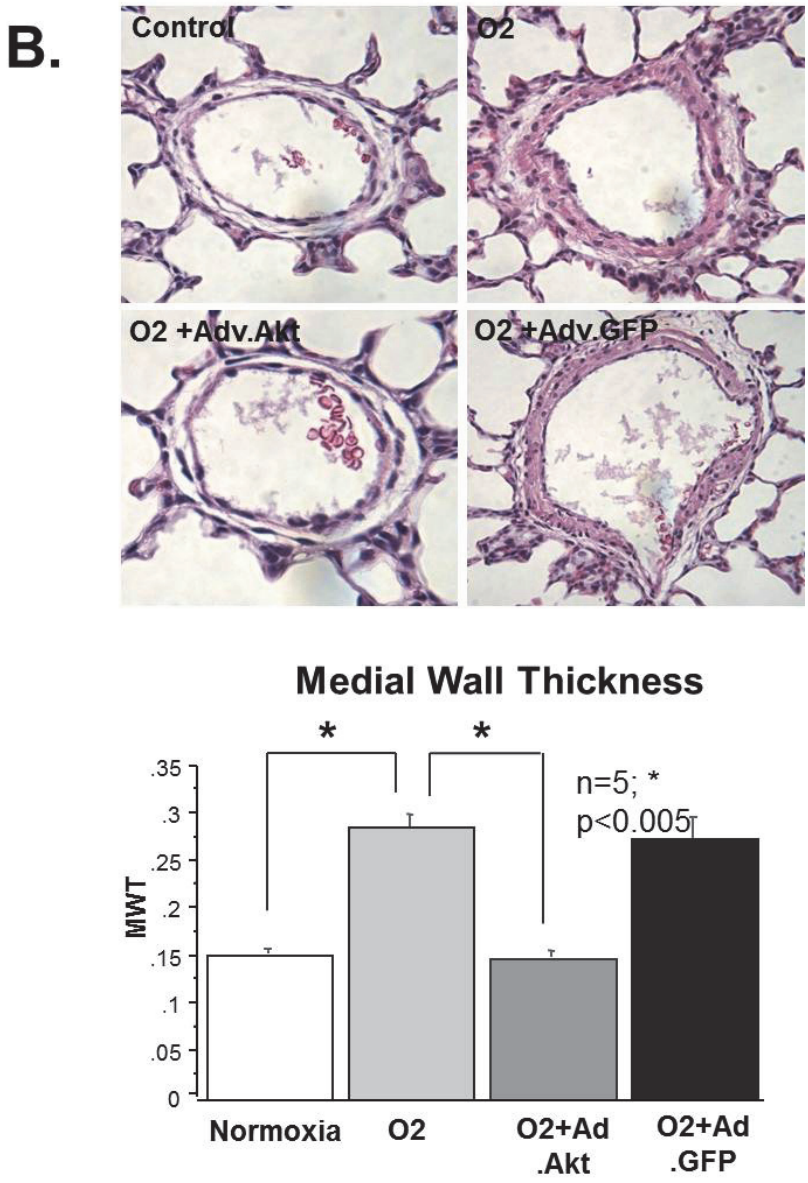


Representative immunoblots of active caspase 3 and alpha-actin in the 4 experimental groups. Hyperoxia exposed lungs showed increased active caspase 3 expression and this was attenuated by *in vivo* intratracheal Ad.Akt gene therapy (n=3/group, *P<0.05). Lungs of the Ad.GFP treated group, however, showed apoptotic activity comparable with hyperoxic controls.

Figure 3-7 *In vivo* Adenovirus-mediated Akt gene transfer decreases RVH and medial wall thickness in a model of BPD.



Hyperoxia-exposed rats had significant right ventricular hypertrophy (RVH) as indicated by the increase in RV/LV+S ratio compared to normoxic controls.



In vivo intratracheal Ad.Akt gene therapy in rat pups housed in hyperoxia significantly reduced RVH (n=4-8/group, *P<0.05) and small pulmonary arterial MWT (n=5/group, *P<0.05) in comparison with untreated hyperoxic controls.

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Chapter Four: **Existence, Functional Impairment and Therapeutic Potential of Endothelial Colony Forming Cells in Oxygen-Induced Arrested Alveolar Growth**

Contributions:
The majority of the experimental work described in this paper was performed by ARSA, with technical assistance from Vadivel A, Fung M, Eaton F, Marshall T and Fan F. The manuscript was written by ARSA and edited by BT.

4.1 Introduction

Vast strides in perinatal care in the last couple of decades have shifted the limit of viability towards gestational ages as low as 23-28 weeks (or 500-1000 g birth weight) (Saigal and Doyle, 2008). However, such extremely preterm neonates are at a high risk of developing chronic lung disease of prematurity (also termed bronchopulmonary dysplasia, BPD), following ventilator and/or oxygen therapy for survival (Jobe, 2011; Kinsella et al., 2006). In addition to prolonged hospitalization and oxygen dependence at 36 weeks of corrected age, BPD leads to protracted respiratory (Allen et al., 2003; Baraldi and Filippone, 2007) and neuro-developmental (Gaddlin et al., 2008; Lemons et al., 2001) consequences that extend into adulthood. Upon onset of the disease process, the currently available management strategies fail to revert alveolar growth arrest or even significantly alter the course of BPD (Gien and Kinsella, 2011). A better understanding of how alveoli and the underlying capillary network develop during normal lung organogenesis and how these mechanisms are disrupted in disease are critical for formulating newer and more effective therapies for BPD.

Evidence suggests a definitive contribution of distal lung vasculature to the development and maintenance of functional alveolar architecture (Stenmark and Abman, 2005; Thebaud and Abman, 2007). Closely simulating human BPD, oxygen or ventilation induced alveolar growth arrest in newborn mammals is consistently associated with decreased distal lung capillary vasculature and extensive pulmonary vascular remodeling (Abman, 2001; Tomashefski et al.,

1984). Strategies promoting lung vascular growth, on the other hand, have preserved alveolar growth in various models of impaired lung development (Jakkula et al., 2000; McGrath-Morrow et al., 2005; Tang et al., 2004; Thebaud et al., 2005). For example, we have shown that the pro-angiogenic factor VEGF in combination with angiopoietin-1 preserves lung alveolarization via promoting angiogenesis in a rat model of oxygen-induced experimental BPD (Thebaud et al., 2005). Conversely, endothelial monocyte activating polypeptide-II (EMAP-II), a negative regulator of vessel formation, has been shown to contribute to BPD evolution (Quintos-Alagheband et al., 2004). These findings, in turn, seem to justify an indispensable vascular component underlying the pathogenesis of BPD.

Extending this understanding, if angiogenic growth factors and adequate lung vascularization contribute to lung development and integrity, then vascular progenitor cells (termed "endothelial progenitor cells" or EPCs) are appealing candidate cells likely to be involved in the same mechanisms. Interestingly, strategies involving mobilization of endogenous EPC from the bone marrow (Ishizawa et al., 2004) or supplementation with exogenous EPC (Balasubramaniam et al., 2010; Takahashi et al., 2004; Zhao et al., 2005) have proved successful in treating experimentally induced lung injury. However, a detailed understanding of the role of EPCs in lung development still remains to be established.

One major limitation in studying EPCs is the lack of a defined set of markers that can unambiguously identify and allow the isolation of this cell type (Timmermans et al., 2009). To date, at least three different methodologies exist

for the isolation of EPCs and consequently EPCs are shown to represent two distinct cell populations- the minimally proliferative hematopoietic or macrophage-like phenotype (that include circulating angiogenic cells and colony forming unit-Hill colonies) and the highly proliferative non-hematopietic phenotype (known as endothelial colony forming cells or ECFCs) (Kirton and Xu, 2010; Yoder et al., 2007). Based on their timing of emergence in culture, these populations have also been described as 'early' and 'late' outgrowth EPCs, respectively (Hur et al., 2004; Yoon et al., 2005). ECFCs, which are phenotypically indistinguishable from cultured endothelial cells, demonstrate a very high potential for proliferation, the capacity for self-renewal and the ability to form blood vessels, *de novo* (Ingram et al., 2004; Yoder et al., 2007). This suggests that ECFCs could be the primary progenitor population participating in neovasculogenesis and in turn makes them putative candidates contributing to the pathogenesis of diseases involving impaired vascular growth, such as BPD. Clinically, ECFC are lower in number in the cord blood of preterm infants who subsequently develop BPD (Borghesi et al., 2009) and these ECFC are seemingly highly vulnerable to oxidative damage (Baker et al., 2009). A thorough study of the contribution of ECFC-dependant vasculogenesis to normal and impaired lung development is necessary for therapeutically harnessing the potential of this newly described cell type.

In the present study, we hypothesized that ECFC-driven angiogenesis is impaired in experimental oxygen-induced BPD and therapeutic ECFC supplementation restores disrupted lung alveolar and vascular growth. Examining

this hypothesis, firstly, we show that ECFCs exist in the distal vasculature of the developing rat lungs. This was done by isolating and carefully characterizing pulmonary vascular (PV)-ECFCs from the distal rat lungs using a newly developed procedure. Secondly, we show that the viability, colony-forming capacity and tube-like network forming potential of PV-ECFCs are decreased in rats with oxygen-induced BPD, compared to controls. Finally, we demonstrate that therapeutic administration of ECFCs derived from human cord blood or its conditioned medium restores alveolar development and attenuates pulmonary hypertension (PHT) in newborn mice subject to hyperoxia-mediated lung injury. These findings suggest that impaired ECFC functionality could, at least in part, underlie arrested alveolar growth in BPD and therapeutic ECFC supplementation might reverse hyperoxia-induced neonatal lung injury.

4.2 Materials and Methods

All procedures were approved by the Animal Health Care Committee of the University of Alberta.

A description of the basic methodologies used across the different projects on this thesis can be found in the relevant section of chapter 2. The following sections concern methods specific to this study and not described elsewhere in this thesis.

4.2.1 Collection of lung tissue samples

To obtain rat lung tissue samples, P14 rats were sacrificed using sodium phenobarbitol (65 mg/kg, i.p.). The anterior chest and abdomen was sprayed liberally with 70% ethanol and the chest was opened with anatomical scissors. The whole lungs along with the heart were removed en bloc and transfered into 50

mL conical tubes containing basal DMEM plus 2% antibiotics. Under aseptic conditions, the peripheral rims of the rat lung lobes were carefully cut out and transferred to Petri dishes containing basal Dulbecco's Modified Eagle Medium (DMEM) plus 2% antibiotics and stored on ice. The harvested rat lungs were processed on the same day.

4.2.2 ECFC isolation and culture

Under strictly aseptic conditions, the lung tissue pieces were chopped using surgical blades and scissors into small pieces (approximately 1-2 mm^2). The pieces were suspended in collagenase/dispase digestive solution (0.1 U collagenase & 0.8 U dipase /mL) (Roche Applied Science, Laval, QC) at 37^oC for 1 hr with intermittent shaking. The lung digest was strained through the 70 mm and 40 mm cell strainers in tandem and washed twice with DMEM plus 10% fetal calf serum (FCS), at 300 g and 4^oC for 10 min. After washing, the cells were resuspended in phosphate buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin (BSA) (termed as MACS buffer) and incubated with streptavidin tagged dynabeads (Dynal, Invitrogen, Burlington, ON) that were pretreated with biotinylated anti-rat or anti-human CD31 antibody (Abcam, Cambridge, MA), as appropriate. The dynabead tagged CD31 positive cells were selected out using a magnetic separator and plated in a 6 well plate (approx. 4000-5000 cells/well) precoated with rat tail collagen type I and placed in a 37^oC, 5% CO_2 humidified incubator. After 24 hrs of culture, nonadherent cells and debris were aspirated, adherent cells were washed once and added with complete Endothelial Growth

Medium-2 (cEGM-2), which was prepared by supplementing Endothelial Basal Medium (EBM)-2 with EGM-2 singlequots (Lonza, Basel, Switzerland), 10% (v/v) fetal bovine serum and 2% (v/v) penicillin/streptomicin (Invitrogen), and 0.25 μg/mL amphotericin B (Invitrogen). Medium was changed daily for 7 days and then every other day up to 14 days. Well circumscribed 'cobblestone-like' ECFC colonies were identified from day 5 by visual inspection using an inverted microscope (Olympus, Lake Success, NY), under 10X magnification. Individual ECFC colonies were marked with a fine tipped marker and isolated using cloning cylinders (Fisher Scientific, Ottawa, ON) and plated in 25-cm^2 flasks pretreated with collagen type I. Upon confluence, PV-ECFCs were plated and expanded in type I collagen coated 75-cm^2 flasks. PV-ECFCs betwen passages 4-8 were used for all experiments. Details on isolation and characterization of PV-ECFCs can be found in Chapter 6.

4.2.3 DiI-acLDL uptake and Ulex europaeus-lectin binding

To assess the ability of the ECFC to incorporate DiI-acetylated-low density lipoprotein (DiI-Ac-LDL), attached cells at passage 4-5 were incubated with 20 μg/mL DiI-Ac-LDL (Biomedical technologies, Stoughton, MA) in cEGM-2 media for 4 hrs at 37 °C, 5% CO_2 in a humidified incubator. Cells were washed several times and fixed with 2% paraformaldehyde for 10 min. After washing with PBS, the cells were reacted with 10 μg/ml fluorescein tagged Ulex europaeus-lectin (Vector Laboratories, Burlingame, USA) for 1 h. Following nuclear counterstaining with Hoechst 33258 the DiIAc-LDL+/FITC-Lectin+

(double-positive) cells were observed with an inverted fluorescence microscope (Leica Microsystems, Richmond Hill, ON, Canada) (Ando et al., 2006).

4.2.4 Immunophenotyping of ECFCs

Early passage (4-5) PV-ECFCs (0.25-0.5 million per sample) were washed in flow buffer (phosphate buffered saline [PBS] containing 0.05% sodium azide and 0.1% bovine serum albumin) and blocked by incubation for 1 hr in 100 μL of flow buffer containing 5% milk. After washing with flow buffer, ECFCs were incubated at 4^{o}C for 30-60 minutes in dark with appropriate concentrations of primary or isotype control antibody, as outlined below, in 50 μL of 5% milk-flow buffer. After washing, the cells were incubated in dark, with comparable concentrations of secondary antibody in 50 μL of 5% milk-flow buffer (this step was skipped if the primary antibody was fluorescence labeled). After washing, antibody labelled ECFCs were analyzed by fluorescence-activated cell sorting (FACS) (FACSCalibur, BD Biosciences, San Diego, CA) (Mead et al., 2008; van Haaften et al., 2009).

For immunophenotyping of rat PV-ECFCs, we used rabbit polyclonal antibodies against rat von Willebrand factor, rat CD133 and rat VEGF receptor 2, which were identified by FITC-conjugated goat polyclonal secondary antibody against rabbit IgG (Abcam, Cambridge, MA). Goat polyclonal antibody was used against rat CD14 along with FITC-conjugated donkey anti-goat IgG secondary. Secondary antibodies, in the absence of their respective primaries, were used as isotype controls, wherever appropriate. FITC-tagged mouse monoclonal

antibodies were used against rat CD31 (Abcam, Cambridge, MA) and rat CD45 (BD Pharmingen, Mississauga, ON). Mouse IgG1,κ antibody conjugated with FITC was used as isotype control.

4.2.5 Retroviral mediated eGFP labelling of PV-ECFCs

By screening multiple Human Immunodeficiency Virus (HIV)-1 based lentiviral vectors for optimal transduction efficiency to ECFCs, we selected a vesicular stomatitis virus (VSV)-pseudotyped lentiviral vector harboring enhanced Green fluorescence protein (eGFP) under the control of ubiquitous Elongation factor (EF)-1 promoter, which we denote as LV-EF-eGFP. To package lentiviral vectors, 293T cells were co-transfected with lentiviral expression plasmid pWPI, packaging plasmid pDelta 8.74 and pMD2G by polyethylenimine (PEI)-based transfection. For transduction, ECFCs in passage 4-5 were incubated overnight with 2 x 106 TU/ml of lentiviral vector in the presence of 7 μg/ml protamine sulfate in cEGM-2. Fresh cEGM-2 was replaced the next day and the wells were examined under a fluorescent microscope for green fluorescence. ECFCs in wells with uniform green fluorescence were trypzinized, expanded and sorted for GFP fluorescence using FACS.

4.2.6 Measurement of tube-like network formation in Matrigel

The formation of cord-like structures by ECFCs was assessed on Matrigel (BD Biosciences, Mississauga, ON) coated 96-well tissue culture plates. 30 μL/well of completely thawed Matrigel was added into the required number of wells and the culture plate was incubated at 37°C for 10 minutes to allow Matrigel

to polymerize. PV-ECFCs from the hyperoxic and RA (control) groups that were maintained in identical culture conditions were seeded in equal numbers (10,000 cells/well) into the Matrigel-coated wells and the plate was incubated for 8-12 hrs in regular cell culture conditions (5% CO_2 and 37°C, in a humidified incubator). The plates were observed using an inverted phase contrast microscope (Leica Microsystems, Richmond Hill, ON, Canada) at 2 hour intervals for capillary-like tube formation. In our experiments, the continuous lattice-like networks usually formed between 6-8 hrs. The capillary like networks were quantified by measuring the number of intersects and the total length of structures, in random fields from each well, using OpenLab (Quorum Technologies Inc, ON, Canada) software (van Haaften et al., 2009).

4.2.7 Estimation of single cell clonogenicity

Pure cultures of rat PV-ECFCs were trypsinized (using Trypsin-EDTA [0.25%], Invitrogen) and made into a suspension in cEGM-2. The FACSAria cell sorter (BD Biosciences, Mississauga, ON) was used to place one ECFC per well in a flat-bottomed 96-well tissue culture plate precoated with type I collagen and containing 200 µL complete EGM-2 media. Cells were cultured at 5% CO_2 and 37 °C in a humidified incubator and culture media was replaced twice/week. At day 14, Hoechst 33258 (Sigma) was added at 3 µg/mL to each well for 10 min, for nuclear detection. The culture plate was examined with a fluorescent microscope at 20x magnification, well by well, for the growth of endothelial cells. Wells with 2 or more endothelial cells were scored as positive. The number of cells per well

was enumerated by visual inspection at 40x magnification. For counting wells containing more than 50 cells, 5 random 40x images of the cells were captured. After making a careful visual estimate of the proportion of the well floor that was covered with cells (P) the following formula was used to calculate the cell counts:

Cell count = N x P(As/Af)

Where N = total number of cells counted; P = percent area of the cell culture surface of a well that is covered by the ECFC colony; As = area of the cell culture surface of a single well of the 96-well plate in cm^2 (0.316 cm^2, supplied in technical data sheet of BD Biosciences falcon); Af = total area of the evaluated microscopic frames under 40x magnification. Colonies with more than 500 cells were trypsinized and resuspended in cEGM-2. Based on the number of cells in that colony, an appropriate amount of cEGM-2 was added and mixed well such that each 200 μL would approximately contain one ECFC. From this suspension, cells were again seeded at single cell density in 96-well plates precoated with collagen type-I, cultured for 2 weeks and evaluated for secondary colonies (or second generation colonies), as described earlier. ECFCs from colonies with more than 500 cells were serially passaged in 24-well and 6-well tissue culture plates followed by 25-cm^2 and 75-cm^2 tissue culture flasks.

4.2.8 Intra-jugular cell administration in mice

After halothane anesthesia, the neck was dissected with a midline incision and the internal jugular vein was located and prepared. Using a 30-gauge needle (Becton-Dickinson, Mississauga, ON, Canada) 100 μL of DMEM containing

100,000 ECFCs was slowly and directly injected into the jugular vein. After closing the incision with biological glue (3 mol/L, Vetbond, St. Paul, MN), the mice were allowed to recover.

4.2.9 Culture of control cells

Human neonatal dermal fibroblasts (hND-Fibs) and human umbilical vein endothelial cells (HUVECs) (ATCC, Manassas, VA, USA) were used as cell controls for *in vivo* conditioned media experiments. Human neonatal dermal fibroblasts (hND-Fibs) were grown in fibroblast basal medium supplemented with FGM growth factor bulletkit (Lonza, Basel, Switzerland). HUVECs were cultured on gelatin-coated culture surfaces using ATCC-formulated Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) fetal bovine serum and 2% (v/v) penicillin/streptomicin, and 0.25 μg/mL amphotericin B. Both cell types were growth in regular cell culture conditions (37^{o}C, 5% CO_2, in a humidified incubator).

4.2.10 Conditioned media preparation

Human umbilical cord blood (hUCB)-ECFCs in passages 4-6 were grown in 75-cm^2 flasks up to 90% confluence in cEGM-2. Following removal of the respective culture medium, the cells were rinsed 3 times with PBS and serum free DMEM was added. After 24 hours, supernatants were collected, concentrated (25x) and desalted by centrifugal filtration (Amicon - Millipore, Billerica, MA) (Ionescu et al., 2011). Upon preparation, CdM was pooled, frozen at –80^{o}C and

thawed right before use. Control cell CdM was obtained from hND-Fibs, by identical technique.

4.2.11 In Vivo CdM Administration

CdM from ECFCs or control cell CdM from hND-Fibs was administered daily intraperitoneally (i.p.) at the dose of 7 μL/g from P4 to P21 (preventive treatment strategy). Animals were harvested on P22.

4.2.12 In vitro wound healing assay

Freshly isolated fetal rat AT2 cells were plated densely on uncoated plastic and incubated overnight in DMEM with 10% serum. On the next day, the confluent monolayers were washed to remove unattached cells and isolation debris. Then a 200 μL pipette tip was drawn across the monolayer, which was rewashed to remove damaged cells. The damaged area was examined microscopically to confirm complete removal of cells and the cultures were incubated at 37^{o}C in plain DMEM, hUCB-ECFC CdM or rat lung fibroblast CdM. Plates were incubated for 12 hours and the plates were observed using an inverted phase contrast microscope (Leica, Richmond Hill, ON, Canada) and images of the wounded surface were captured at 2 hour intervals (Buckley et al., 2008). The images were analyzed with Openlab imaging software (Improvision, Coventry, UK) and the percent wound closure was calculated with the following formula: [(Change in wound area ÷ Initial wound area) x 100] % (van Haaften et al., 2009).

4.2.13 Exercise Capacity

Exercise capacity, using a graduated treadmill exercise protocol by a blinded observer. 10 month old rag-/- mice were run on the rodent treadmill (Columbus Instruments, Columbus, OH) as follows: 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 (van Haaften et al., 2009).

4.3 Results

4.3.1 ECFCs exist in the developing mammalian lung

ECFCs harbored in the peripheral microvasculature of the rat lungs were isolated via enzymatic disintegration of lungs and selecting out CD31 expressing subset of cells. Plating CD31+ cells in type I collagen precoated culture surfaces, in endothelial-specific culture conditions, yielded 'cobblestone-like' colonies (Figure 4-1A) between 4 to 14 days in culture. These ‘late-outgrowth’ colonies were morphologically identified, separated out using cloning cylinders and expanded over 2-4 passages to yield pure cultures of PV-ECFCs. The cultured ECFCs demonstrated basic endothelial cell characteristics such as ingestion of acetylated low-density lipoprotein (Dil-acLDL), binding Ulex europaeus-lectin and tube formation in Matrigel (Figure 4-1A). Rat PV-ECFCs were further phenotypically characterized for the expression of endothelial-specific cell surface

markers such as CD31, vascular endothelial growth factor receptor (VEGFR)-2 and von Willebrand Factor (vWF) (Figure 4-1B) (Ingram et al., 2004; Mead et al., 2008). PV-ECFCs were negative for hematopoietic cell specific CD45 and monocyte/macrophage specific CD14. They were also negative for CD133, a cell surface antigen previously identified on hematopoietic and endothelial progenitor cells (Figure 4-1B) (Rafii and Lyden, 2003).

We assessed the high proliferative and self-renewing capacity of isolated PV-ECFCs reflected by their ability to form colonies when plated at a single cell density. By definition, clonogenic potential is one of the indispensable properties of ECFCs and has been widely demonstrated in ECFCs isolated from other sources such as peripheral blood and cord blood (Ingram et al., 2004). Rat PV-ECFCs demonstrate the ability to generate colonies from single plated cells. Of single plated PV-ECFCs, 17 ± 10.4 % and 26.6 ± 3.0 % of cells formed colonies with 50-500 cells and more than 500 cells, respectively, in 6 independent experiments (Figure 4-1C). When the colonies with 500 or more cells were trypsinized and serially replated as single cells, PV-ECFCs generated secondary and tertiary generations of ECFC colonies. This further testifies the high proliferative and self-renewing potential of these cells. These investigations together confirm the existence of resident vascular ECFCs in the peripheral microvasculature of the developing rat lung.

4.3.2 ECFCs are functionally impaired in hyperoxia-exposed newborn rats.

We isolated ECFCs from the lungs of hyperoxia-exposed and control rats on PD-14 and assessed their survival in culture, clonogenic potential and ability to form 'tube-like' networks in Matrigel (Figure 4-2A). When plated at equal densities and cultured in identical conditions for 3 weeks, PV-ECFCs from the hyperoxia-exposed rats showed reduced survival and cell growth (Figure 4-2B). To more closely evaluate their ability to proliferate and generate colonies at a single cell level, we plated ECFCs obtained from hyperoxia-exposed and control lungs, one cell per well in 96 well plates, and measured the percentage of single ECFCs capable of generating colonies after 14 days in culture. In 3 independent observations, 23.6 ± 2.5% of single plated ECFCs from the control group were capable of forming colonies containing more than 500 cells each. On the other hand, only 9.3 ± 3.2% of ECFCs form the hyperoxia group formed colonies with more than 500 cells ($p<0.05$). No significant difference between the groups was observed in the percentage of cells that formed colonies containing fewer than 50 cells or 50 to 500 cells (Figure 4-2C). However, the numbers of late outgrowth ECFC colonies formed from every 500 CD31+ lung cells plated per sq-cm were comparable between hyperoxia-exposed and control groups, with no significant difference from 5 independent trials (Figure 4-2E). We also evaluated the ability of rat PV-ECFC, from both groups, to form 'tube-like' networks in Matrigel. PV-ECFCs from both groups were suspended in Matrigel, and cultured in room air (21% O_2). Quantitative assessment of the tube-like structures revealed that ECFCs from the hyperoxia-exposed lungs, in comparison with controls, formed networks

decreased in total cord length by 32.7 ± 11.8% ($P < 0.05$) and number of branching points by 18.2 ± 15.3% ($P<0.05$) (Figure 4-2D). These results indicate that the potential of ECFCs to contribute to new vessel formation may be compromised in the lungs of animals exposed to hyperoxia. Together, the above findings suggest a functional deficiency in the ECFCs harbored in the lungs of hyperoxia-exposed newborn rats.

4.3.3 Intra-jugular ECFC therapy reverses alveolar growth arrest and preserves lung vascularity in hyperoxia-induced lung damage.

Exposure of newborn rag-/- mice to hyperoxia (85% O_2) from PD5 to PD14 (Figure 4-3A) induced a histological pattern reminiscent of human BPD, characterized by fewer, enlarged alveolar structures (mean linear intercept [MLI] 41 ± 2.3 vs 25.6 ± 1.9 in RA controls, [$P<0.05$]) (Figure 4-3B). This was associated with increased lung compliance (static and dynamic lung compliances, respectively, 82.8 ± 34.9% and 85.5 ± 22.5% more than control animals, [$P<0.01$]) (Figure 4-3C). In this model, we tested the ability of ECFCs to reverse established alveolar growth arrest, a potentially important perspective for unpredictable and incurable diseases other than BPD, such as pulmonary emphysema. Intra-jugular administration of hUCB-ECFCs (105 cells per animal) at PD14 after established alveolar growth arrest, restored normal alveolar architecture (MLI, 30.3 ± 1.6 vs 41 ± 2.3 in untreated hyperoxia-exposed animals, [$P<0.05$]) (Figure 4-3B) and almost normalized lung compliance (static and dynamic lung compliances, respectively, 25.5 ± 10.9 [$P<0.01$] and 25.6 ± 15.35 [$P<0.05$] less than the untreated hyperoxia-exposed group) (Figure 4-3C). In

addition, we observed that ECFCs had no adverse effect on lung function and structure in room air-housed control animals.

PHT, is an important co-morbidity frequently associated with severe BPD. This manifestation is recapitulated in hyperoxia-induced experimental BPD and is measurable using echocardiography (pulmonary artery acceleration time [PAT] 11.4 ± 0.4 vs 14.9 ± 1.6 in RA controls, [$P<0.05$]) (Figure 4-4A) and cardiac morphology (right ventricular hypertrophy [RVH]; RV/LV-S ratio 0.35 ± 0.05 vs 0.22 ± 0.06 in RA controls, [$P<0.01$]) (Figure 4-4B). ECFC therapy reverted PAT almost to control levels (14.1 ± 2) and significantly reduced RVH (RV/LV-S ratio 0.026 ± 0.03) (Figures 4-4A & B).

4.3.4 ECFCs display long-term safety and efficacy

Intra-jugular delivery of hUCB-ECFCs at PD14 was safe and efficient up to 10 months of life. Serial histological sections of major organs such as brain, lung, heart, liver, spleen and kidneys showed no suspicious images in both RA and O_2-exposed mice treated with hUCB-ECFCs (n=4/group). Exercise capacity, assessed using a graduated treadmill exercise protocol, was significantly decreased in untreated O_2-exposed mice (Figure 4-5A). Mice treated with ECFCs exhibited significantly improved exercise capacity (Figure 4-5A) compared to untreated O_2 animals, at 10 months of age. Significant decrease in pulmonary arterial acceleration time (PAT), indicative of PHT, was persistent in the 10 month old O_2-exposed mice. PAT was maintained at almost control levels in the animals treated with ECFCs (Figure 4-5B). 10 month old RA control animals

treated with whole cell therapy showed no adverse reactions in exercise capacity (Figure 4-5A) or lung structure (Figure 4-5B).

4.3.5 ECFC conditioned medium accelerates AT2 cell wound closure and preserves network-formation by lung ECFCs in Matrigel.

Evidence suggests that EPCs act in a paracrine fashion, secreting factors that promote tissue repair and healing (Di Santo et al., 2009; Kim et al., 2010). We hypothesized that ECFCs could also have a paracrine arm in mediating their therapeutic effects. To verify this hypothesis, we assessed the protective effects of ECFC-CdM using two experimental models representing the epithelial and endothelial compartments of lung alveoli. First, confluent monolayers of AT2 cells were subject to a pipette tip inflicted scratch injury and allowed to close by migration and proliferation in the presence of plain DMEM or hUCB-ECFC derived CdM. At 12 hours, AT2 cell wound closure was significantly higher with ECFC-CdM compared to plain DMEM controls (77.1 ± 7.9 vs 43.7 ± 11.5 in controls; [$p<0.01$]) (Figure 4-6A). Second, human PV-ECFCs in passage 5 of primary culture, were suspended in serum-free matrigel overlaid with plain DMEM or ECFC-CdM and incubated in room air or 95% oxygen. At 6 hrs, the formation of endothelial tube-like networks was assessed by measuring the total cord length and counting the total number of intersections in the networks. Hyperoxia significantly decreased endothelial cord-like structure formation when bathed in plain DMEM, whereas ECFC-CdM significantly counteracted the effect of hyperoxia and preserved network formation (Figure 4-6B). In both assays,

human fibroblast-derived CdM (Fib-CdM) did not demonstrate any beneficial effect observed with ECFC-CdM.

4.3.6 Treatment with ECFC-conditioned media preserves alveolar development and lung vascularity in hyperoxia-induced lung damage.

Exposure of newborn rats to 95% oxygen from P4 to P14 resulted in distal air space enlargement and alveolar simplification (MLI 83.8 ± 12.9 vs 52.8 ± 7.2 in RA controls, [$P<0.001$]) (Figure 4-7) and decreased distal lung vascular density compared to RA animals (MVD 17.7 ± 2.3 vs 37 ± 6.7 in controls, [$P<0.01$]) (Figure 4-8). CdM harvested from serum free cultures of hUCB-ECFCs was administered prophylactically, as daily intra-peritoneal (i.p., 7 ml/g) injections from PD4 to PD21. CdM therapy preserved alveolar architecture (MLI 51.5 ± 3.1) (Figure 4-7) and lung vascularity (MVD 25.5 ± 2.7) (Figure 4-8) to an extent therapeutically comparable to administration of whole cells. ECFC-CdM was also effective in preventing PHT (PAT 20 ± 3 vs 14.6 ± 2.2 in hyperoxia group treated with DMEM [$P<0.05$]; PAT was 21.6 ± 4.3 in RA controls) (Figure 4-9A) and RVH (Figure 4-9B). In order to verify whether the therapeutic effects were specific to ECFC-CdM, we treated a subset of O_2-exposed animals to CdM derived form hND-Fibs (Fib-CdM) and HUVECs (HUVEC-CdM). Alveolar structure, lung vascularity and PAT in the animals treated with either Fib-CdM of HUVEC-CdM was no different from DMEM treated O_2 rats (Figure 4-8 & 4-9A). In addition, it was also clear that CdM from hUCB-ECFCs had no adverse effects on lung structure and lung vascularity in RA controls.

4.4 Discussion

The primary findings in this study are: (a) ECFCs exist in the distal vasculature of the developing rat lung and their functionality, defined by their- (i) survival and viability in culture (ii) capacity to generate colonies from plated single cells (iii) ability to form tube-like networks in matrigel, is impaired in experimental oxygen-induced lung injury. (b) Therapeutic administration of hUCB-ECFCs rescues arrested alveolar growth and maintains normal lung vascularity in oxygen-mediated lung damage. (c) ECFCs are capable of secreting therapeutically potent paracrine factors that prevent impaired alveolar growth and sustain lung vascularization in oxygen-induced lung injury. (d) Therapeutic benefit of ECFC whole cell administration persisted up to 10 months in rats, with no serious adverse effects.

BPD currently affects preterm infants born as early as 24 weeks of gestation, still in their late canalicular or early saccular stage of development (Bourbon et al., 2005). The extreme immaturity of the lungs in these preemies, in conjunction with their need for oxygen therapy and assisted ventilation, predisposes them to this chronic lung disease (Gien and Kinsella, 2011). Alveologenesis involves budding of septal crests, which is followed by elongation of the septal walls to form individual alveoli. This process of septation is disrupted in BPD resulting in alveolar simplification and enlargement (Coalson, 2003). Coupled with impaired alveologenesis, alveolar angiogenesis is also interrupted in BPD, resulting in striking distal lung microvascular paucity (Bhatt et al., 2001). Previously it was believed that newly formed blood vessels in the

lungs passively follow developing airways (Hall et al., 2000). However, this has been increasingly challenged by recent research testifying the interconnectedness between alveolarization and angiogenesis (Abman, 2001; Thebaud and Abman, 2007). Secondary abnormalities occur in one process when the other is primarily affected (Jakkula et al., 2000; McGrath-Morrow et al., 2005). Alternatively, improving lung vascularity effectively limits impairment in alveolarization (Kunig et al., 2005; Thebaud et al., 2005). A closer understanding of the mechanisms involved in lung vascular development, therefore, can offer newer tools to contain the incidence and severity of BPD.

Currently, two processes are known to primarily contribute to new blood vessel formation- vasculogenesis (*de novo* formation of vessels from primitive hemangioblasts) and angiogenesis (sprouting of new vessels from the already existing vessels) (Risau, 1997). The latter has so far been considered as the only mechanism active in postnatal life. The discovery of circulating EPCs by Asahara et al in 1997 (Asahara et al., 1997), followed by two decades of research in this direction, has established that vasculogenesis also occurs in postnatal life via in situ incorporation, differentiation and/or proliferation of EPCs (Ribatti et al., 2001). Clinically, evidence correlates higher circulating EPC numbers with improved outcomes in acute lung injury (Burnham et al., 2005), bacterial pneumonia (Yamada et al., 2005) and sepsis (Rafat et al., 2007) and with a reduced risk for cardiovascular disease (Hill et al., 2003). A subsequent set of studies have tested therapeutic targeting of circulating EPC numbers to restore various organs such as the heart (Kocher et al., 2001; Schuster et al., 2004), brain

(Zhang et al., 2002), retina (Otani et al., 2002), and ischaemic hindlimb (Kalka et al., 2000; Sasaki et al., 2006), after experimental injury. Similar strategies have also been successfully explored in models mimicking newborn and adult lung diseases (Ishizawa et al., 2004; Takahashi et al., 2004; Zhao et al., 2005). More specifically, a recent study demonstrates that treatment with bone marrow-derived angiogenic cells restores lung structure in a model of hyperoxia-induced BPD (Balasubramaniam et al., 2010). Nevertheless, the characteristics defining the various angiogenic cell populations and the significance of their contribution to new vessel formation in lung development and repair are still not established.

Recent advances in EPC research has drawn in fresh insight into the identity of the different cell types that have been described to contribute to new vessel formation (Kirton and Xu, 2010). Among them, ECFCs, a population of late outgrowth EPCs, have received widespread attention as lineage-specific endothelial progenitors participating in neovasculogenesis (Ingram et al., 2004; Steinmetz et al., 2010; Yoder, 2010). ECFCs reside throughout the vascular endothelium contributing to generalized vascular integrity (Ingram et al., 2005) and are also believed to be mobilized into a circulating pool of endothelial progenitors, putatively involved in neovasculogenesis via tissue recruitment and homing (Yoder et al., 2007). Thanks to the growing evidence on the role of ECFCs in neovasculogenesis, it is very likely that these cells underlie the pathogenesis of diseases such as BPD, characterized by disrupted vascular growth. A systematic evaluation of the behavior of circulating and tissue resident

ECFCs is paramount to understanding the origin and evolution of BPD and hence, evolving an effective therapeutic solution.

Evidence does indicate an existing association between circulating ECFC numbers and their susceptibility to hyperoxia in preterm neonates at risk of contracting BPD. A study on cord-blood samples of preterm infants with gestational age of less than 32 weeks (or birth weight <1500 g) indicate that extremely preterm infants have lower numbers of circulating ECFCs at birth and are proportionately at a higher risk of developing BPD (Borghesi et al., 2009). Another study recovered higher numbers of ECFCs from the cord-blood of preterm infants at 28-35 weeks gestation compared to term cord-blood (Baker et al., 2009). However, the ECFCs from the preterm cohort had an increased susceptibility to hyperoxia compared with term ECFCs (Baker et al., 2009). Even though earlier studies suggest the existence of a subset of highly proliferating resident EPCs in the pulmonary microvasculature of adult rats (Alvarez et al., 2008), specific details about their presence and their response to injury in the developing mammalian lung have so far not been rigorously investigated. The current study addresses this by first, isolating and characterizing ECFCs that are resident in the microvasculature of the developing rat lung; second, assessing the functionality of PV-ECFCs from rats exposed to hyperoxia-mediated lung damage.

Rat PV-ECFCs were isolated from enzymatically disintegrated distal lung tissue of PD-14 rats, using a novel procedure developed based on the preexisting protocol for recovering circulating ECFCs from peripheral blood (Mead et al.,

2008). The isolated rat PV-ECFCs demonstrated all properties that have been described earlier (Ingram et al., 2005; Mead et al., 2008) to characterize ECFCs. PV-ECFCs were essentially endothelial in nature and demonstrated acLDL uptake, bound Ulex-lectin and formed tube-like networks in Matrigel. They expressed endothelial-specific surface markers (CD31, VEGFR2 and vWF) and were negative for CD14 and CD45 expression, indicating their non-myeloid/non-hematopoietic origin. In addition, ~25% or more of the isolated PV-ECFCs were able to generate colonies with 500 or more daughter cells, when plated as single cells. Cells from those colonies could also be replated into secondary and tertiary generations of ECFC colonies with comparable cell numbers. This clonogenic property specifically characterizes ECFCs, distinguishing them from mature endothelial cells (Ingram et al., 2005).

Suggested by earlier observations on the susceptibility of circulating ECFCs to oxidant-damage (Baker et al., 2009; Ingram et al., 2007), we suspected a functional deprivation in resident PV-ECFCs in the hyperoxia-injured lungs. We evaluated ECFC functionality in terms of their viability in culture, production of colonies from cultured single cells and formation of endothelial networks in Matrigel. ECFCs from the hyperoxia-injured lungs were significantly deprived of their ability to survive and grow in culture, generate colonies with more than 500 daughter cells per seeded cell and produce tube-like networks in Matrigel. However, contrary to earlier observations on lung EPCs (Balasubramaniam et al., 2007; Irwin et al., 2007), we did not observe any differences in the numbers of ECFC colonies obtainable by plating equal numbers of whole lung cells, between

the hyperoxia-exposed and control groups. This could be a possible limitation of the currently available technique of isolating and obtaining pure cultures of PV-ECFCs. Identification of more robust ECFC markers facilitating easier isolation can obviate this shortcoming in future studies.

We then speculated that upon therapeutic supplementation, ECFCs could rescue the lungs from O_2-mediated BPD-like damage. To demonstrate this repair potential, we injected hUCB-ECFCs therapeutically into a neonatal mouse model of oxygen-induced arrested alveolar growth. ECFCs restored lung function and alveolar architecture after established lung injury. ECFCs promoted neovascularization in the oxygen-injured lungs and effectively prevented PHT and RVH, co-morbid conditions commonly associated with BPD. The observed therapeutic effectiveness of ECFCs in restoring lung structure and function was comparable with that of approaches using bone-marrow derived mesenchymal stem cells (van Haaften et al., 2009) or early outgrowth EPCs (Zhao et al., 2005), in closely related models of lung injury. It is noteworthy that the cord blood source of therapeutic cells described in this study is clinically relevant and readily available from the perspective of treating neonatal diseases. More interestingly, we also observed an extended therapeutic benefit that lasted in the ECFC-treated mice for up to 10 months, demonstrable by preserved exercise capacity and pulmonary hemodynamics. No tumors were detectable by examining serial histological sections of major organs such as brain, lung, heart, liver, kidney and spleen at 10 months post-ECFC injection. No other adverse effects were noticed in the cell treated animals.

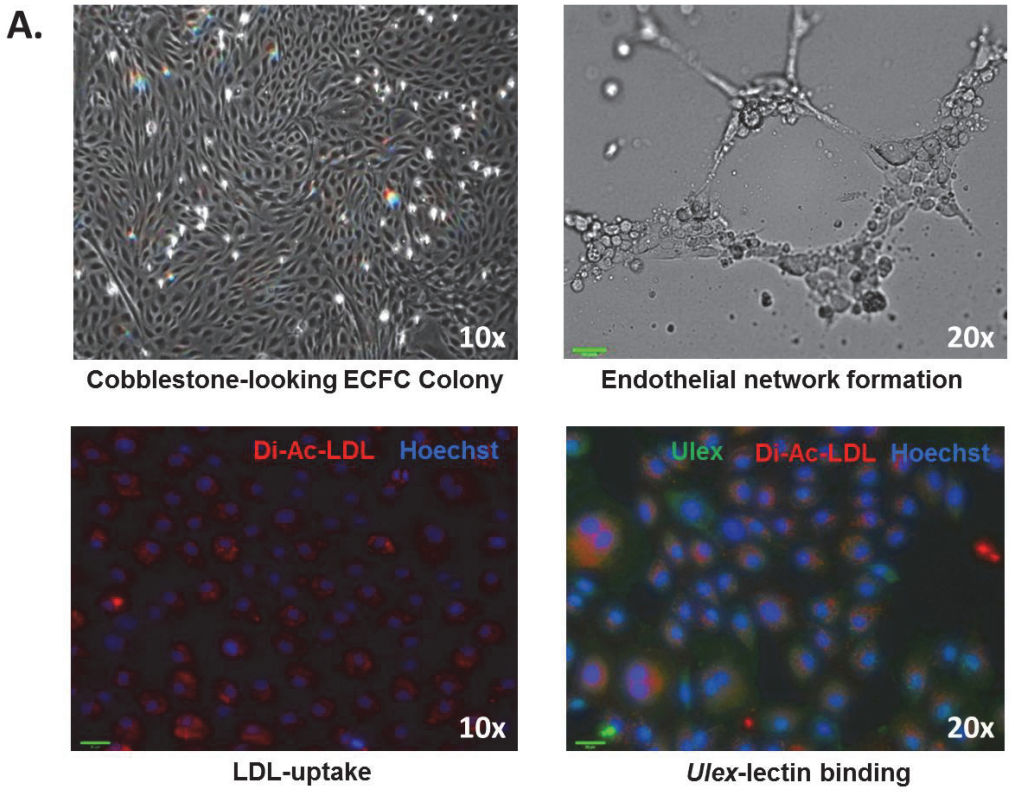
Furthermore, a growing body of research suggests that most of the beneficial effects of stem cell therapy are accounted for by stem cell-derived paracrine mediators (Lee et al., 2011; Prockop et al., 2010; Weiss et al., 2008). In particular, studies testing EPC-based treatment strategies, demonstrate therapeutic equivalence between whole cells and EPC-conditioned media (Di Santo et al., 2009; Krenning et al., 2009; Yang et al., 2010). We therefore speculated the possibility of a paracrine effect underlying the therapeutic benefit observed with ECFC treatment. To test this we examined the protective effect of ECFC-CdM using *in vitro* and *in vivo* models of lung injury. *In vitro*, we observed that ECFC-CdM accelerates alveolar epithelial wound healing and prevents hyperoxia-mediated inhibition of endothelial cord-like network formation in Matrigel. *In vivo*, very similar to our experience with CdM from bone marrow-derived mesenchymal stem cells (Waszak et al. [in press]; Pierro et al. [unpublished observations]), daily i.p. injections of ECFC-CdM effectively prevented lung alveolar and vascular growth disruption in the established newborn rat model of experimental BPD. This observation clearly demonstrates an additional paracrine arm underlying the therapeutic effect of hUCB-ECFCs. However, more studies are necessary to elucidate the "active ingredient(s)" in the ECFC-CdM that is/are responsible for the observed therapeutic benefits.

In conclusion, we show for the first time that ECFCs exist in the distal vasculature of the developing mammalian lung and their functional capacity is impaired in O_2-induced experimental BPD. We also show that therapeutic supplementation with hUCB-ECFCs represents a new and safe cell based therapy

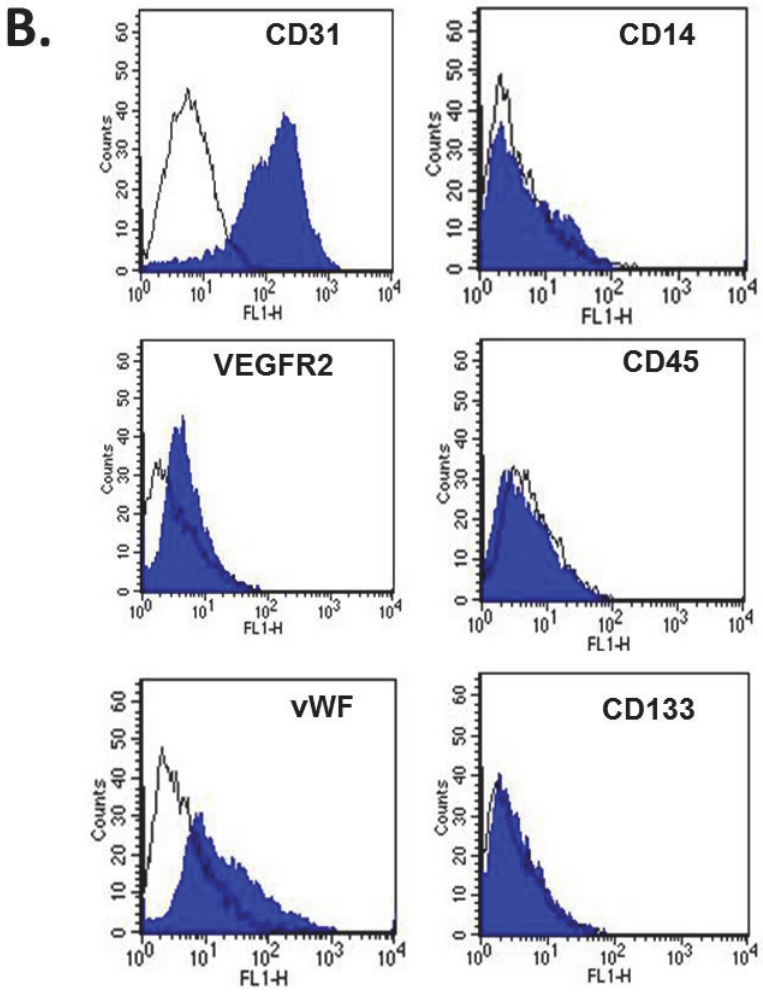
to prevent and treat lung diseases such as BPD, characterized by alveolar damage and impaired lung vascularity. In addition, observations in this study suggest a potential paracrine mechanism which, at least in part, explains the observed therapeutic benefits attributable to ECFCs. More studies in this direction are clearly indicated to definitively elucidate the contribution of ECFCs to mammalian lung development and hence evolve more effective therapies to contain the incidence and progression of BPD.

Figures

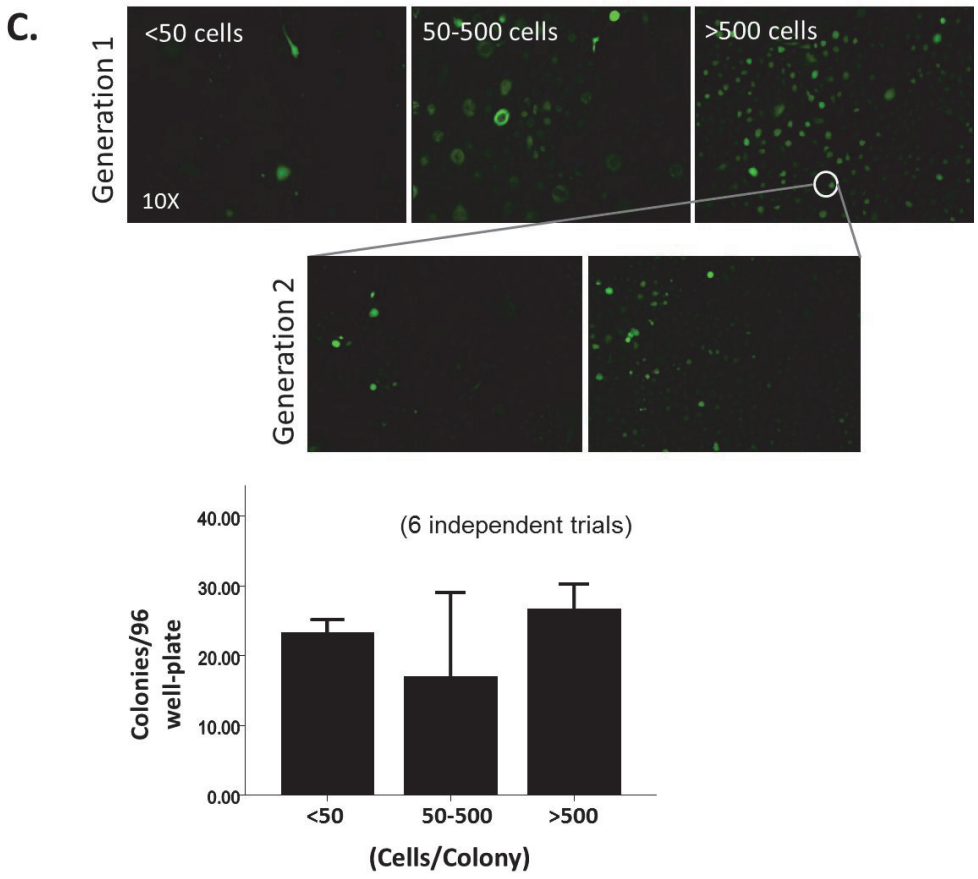
Figure 4-1 ECFCs exist in the newborn rat lung



Phenotypic analysis of rat PV-ECFCs was performed using morphological and functional characteristics. PV-ECFCs formed 'cobblestone' colonies when cultured as a monolayer. The cells were visualized by phase contrast microscopy at 10x magnification. PV-ECFCs formed tube-like structures when suspended in matrigel and were observed by light microscopy (20X magnification). PV-ECFCs ingested Dil-acLDL (red) following 4 hrs of incubation. Nuclei were counterstained with Hoechst 33342 to confirm that all adherent cells were positive for LDL-uptake. PV-ECFC bound *Ulex europaeus*-lectin (green) following 1 hr of incubation after fixation. Nuclei were counterstained with Hoechst 33342 to confirm that all adherent cells are positive for *Ulex* lectin-binding.

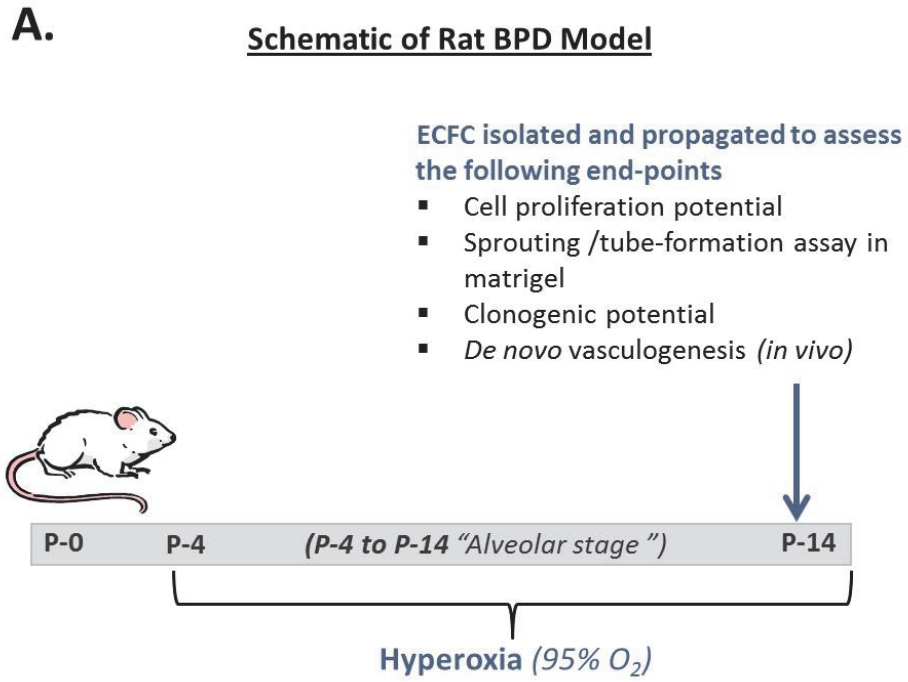


Rat PV-ECFCs were analyzed for their surface marker expression. PV-ECFCs were positive for endothelial-specific cell surface antigens- CD31, vascular endothelial growth factor receptor (VEGFR)-2, von Willebrand Factor (vWF) and negative for hematopoietic cell specific CD45 and monocyte/macrophage specific CD14. PV-ECFCs are also negative for CD133, a marker of cell immaturity. Filled 'blue' histograms represent antigen staining with negative isotype controls overlaid in white. All experiments were performed in triplicate.

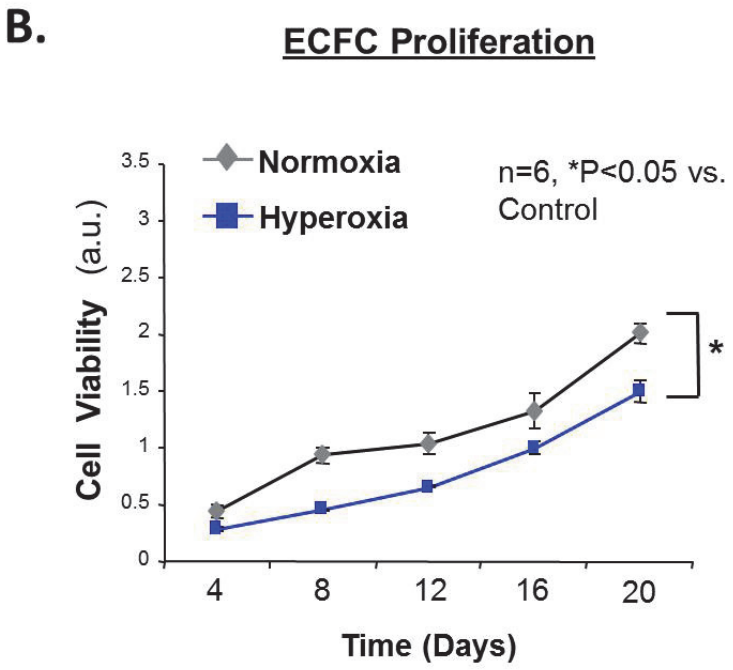


Rat PV-ECFC are capable of giving rise to clusters (up to 50 cells) or colonies 50-500 cells or more than 500 cells in 96-well plates when plated at single-cell seeding density. PV-ECFCs were marked via lentivirally transduced eGFP expression and plated one cell per well by FACS sorter. Results represent the mean ± standard error of mean (SEM) of 6 independent experiments. When the colonies with 500 or more cells were trypsinized and plated over again at single cell density, the PV-ECFCs demonstrated the ability to be serially passaged into successive generations of ECFC colonies with similar cell numbers (data not shown).

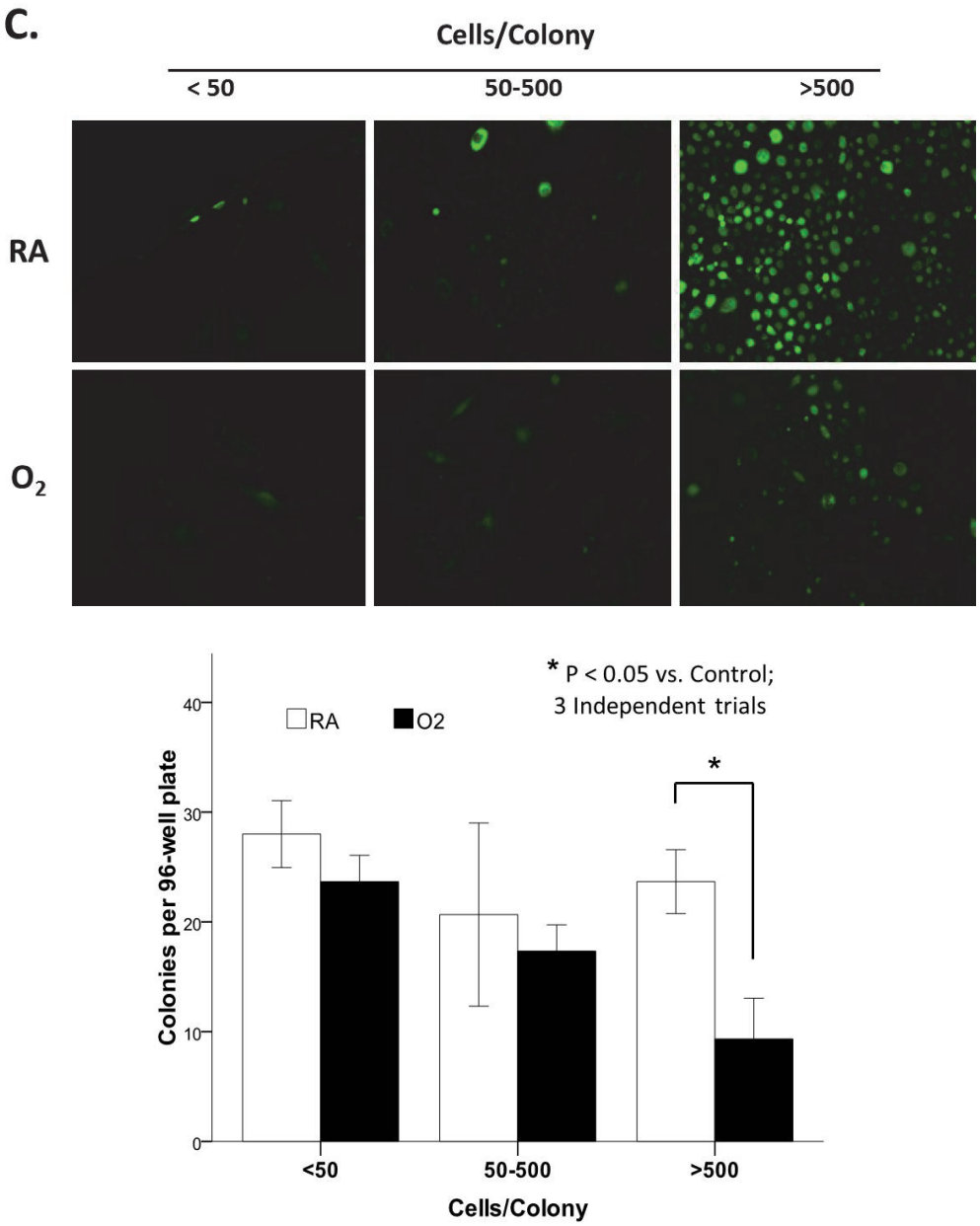
Figure 4-2 Proliferation, colony forming and 'vessel-forming' potentials of PV-ECFCs are impaired in hyperoxia-exposed newborn rats



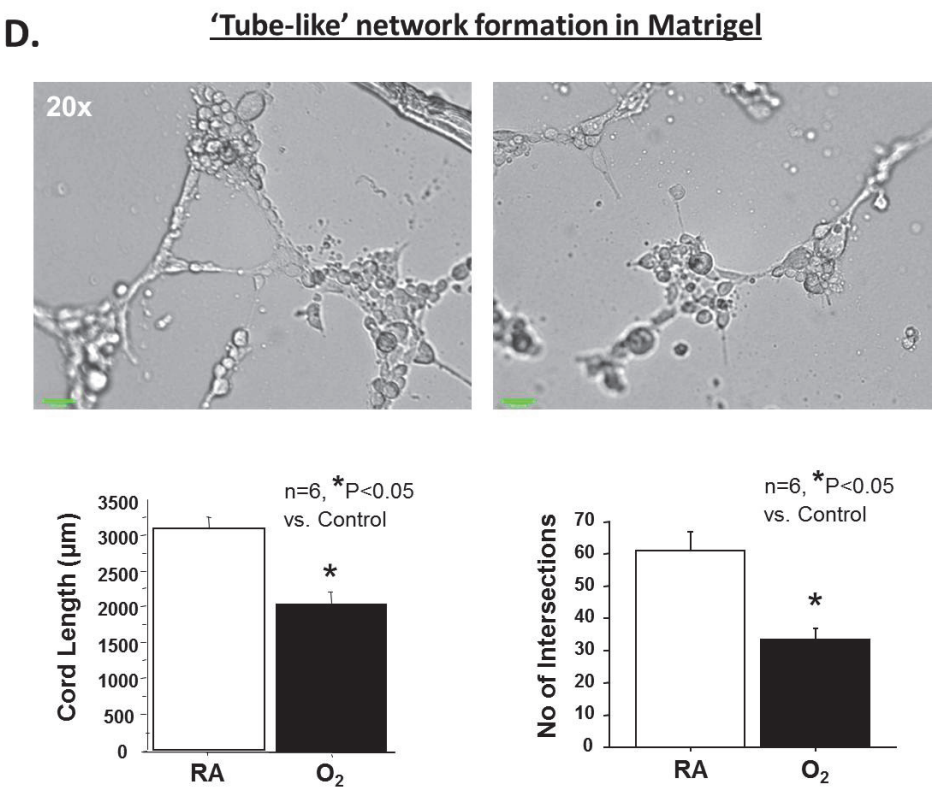
The schematic describes the rat model of hyperoxia-induced BPD where newborn rats are housed in 95% O_2 from portnatal day (PD)-1 to PD-14 and studied in comparison with room air (RA) raised control rats. Exposure of rat pups to hyperoxia during the critical alveolar stage of lung development results in arrested alveolar growth (data not shown). PV-ECFCs were isolated from the hyperoxia-exposed and control rats on PD-14 and their proliferative, clonogenic and 'tube-like' network forming potentials were assessed *in vitro*.



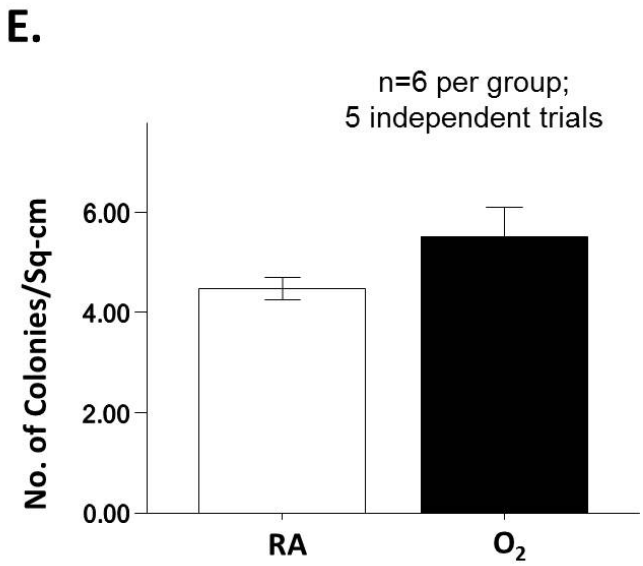
PV-ECFCs form the control and hyperoxic rats were plated at equal cell densities and cultured under identical culture conditions. PV-ECFCs from the hyperoxia-exposed rats show lesser survival and cell growth over the observed 3 week time period. Cell viability measurements were performed using MTT assay on days 4, 8, 12, 16 and 20. Results represent the mean ± SEM of 6 independent trials.



The ability of rat PV-ECFC to form cord-like endothelial networks was assessed by plating cells from control and hyperoxic groups in Matrigel. Quantitative assessment of the 'tube-like' structures reveal a significant decrease in total cord length and number of branching points in the hyperoxia-exposed group, compared to controls. Results represent the mean ± SEM of 6 independent trials.

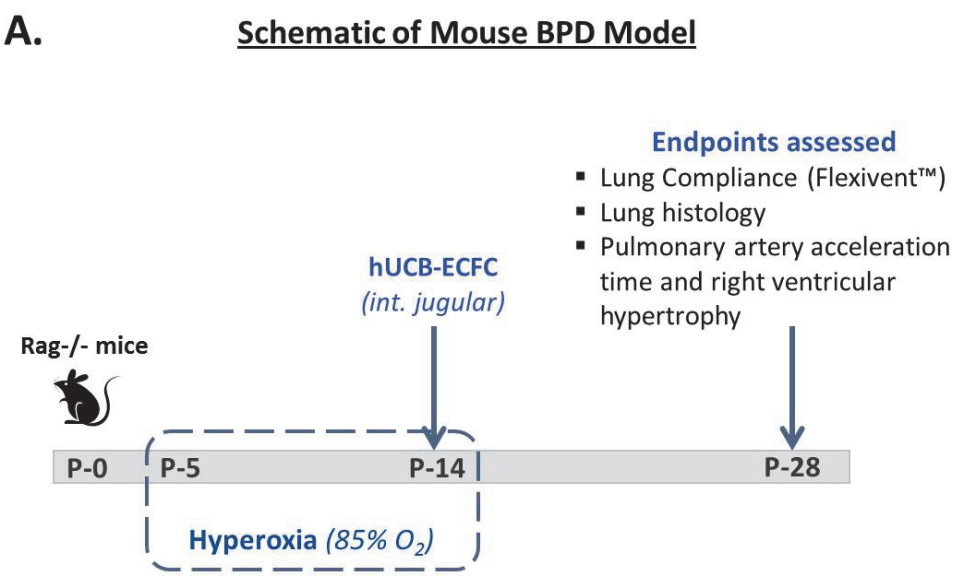


Colony forming potential of single plated rat PV-ECFCs was assessed by measuring the percentage of single ECFCs capable of generating colonies after 14 days in culture. A significantly lesser percentage of PV-ECFCs from the hyperoxia-exposed group, plated as single cells, were capable of generating colonies with 500 or more cells in comparison with normoxic controls.



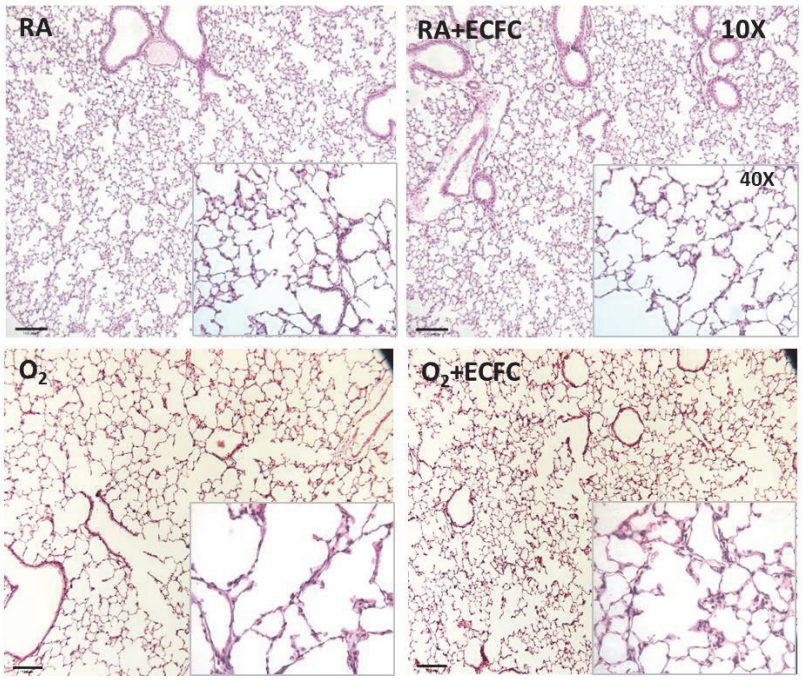
Number of late outgrowth ECFC colonies formed from every 0.5 x 10^3 CD31+ lung cells plated per sq-cm was evaluated for hyperoxia-exposed and control groups. Both control and hyperoxia-exposed lungs yield comparable numbers of late outgrowth ECFC colonies and there was no significant difference from 5 independent trials.

Figure 4-3 ECFC therapy reverses alveolar growth arrest in hyperoxia-exposed newborn mice

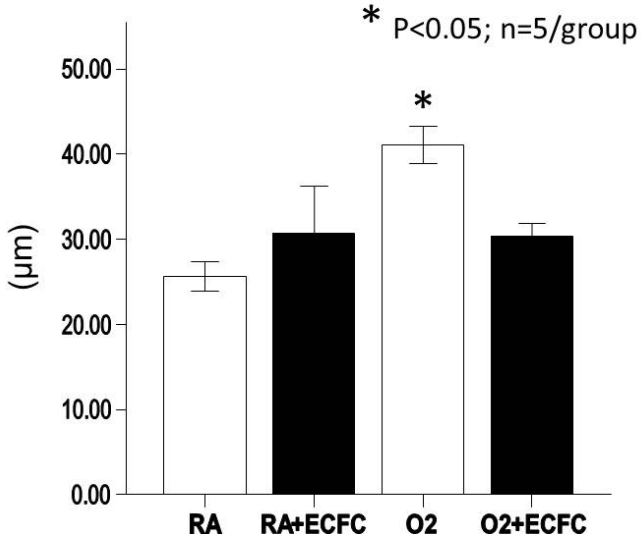


Schematic that describes the mouse model of hyperoxia-induced BPD where newborn rag-/- mice are housed in 85% O_2 from portnatal day (PD)-5 to PD-14 and studied in comparison with RA raised control mice. The mice were treated with intra-jugular injections of hUCB-ECFCs on PD-14 and housed in RA until assessment of the listed end points on PD-27 and 28.

B.



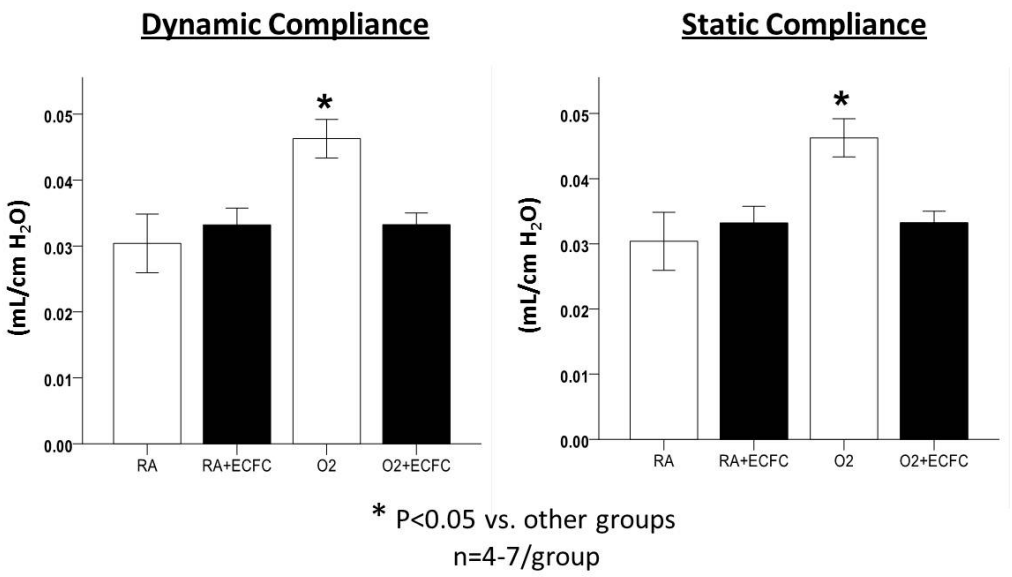
Mean Linear Intercept



(Figure legend overleaf)

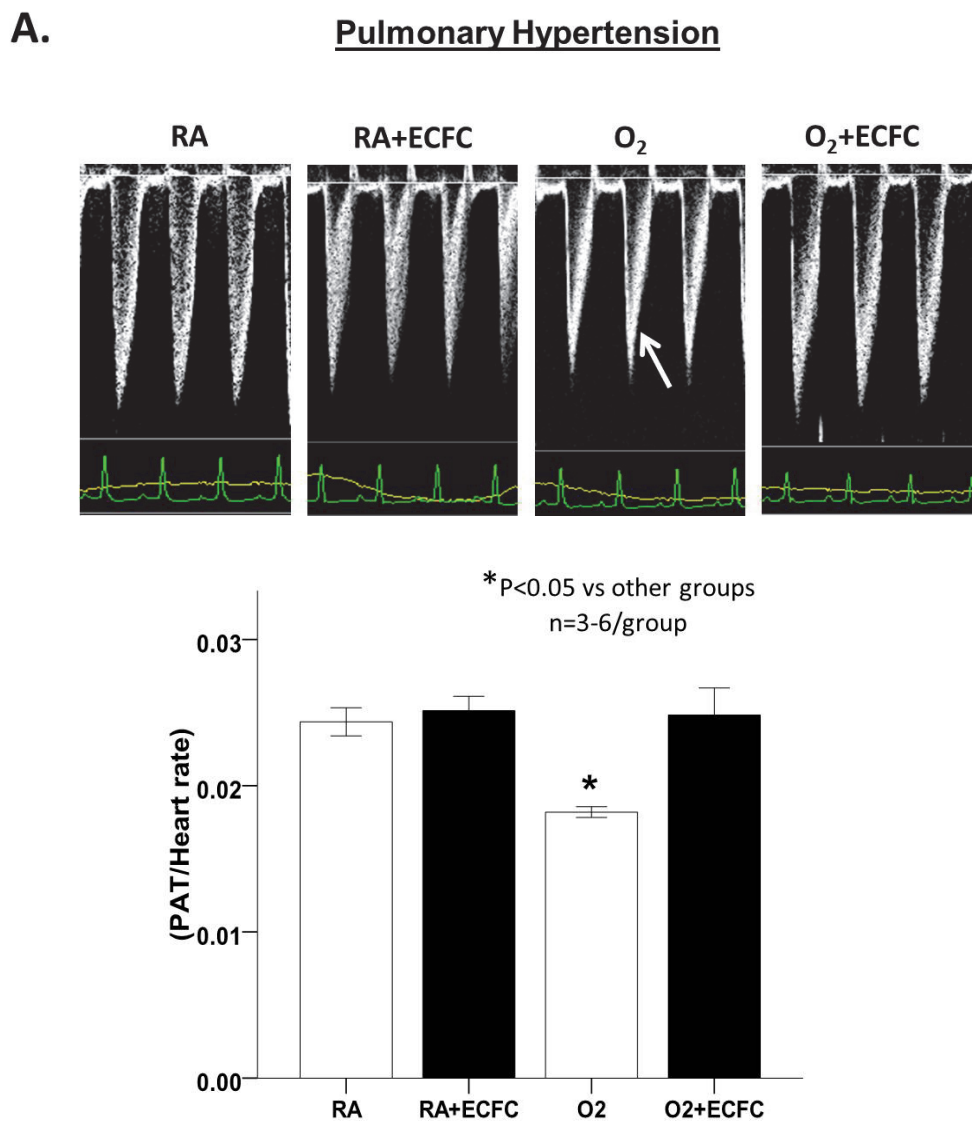
H&E–stained lung sections showing larger and fewer alveoli in hyperoxia-exposed (O_2 group) lungs as compared to lungs from RA housed mice and RA animals treated with hUCB-ECFCs (RA+ECFC group). Intra-tracheal (i.t.) administration of hUCB-ECFCs (O_2+ECFC group) in hyperoxia-exposed animals, preserved alveolar growth. Quantitative evaluation was performed by measuring the mean linear intercept (MLI). Results represent the mean ± SEM of 5 animals per group.

C.

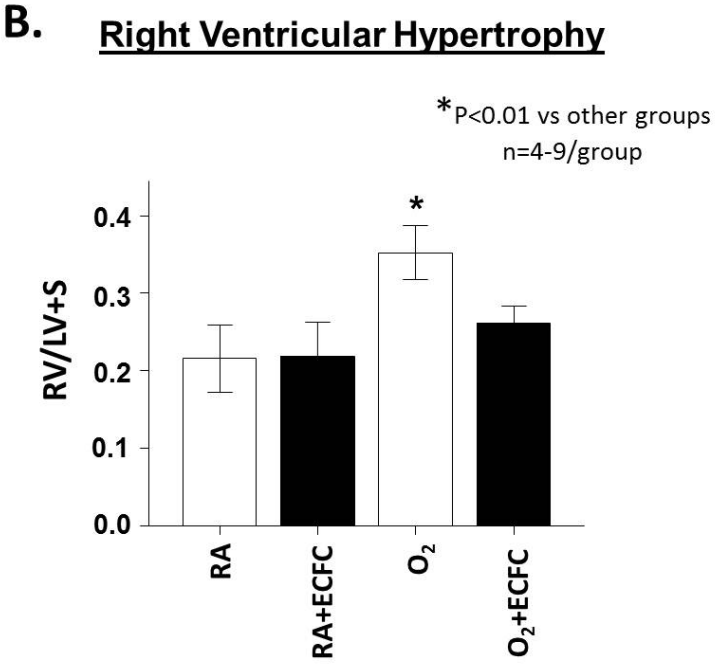


Invasive lung function testing shows increased lung compliance in untreated hyperoxia-exposed mice compared to untreated RA and ECFC-treated RA groups. Compliance was significantly restored closer to RA controls in hyperoxia-exposed animals treated with ECFCs. Results represent the mean ± SEM of 4-7 animals per group.

Figure 4-4 ECFCs prevent features of PHT in hyperoxia-exposed newborn mice

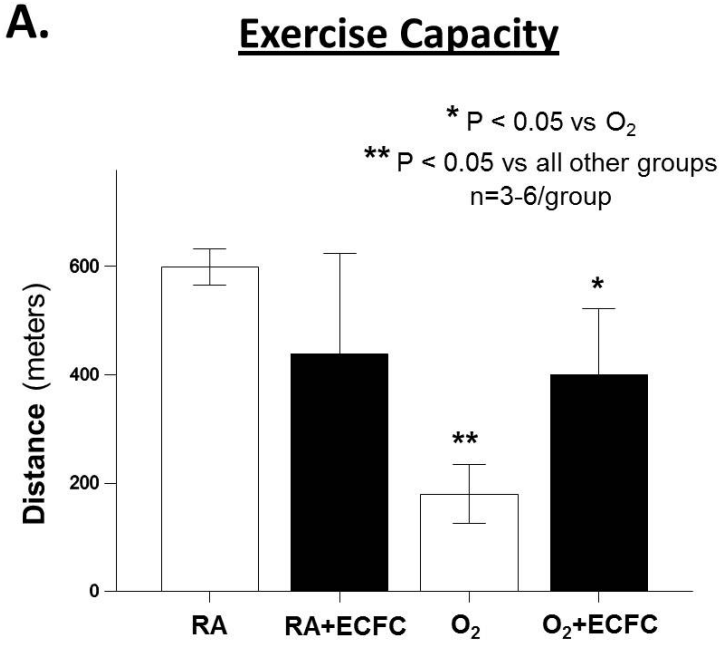


Intra-jugular hUCB-ECFC administration prevents PHT associated with hyperoxia-induced lung injury. Pulmonary arterial acceleration time (PAT) was significantly decreased in chronic hyperoxia-induced lung injury and showed a characteristic notch indicating PHT (arrow). Intra-jugular hUCB-ECFCs, restored the PAT almost to control levels. Results represent the mean ± SEM of 3-6 animals per group.

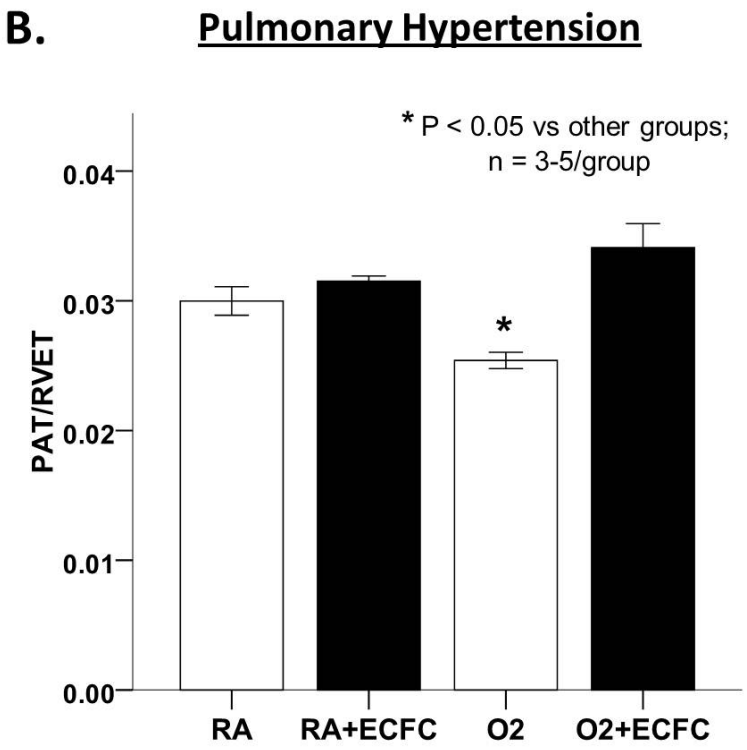


The pulmonary outflow Doppler findings were further confirmed by assessing right ventricular hypertrophy (RVH) via measurements of right ventricle/left ventricle plus septum (RV/LV-S) ratio (Fulton Index). Hyperoxia-exposed rats had significant RVH as indicated by the increase in RV/LV+S ratio compared with normoxic controls. Intra-jugular hUCB-ECFCs significantly attenuated RVH. Results represent the mean ± SEM of 4-9 animals per group.

Figure 4-5 Long term (10 month) safety and efficacy of ECFC cell therapy.

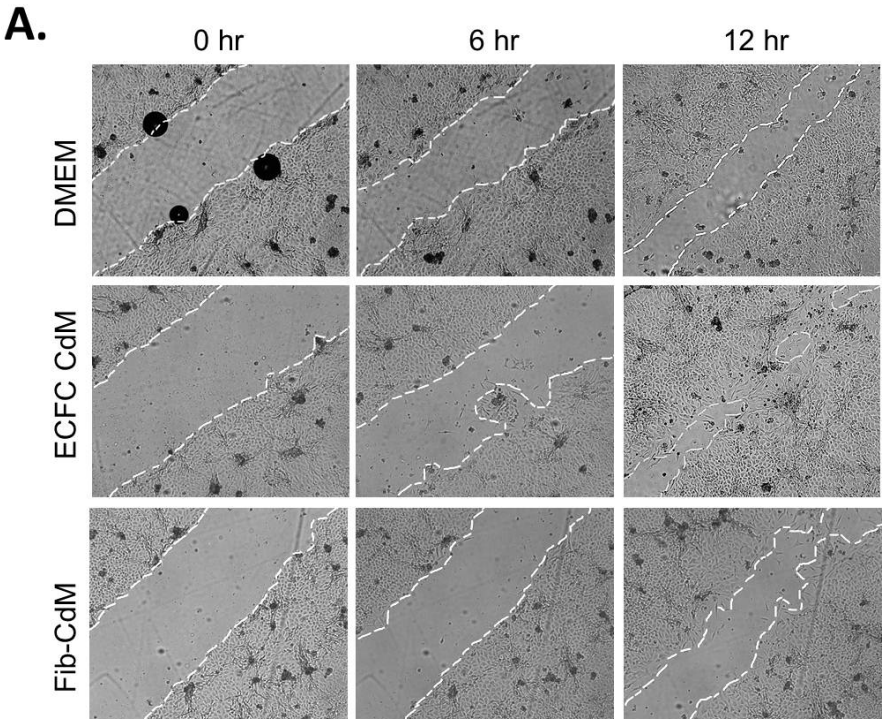


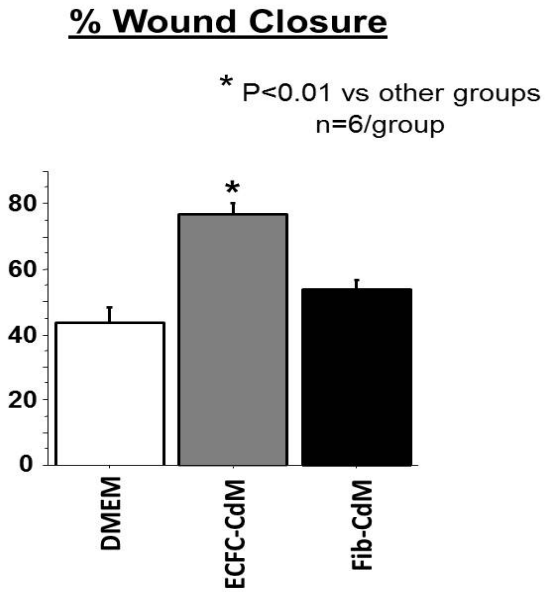
Exercise capacity of the long-term 10 mo old mice was assessed using a graduated treadmill exercise protocol done by a blinded observer. Oxygen exposed mice experienced reduced exercise capacity (expressed as total distance in meters covered on the treadmill), at 10 months of age compared to RA housed animals. Among them, animals treated with ECFCs had improved exercise capacity. Results represent the mean ± SEM of 3-6 animals per group.



Significant decrease in pulmonary arterial acceleration time (PAT), indicative of PHT, was persistent in the 10 month old hyperoxia-exposed mice. PAT was maintained at almost control levels in the animals treated with ECFCs. Results represent the mean ± SEM of 3-5 animals per group.

Figure 4-6 ECFC conditioned medium accelerates AT2 cell wound closure and preserves network-formation by lung ECFCs exposed to hyperoxia.





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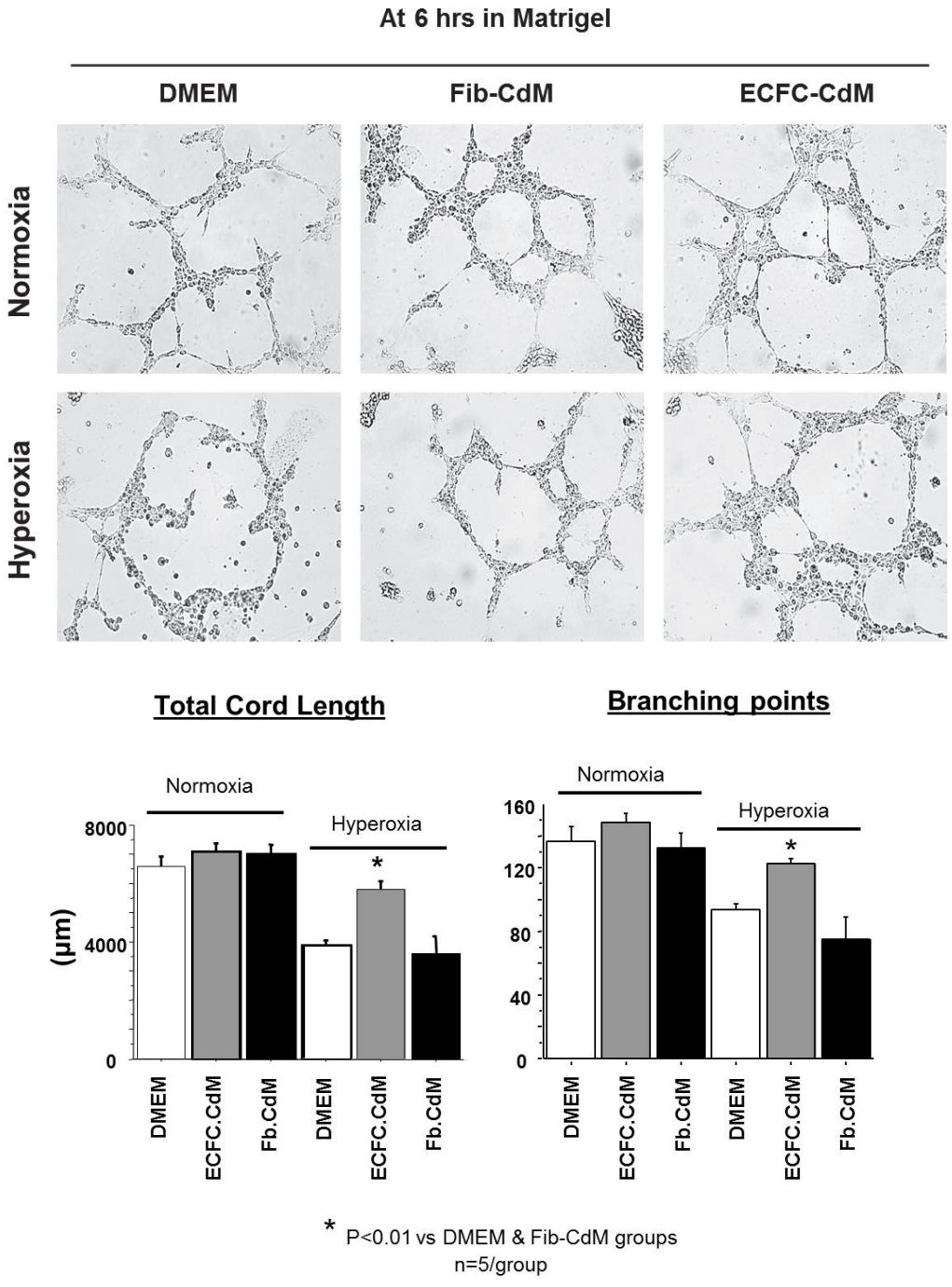
ECFC conditioned media accelerates the closure of pipette tip inflicted wound on confluent monolayers of cultured alveolar type (AT)2 cells. Rate of closure of cells bathed in rat lung fibroblast (RLF) CdM was not different from that of DMEM (vehicle) controls. Shown are representative photomicrographs and mean value (± SEM of 6 independent trials) of wound closure rate of confluent monolayers of AT2 cells damaged using a pipette tip, washed to remove damaged cells and treated with vehicle (DMEM), ECFC-CdM or RLF-CdM.



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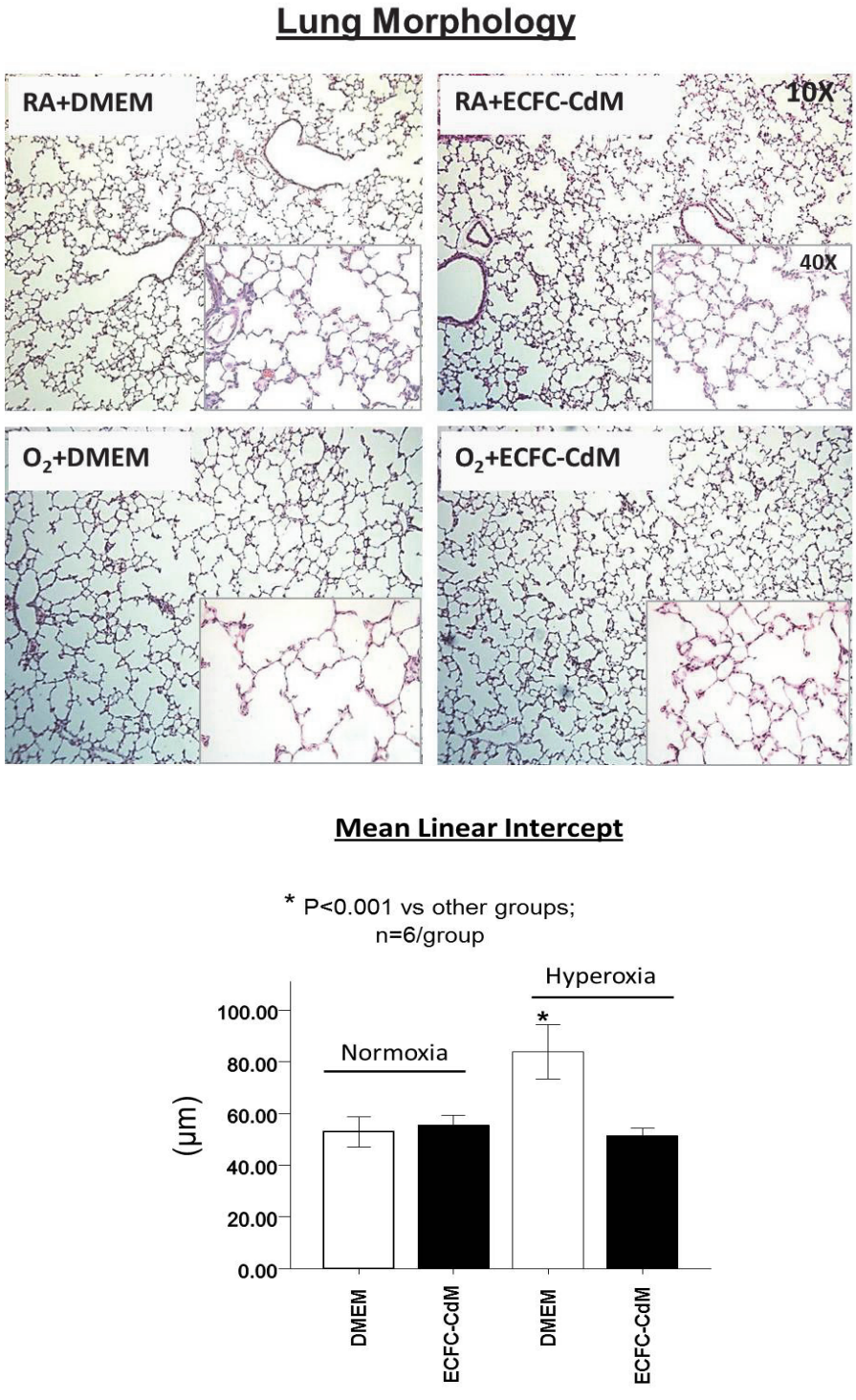
Complexity of endothelial cord-like networks formed by fetal hL-ECFC in Matrigel, assessed by cord length and number branching points, was significantly decreased in cells incubated in hyperoxic conditions (95% O_2) compared to RA controls. Treatment with cord blood derived-ECFC-CdM, and not RLF-CdM, preserved the ability of human PV-ECFCs to form complex endothelial networks in Matrigel. Results represent the mean ± SEM of 5 independent trials.

Figure 4-7 ECFC-conditioned media prevents alveolar growth arrest in hyperoxia-exposed newborn rats.



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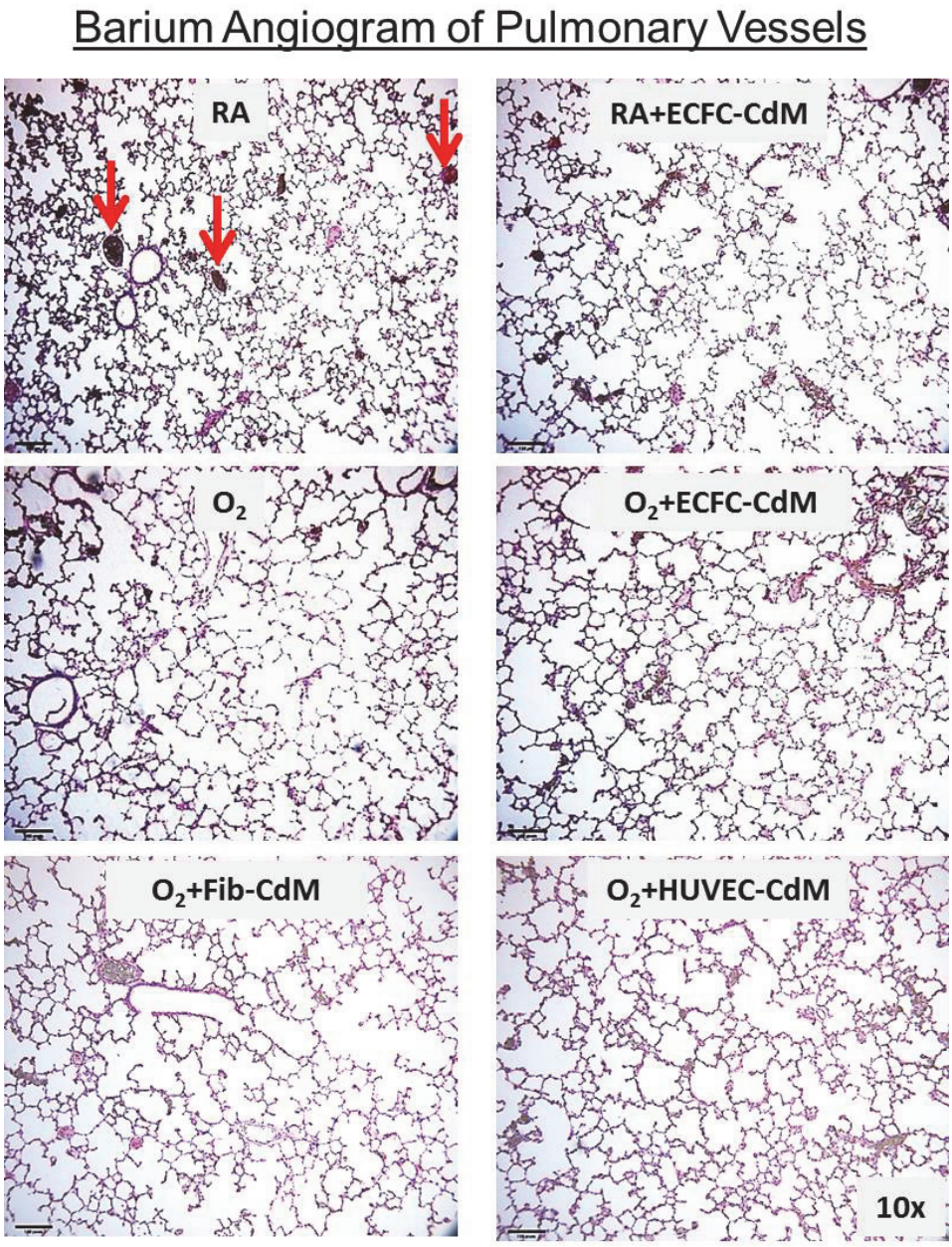
Representative H&E–stained lung sections showing larger and fewer alveoli in hyperoxia-exposed lungs as compared to lungs from rats housed in RA and RA animals treated with hUCB-ECFC conditioned medium (Cdm). Daily intra-peritoneal (i.p.) administration of ECFC-CdM in oxygen exposed animals preserved alveolar growth. Quantitative confirmation is provided by MLI measurement. Results represent the mean ± SEM of 6 animals per group.

Figure 4-8 ECFC-conditioned medium maintains lung angiogenesis in hyperoxia-exposed newborn rats



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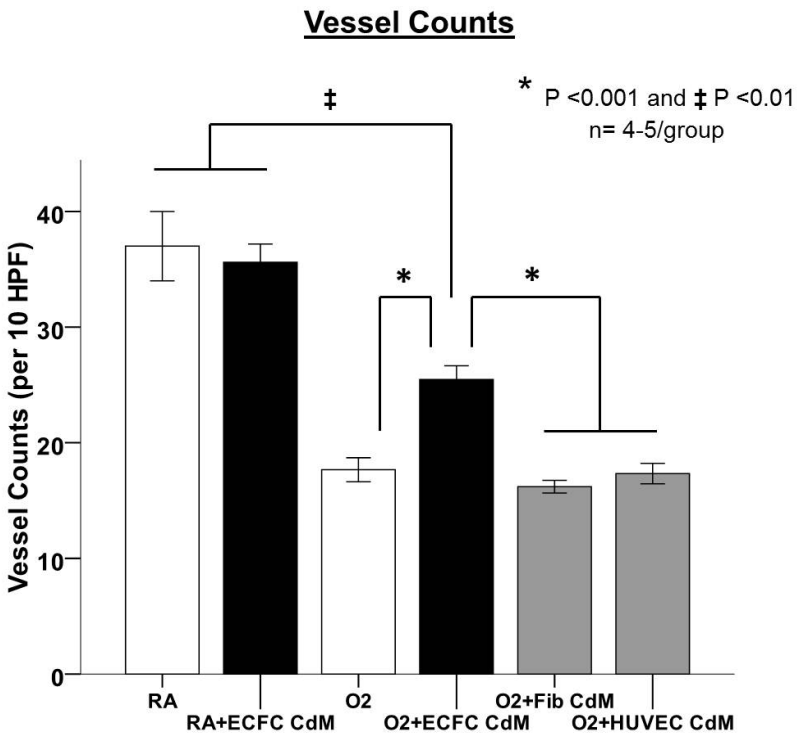
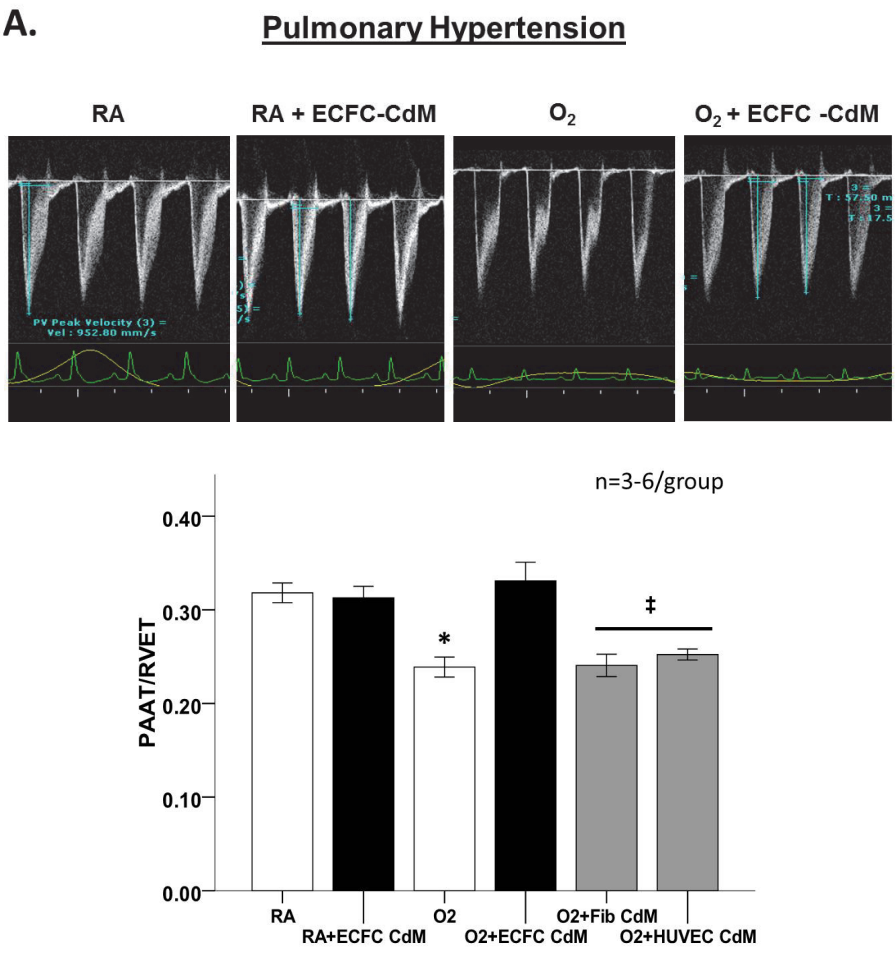
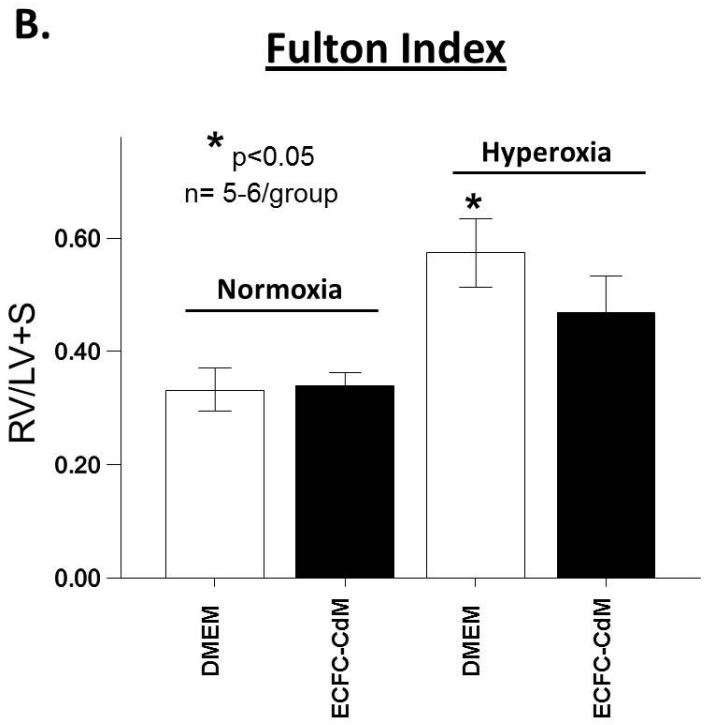


Representative H&E-stained lung sections with barium filled pulmonary vessels (red arrows). Mean vessel density (MVD) of 30-100 μm sized blood vessels per 10 high power fields (40x) was significantly decreased in the lungs of O_2-exposed animals. Daily intra-peritoneal (i.p.) injections of hUCB-ECFC conditioned media (CdM) preserved lung vessel density. No difference from the untreated O_2 group was observed in the O_2-exposed animals treated with human neonatal dermal fibroblast (Fib)- or human umbilical vein endothelial cell (HUVEC)-CdM. Results represent the mean ± SEM of 4-5 animals per group.

Figure 4-9 ECFC-conditioned medium prevents PHT in hyperoxia-exposed newborn rats



Daily i.p. injections of ECFC-CdM prevents PHT associated with hyperoxia-induced lung injury. Significant decrease in pulmonary arterial acceleration time (PAT) observed in chronic hyperoxia–induced lung injury was restored almost to control levels in the animals treated with ECFC-CdM. No difference from the untreated O_2 group was observed in the O_2-exposed animals treated with Fib- or HUVEC-CdM. Results represent the mean ± SEM of 3-6 animals per group. * refers to $P < 0.01$ vs RA, RA+ECFC CdM & O_2+ECFC CdM groups. ‡ refers to $P<0.05$ vs RA, RA+ECFC CdM & O_2+ECFC CdM groups.



The pulmonary outflow Doppler findings were further confirmed by RV/LV-S ratio measurements. Demonstrable RVH observed in hyperoxia-exposed rats in comparison with normoxic controls was significantly attenuated in the ECFC-CdM treated group. Results represent the mean ± SEM of 5-6 animals per group.

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Chapter Five: **Role and Therapeutic Potential of Endothelial Progenitor Cells in Lung Hypoplasia and Pulmonary Hypertension**

Contributions:
The majority of the experimental work described in this paper was performed by ARSA, with technical assistance from Vadivel A, Eaton F and Fan F. The manuscript was written by ARSA and edited by BT.

5.1 Introduction

Congenital diaphragmatic hernia (CDH) is a common congenital malformation (1/2500 live births) (Colvin et al., 2005) with a high mortality rate (~50%) (Stege et al., 2003). Typically, CDH is a defect in the diaphragm with displacement of the abdominal viscera into the thorax, with resulting lung hypoplasia and pulmonary vascular maldevelopment (Lally, 2002). Lung hypoplasia is characterized by smaller number and generations of airways, thickened alveolar septa, and abnormal architecture of the alveolar spaces. Pulmonary vascular maldevelopment, on the other hand, is marked by decreased vessel numbers and increased adventitia and medial thickness (Rottier and Tibboel, 2005). These vascular changes resulting in pulmonary hypertension (PHT) together with lung hypoplasia are the key determinants of CDH associated mortality (Lally, 2002; van den Hout et al., 2009).

At birth, a drastic cardiopulmonary transition occurs as the lung assumes the vital gas exchange function for the first time. This transition involves a rapid fall in pulmonary vascular resistance and pulmonary arterial pressure, and a dramatic rise in pulmonary blood flow. The hypoplastic vasculature characterizing congenital diaphragmatic hernia does not allow normal cardiopulmonary transition, resulting in persistent pulmonary hypertension of the newborn (PPHN) (Steinhorn, 2010). The ensuing hemodynamic complications result in severe and clinically unresponsive hypoxemia. Although inhaled nitric oxide (NO, a selective pulmonary vasodilator) has simplified the management of these newborns, still a majority of them remain refractory and do not respond to

any treatment (Steinhorn, 2008). As a result, infants with severe PPHN and hypoplastic lungs will not survive (~50%) or suffer significant morbidity.

Even amongst survivors the prognosis is guarded. Late PHT (weeks to months after birth) and chronic PHT (months to years after birth) are increasingly recognized in CDH survivors (Keller et al., 2006; Kinsella et al., 2005a). This new emerging pattern of late and chronic PHT extending beyond the immediate neonatal period significantly impedes quality of life and increases the risk of death. The refractory response to inhaled NO to treat PPHN in CDH and the emergence of the late and chronic variants of PHT may reflect the degree of underlying pulmonary vascular hypoplasia. Consequently, the ultimate therapeutic goal, in order to improve survival of infants affected with this devastating malformation, is to promote lung vascular growth and thereby limit or avert PHT (Greer et al., 2003).

Evidence indicates that proangiogenic growth factors can be used therapeutically to promote lung vascular growth and preserve alveolar integrity (D'Angio and Maniscalco, 2002; Thebaud, 2007; Thebaud and Abman, 2007). In addition to reinstating the interdependence of lung alveolar and vascular growth, these findings imply that vascular progenitor cells may represent a newer and possibly more potent therapeutic option. Indeed, recent research shows that circulating endothelial progenitor cells (EPCs) contribute to lung repair (Borghesi et al., 2010), are dysfunctional in human PHT (Fadini et al., 2007) and improve PHT in experimental models (Toshner and Morrell, 2010). Even more recently, a novel hierarchy of EPCs termed endothelial colony forming cells (ECFCs), have

been described to exist in human peripheral/cord blood and in the vascular endothelial lining (Ingram et al., 2005; Ingram et al., 2004). ECFCs display a robust proliferative potential, express mature endothelial cell markers and form capillary-like structures *in vitro* and vessels *in vivo* (Yoder, 2010; Yoder et al., 2007). Importantly, these cells do not express hematopietic/myeloid markers such as CD14, CD45 and CD115 nor do they display phagocytic activity (Yoder et al., 2007). Suggested as unipotent progenitor cells primarily supporting neovasculogenesis, ECFCs may to contribute to new vessel formation during lung organogenesis. They are also likely candidates underlying postnatal lung repair and maintenance. In a recent set of studies, we showed that ECFCs exist in the developing mammalian lung (Chapter 4). We have also demonstrated that pulmonary vascular (PV)-ECFCs are functionally impaired in neonatal experimental oxygen-induced lung injury and ECFC therapy reverses lung damage and restores alveolar growth (see Chapter 4). In the current study, we examined the potential role of ECFCs in the pathogenesis and treatment of CDH associated lung hypoplasia and PHT.

We hypothesized that disruption of ECFC-driven angiogenesis underlies CDH-associated lung hypoplasia and ECFC therapy restores normal lung growth and prevents PHT. We tested our hypothesis using a new neonatal rat monocrotaline (MCT)-induced model of lung hypoplasia and PHT. This model that simulates the late and chronic PH, currently reported in CDH survivors. Firstly, we show that the survival, colony forming and vessel-like network forming potential of PV-ECFCs is impaired in MCT-exposed newborn rats.

Secondly, we show that *in vitro* exposure to CDH-inducing teratogen, nitrofen, blunts growth and lessens the colony forming capacity of rat PV-ECFCs. Finally, we demonstrate that therapeutic administration of human umbilical cord-blood (hUCB)-derived ECFCs or its conditioned media (CdM) attenuates PHT and preserves alveolar development in MCT-exposed newborn rats. These findings suggest that impaired ECFC functionality, at least in part, may underlie CDH-associated lung hypoplasia/PHT and this can potentially be reversed with ECFC supplementation.

5.2 Materials and Methods

All procedures were approved by the Animal Health Care Committee of the University of Alberta.

Some of the methodologies used in this paper have already been described in chapter 4 and are not repeated here. A description of the basic methodologies used across the different projects on this thesis can be found in the relevant sections of chapter 2. The following sections concern methods specific to this study and not described elsewhere in this thesis.

5.2.1 MCT-induced model of lung hypoplasia and PHT

Lung lesions mimicking CDH-associated lung hypoplasia and vascular dysgenesis were induced in newborn rats by sub-cutaneous administration of monocrotaline (MCT; Sigma-Aldrich, Oakville, Ontario). Dosage schedules and time-lines for MCT administration was developed based on the existing protocol for inducing PHT in adult rats (Mathew et al., 2004). Newborn rats were treated with a single sub-cutaneous (s.c.) injection of 60 μg/g body weight on post-natal day (PD)-6. A week or later following the injection, lungs of the treated rat pups

demonstrated the characteristic lesions of MCT-induced lung vascular damage and associated hemodynamic changes. Control rats were treated by sham s.c. injections with 0.9 % saline.

5.2.2 In vitro Nitrofen Exposure

Stock solutions of nitrofen were made up in a 10% ethanol solution at a concentration of 10 mM. This was then diluted with the appropriate quantities of cEGM-2 to obtain the final concentrations used in the experiments (10 μM, 25 μM or 100 μM of nitrofen) (Clugston et al., 2010). The highest ethanol concentration in any group was 0.1%. Prior pilot experiments were done to ensure that exposure of ECFCs to this concentration of ethanol vehicle had no significant impact on their viability.

5.2.3 Intra-jugular cell administration in nude rats

Cells were administered as already described for mice, in chapter 4.

5.2.4 In Vivo CdM Administration

CdM form ECFCs was administered as daily intraperitoneal (i.p.) injections at the dose of 7 μL/g from PD-7 to PD-21. Animals were harvested a few hours after the last injection.

5.3 Results

5.3.1 MCT-induced newborn rat model of lung hypoplasia and PHT mimics late PHT in CDH survivors:

To model late and chronic PHT observed in CDH survivors and to enable therapeutic testing of ECFCs, the neonatal rat model of MCT-induced lung hypoplasia with PHT was developed by appropriate modification of the existing model of MCT-mediated PHT in adult rodents (Figure 5-1A). In this model, newborn Sprague-Dawley or Nude rats were administered with a sub-cutaneous injection of MCT (60 μg/g body weight) at 6 days of life. When exposed to MCT in the early postnatal stage of lung development, the alveolar growth in these rat pups became stunted resulting in lung hypoplasia and alveolar simplification (data not shown). At PD-14, noticeable rarefaction of distal lung arterial density was observed via scanning electron microscopy of pulmonary vascular casts and barium angiogram of lung vasculature (Figure 5-1B). Hemodynamic changes due to pulmonary hypertension were even more striking in the MCT treated rats with a demonstrably decreased pulmonary arterial acceleration time (PAT) measured by Doppler echocardiography. In MCT exposed animals, pulmonary artery outflow Doppler tracings showed the a characteristic notch indicating pulmonary hypertension (arrow heads in Figure 5-1C). Right ventricular remodeling as a result of pulmonary hypertension was estimated by RVH measurements using Fulton Index. MCT-injected rats had significant RVH as indicated by the increase in RV/LV+S ratio compared to controls ($P<0.05$) (Figure 5-1D).

5.3.2 ECFCs are functionally impaired in MCT-induced lung hypoplasia with PHT:

In the MCT injected newborn rat, PV-ECFCs demonstrated lessened ability to proliferate and diminished capacity to form vessel-like networks in culture. We isolated PV-ECFCs from MCT and control newborn SD-rats on PD-14 and assessed their ability to survive in culture, generate colonies and form vessel-like networks in Matrigel. When plated at equal densities and cultured in identical conditions for 3 weeks, PV-ECFCs from the MCT-exposed rats showed reduced survival and followed a flattened growth curve in comparison to controls (Figure 5-2A). To evaluate their ability to generate colonies at a single cell level, we plated PV-ECFCs of MCT and control groups one cell per well in 96 well plates, and measured the number of single ECFCs capable of generating colonies after 14 days in culture. In 3 independent observations, 24 ± 3.4 % of single plated PV-ECFCs from the control group were capable of forming colonies containing more than 500 cells each as compared to. 11 ± 3.7 ($P<0.05$) from MCT rats. No significant difference between the groups was observed in the percentage of cells that formed colonies containing fewer than 50 cells or 50 to 500 cells (Figure 5-2B). In addition, the numbers of late outgrowth ECFC colonies formed from every 0.5 x 103 CD31+ lung cells plated per sq-cm was significantly lesser in the MCT group (3 ± 0.3 vs. 4 ± 0.4 in controls; $P<0.01$) (Figure 5-2C). As a surrogate indicator of the vessel-forming potential of PV-ECFCs, we investigated the ability of these cells to form cord-like endothelial networks by culturing them in Matrigel. Quantitative assessment of the tube-like structures revealed that

ECFCs from the MCT lungs formed networks with decreased total cord length and fewer interconnections (Figure 5-2D). Together, the above findings suggest that the ability of ECFCs to contribute to vessel formation is compromised in the lungs of animals exposed to MCT.

5.3.3 Nitrofen reduces ECFC survival and clonogenic potential:

Nitrofen is an herbicide capable of reliably inducing CDH in rat fetuses exposed in utero. The lung hypoplasia observed in nitrofen-exposed rat fetuses closely simulates the pulmonary abnormalities seen in human CDH. Since the current study investigates the involvement of PV-ECFCs in CDH associated lung pathology, we examined the response of these cells to nitrofen toxicity. Cultured rat PV-ECFCs were exposed to nitrofen *in vitro* and assessed for cell survival and clonogenic potentials. First, we generated survival plots of ECFCs cultured in the presence of 0 μM (control), 10 μM, 25 μM and 100 μM nitrofen (Figure 5-3A). When cultured in the absence of nitrofen, rat PV-ECFCs produce a typical sigmoidal growth curve with a characteristic lag phase, "logarithmic" growth phase followed by a plateau phase when the culture surface becomes confluent. As seen in Figure 5-3A, nitrofen addition resulted in relatively flattened growth curves indicating retarded slowed growth in these cells. This response was dose dependent with significantly diminished growth potential at 25 μM and 100 μM concentrations of nitrofen. When single plated PV-ECFCs were exposed to 100 μM nitrofen and maintained in culture over a 14 day time period, their ability to generate colonies with 500 or more cells was significantly compromised (17 ± 5.0

vs. 28 ± 1.5 in controls; P<0.05) (Figure 5-3B]. No significant difference, however, was observed in the capacity of nitrofen exposed single PV-ECFCs to produce smaller colonies with a cell density less than 500.

5.3.4 ECFCs prevent the emergence of PHT and its associated changes in MCT rats:

In order to test the potential of cord blood-derived ECFCs to stimulate lung growth and attenuate PHT, we employed the preventive and rescue treatment approaches on the newborn rat model of MCT-induced lung hypoplasia and PHT. In the preventive strategy, MCT-injected newborn nude rats were administered with an intra-jugular bolus of 1x105 hUCB-ECFCs, one day after MCT administration. After 2 weeks following the ECFC injections, the rat pups were euthanized on PD-21, following pulmonary outflow Doppler and invasive lung compliance assessments. Lungs were harvested for morphological examination, and hearts for estimation of Fulton Index (Figure 5-4). ECFC treatment prevented the development of pulmonary hypertension and its associated right ventricular hypertrophy in newborn rats administered with MCT (Figure 5-5A,B). There was a significant decrease in PAT/RVET values in MCT-exposed rats compared to controls (0.17 ± 0.02 vs. 0.28 ± 0.05 in controls; P<0.05). ECFC treated animals showed PAT/RVET values very close to control levels (0.26 ± 0.03) (Figure 5-5A). PAT values are expressed as their ratio to right ventricular ejection time (RVET) to normalize for the individual differences in heart rate among subjects. The Doppler findings were further confirmed by heart weight measurements and calculating RV/LV+S ratios (Fulton Index). The observably elevated RV/LV+S

ratios in the MCT group (0.27 ± 0.05 vs. 0.19 ± 0.01; P<0.01) was significantly attenuated by ECFC therapy (0.16 ± 0.03) (Figure 5-5B). ECFC administration in control rats showed no impact on their PAT or Fulton Index measurements.

Remodeling of small to medium sized pulmonary arteries with characteristic increase in percent MWT is another robust marker of persistent PHT. This change was strikingly evident in MCT exposed rat lungs (90 ± 20.6 vs. 38 ± 4.2 in controls; P<0.01). ECFC treatment prevented pulmonary vascular remodeling and maintained % MWT closer to control levels (53 ± 10.0) (Figure 5-6).

5.3.5 ECFC therapy preserves alveolar growth in MCT-exposed newborn rats:

In addition to pulmonary vascular abnormalities, MCT treatment results in stunted alveolar growth characterized by marked alveolaalr simplification. H&E–stained lung sections from MCT-exposed PD-21 rats showed larger and fewer alveoli as compared to lungs from age-matched control animals. The alveolar architecture appeared more mature with observably greater secondary septation in the ECFC treated MCT rats (Figure 5-7A). Invasive lung function testing using FlexiventTM showed decreased lung compliance in MCT-administered rats, compared to controls (MCT vs. controls: static compliance, 0.13 ± 0.01 vs. 0.19 ± 0.01 & dynamic compliance, 0.07 ± 0.01 vs. 0.09 ± 0.01; P<0.05). ECFCs administered according to the preventive strategy preserved both static and dynamic compliance parameters closer to control levels (static & dynamic

compliances, 0.18 ± 0.01 & 0.13 ± 0.01). ECFC administration in control rats showed no impact on their compliance measurements (Figure 5-7B).

5.3.6 ECFC therapy reverts PHT and restores alveolar development in newborn rats with MCT-induced lung damage:

In order to more closely simulate the clinical setting, we assessed the potential of hUCB-ECFCs to rescue reverse PHT one week after MCT administrationrat pups that were allowed to suffer MCT mediated lung damage for a week. The animals were evaluated 14 days after the ECFC injections (PD-28). MCT-exposed rat pups group were significantly relieved of pulmonary hypertension and its associated right ventricular hypertrophy. Doppler studies indicated that PAT/RVET values observed in MCT-injected rats were restored to almost control levels following ECFC therapy (0.26 ± 0.04 vs. 0.22 ± 0.03 in MCT group [$P<0.05$]; controls, 0.3 ± 0.05) (Figure 5-8A). The Doppler findings were supported by the absent or significantly milder increase in RV/LV+S ratio in MCT animals treated with ECFCs (0.32 ± 0.06). Marked RVH was recorded in the untreated animals of the MCT group (0.41 ± 0.05 vs. 0.26 ± 0.01 in controls; $P<0.01$) (Figure 5-8B). Lung histology depicted larger and fewer alveoli in MCT-exposed lungs (PD-28) as compared to lungs from age-matched controls. In these animals, intra-jugular ECFC injections on PD-14 rescued alveolar architecture (Figure 5-9A). ECFC therapy significantly improved static lung compliance in ECFC treated MCT rats (0.4 ± 0.06 vs. 0.3 ± 0.07 in MCT group [$P<0.05$]; controls, 0.5 ± 0.06). No significant improvement was evident in dynamic lung compliance (Figure 5-9B).

5.3.7 ECFCs promote pulmonary vessel growth in MCT-exposed rats

Promotion of new vessel formation might explain the therapeutic effect of ECFCs in MCT-exposed animals. In order to assess this, at PD 21, we infused barium into the pulmonary arteries of control vs. MCT animals and counted the number of barium-filled small to medium sized pulmonary arteries in lung sections. ECFC therapy preserved mean vascular density of the distal lungs in MCT-exposed rats (31 ± 4.8 in ECFC treated group vs. 21 ± 3.7 in MCT controls) (Figure. 5-10)

5.3.8 ECFC-conditioned media prevents PHT and preserves alveolar growth in MCT-rats:

Evidence suggests that stem cells act in a paracrine fashion, secreting factors that protect the lung. In order to test the presence of such paracrine mechanisms contributing to the therapeutic potential of ECFCs, we administered CdM from hUCB-ECFCs as daily intra-peritoneal (i.p.) doses (1 μL/g body weight) starting a day after the MCT injections. ECFC-CdM was administered for two weeks (PD-7 to PD-21) at the end of which the animals were assessed for RVH and alveolar morphology (Figure 5-11A). Daily i.p. injections of ECFC-CdM significantly attenuated RVH associated with MCT-exposure (0.4 ± 0.1 vs. 0.5 ± 0.07 in MCT group [$P<0.001$]; controls, 0.3 ± 0.02) (Figure 5-11B). Daily i.p. injections of ECFC CdM also preserved alveolar development reflected by more mature alveoli in lung histology sections (Figure 5-11C) and improved lung compliance in the MCT exposed rats (Static compliance: 0.2 ± 0.01 vs. 0.08 ±

0.02 in MCT group [P<0.001]; Dynamic compliance: 0.1 ± 0.01 vs. 0.05 ± 0.01 in MCT group [P<0.005]) (Figure 5-11D).

5.4 Discussion

In this study, we show that rat PV-ECFCs are functionally disrupted in CDH-associated lung hypoplasia and demonstrate the therapeutic potential of cord blood-derived ECFCs to prevent PHT and restore normal lung growth. Our major findings are- (a) PV-ECFC functionality, defined by their capacity for (i) survival, (ii) colonies generation from single plated cells, and (iii) form vessel-like endothelial networks, is impaired in MCT-induced lung hypoplasia; (b) *in vitro* exposure to CDH-inducing teratogen, nitrofen, disrupts growth and colony forming capacity of PV-ECFCs; (c) Administration of hUCB-ECFCs prevents and reverses MCT-induced PHT and restores normal alveolar development; (d) hUCB-ECFCs act via a paracrine effect because cell free CdM prevents MCT-induced PHT and alveolar hypoplasia.

Newborns with CDH are almost invariably afflicted with pulmonary hypoplasia and varying degrees of PHT (Abman, 2007). PPHN, the earliest presentation of PHT in CDH neonates, carries a huge morbidity and mortality (Kinsella et al., 2005b). In fact, one of the greatest strides in the management of neonates with CDH has been the strict attention and priority provided to tackling PPHN associated respiratory failure and hypoxemia, over the need to close the diaphragmatic defect (van den Hout et al., 2009). However, owing to a significant reduction of the pulmonary vascular bed in these infants, PHT tends to persist

beyond the neonatal period in the form of late and chronic variants (Kinsella et al., 2005b), with as yet unidentified sequelae (Peetsold et al., 2009). Consequently, a closer understanding of lung vascular development shall furnish newer tools to improve the outcomes in this growing patient population.

5.4.1 EPCs, ECFCs and Lung Vascular Dysgenesis

The blood vessels in the lung are thought to arise from a combination of angiogenesis and vasculogenesis (Stenmark and Gebb, 2003). Angiogenesis (sprouting of new vessels from preexisting vasculature) is believed to contribute to the formation of the proximal or central pulmonary vessels. Vasculogenesis (*de novo* origin of blood vessels out of mesenchymal precursors), on the other hand, is assumed to generate the distal lung vascular network. The two processes merge at 10 to 11 weeks of gestation in humans in the form of communicating channels that provide continuity of blood flow (deMello and Reid, 2000). Presumably, this assembly of a normal, functional pulmonary vascular network is dependent on a tightly orchestrated balance between the angiogenic and vasculogenic processes, and any disruption of this balance may have adverse consequences during fetal, neonatal, or adult life. In the light of newer evidence supporting the existence of "postnatal vasculogenesis" (Ribatti et al., 2001) and the abundance of research funneled into the role of endothelial cells and their precursors (i.e. EPCs) in this phenomenon (Kirton and Xu, 2010), we are confronted with a whole new direction of investigation into the development and maintenance of blood vessels.

Exploring in this direction is also very pertinent to understanding the pathophysiology of CDH-associated lung vascular dysgenesis.

It is likely that EPC dysfunction may play a central and critical role in the initiation and progression of CDH-associated PHT. Most studies to date, investigating the role of EPCs in lung vascular dysgenesis and PHT, have examined the number and functional state of circulating early-outgrowth EPCs, particularly of bone-marrow origin (Toshner and Morrell, 2010). In PHT patients, circulating EPCs seem to be functionally deprived, showing decreased capacity to migrate and incorporate into tube-like structures *in vitro* (Diller et al., 2008; Junhui et al., 2008). Counter-intuitively, recruitment of bone-marrow derived EPC-like cells into remodeled pulmonary vessel walls has been suggested to aggravate the disease process (Davie et al., 2004; Toshner et al., 2009). Blocking bone-marrow progenitor cell recruitment via inhibiting stromal derived factor-1/CXCR4 axis reversed pulmonary vascular remodeling and PHT (Young et al., 2009). Whatsoever the interpretation of these data, one thing that clearly stands out is the existing disparity in the ways the examined EPC types are defined and evaluated. The current study addresses this limitation by adopting a steadfast definition for the evaluated EPC type and specifically examining their functional state in MCT-mediated lung hypoplasia and PHT.

Thanks to recent advances in our understanding of vascular precursors, ECFCs emerge as the cell type conforming to the currently accepted definition of ‘true’ EPCs (Kirton and Xu, 2010). We isolated the tissue resident ECFCs from enzymatically disintegrated peripheral lungs of MCT-exposed and control rats and

evaluated their ability to survive in culture, form endothelial networks in matrigel and generate of colonies from single plated cells. ECFCs from the MCT-exposed lungs were significantly deprived of their ability to survive and expand in culture, generate colonies with more than 500 daughter cells per seeded cell and form vessel-like networks in Matrigel. More interestingly, numbers of late-outgrowth ECFC colonies per sq-cm obtainable upon plating equal numbers of CD31-positive lung cells was significantly less in the MCT-administered group compared to age-matched controls. Even though earlier studies report increased numbers of bone-marrow derived putative EPCs in regions of vascular remodeling in PHT lungs (Davie et al., 2004; Toshner et al., 2009), we observed a significant drop in the resident lung ECFC density in MCT-exposed PHT lungs. Being the 'true' vessel-forming progenitors, decrease in ECFC numbers correlates with the observed distal lung vascular paucity characterizing MCT-mediated lung hypoplasia.

In utero exposure to nitrofen reliably induces CDH with associated lung hypoplasia in rat fetuses. When healthy PV-ECFCs were cultured in the presence of this teratogen, cell survival and rate of expansion in culture was diminished in a dose dependent manner. More strikingly, a marked fall in the number of ECFCs capable of forming colonies with 500 or more cells was observed. However, attempts to isolate and expand PV-ECFCs from the lungs of control and nitrofen-exposed rat fetuses were not successful. This may be due to a limitation in the sensitivity of the currently available technique of ECFC isolation. The existing method probably requires capturing a threshold number of CD31+ cells for

successful expansion and establishment of pure ECFC cultures. Identification of more robust ECFC isolation techniques can obviate this shortcoming in future studies.

5.4.2 Therapeutic Potential of ECFCs in Lung Hypoplasia and PHT

The earliest studies on EPCs in PHT have centered around their therapeutic potential. Ex vivo expanded early-outgrowth EPCs have been shown to have therapeutic benefit in short term studies in animal models of PAH (Nagaya et al., 2003; Raoul et al., 2007; Takahashi et al., 2004; Zhao et al., 2005). Subsequently, there have been two small randomised trials in humans demonstrating an improved hemodynamic response and exercise capacity following a single infusion of the same cells (Wang et al., 2007; Zhu et al., 2008). Another very recent study by Smadja and colleagues suggests that ECFCs might mediate the clinical benefits of prostanoids in pulmonary arterial hypertension (Smadja et al., 2011). The current study examines the functional state of PV-ECFCs in rats with PHT and evaluates the potential of ECFCs as a viable therapeutic cell type for this disorder.

We recognize that the MCT model involves postnatal vasculotoxic insult to the lungs and does not quite recapitulate the *in utero* developmental failure that results in CDH. In this respect, the rat nitrofen model and the surgical sheep models more closely reflect the diaphragmatic and pulmonary abnormalities of human CDH (van Loenhout, 2009). Despite these limitations, we believe that the MCT model represents a scientifically and economically acceptable surrogate to

study the therapeutic potential of ECFCs to correct life-threatening features of CDH, such as lung hypoplasia and PHT. Post-natal therapeutic approaches cannot be assessed in the nitrofen model, since nitrofen-exposed fetuses succumb within hours after birth to respiratory distress. Likewise, the sheep model requires a neonatal intensive care setting equipped with facilities for intubation, mechanical ventilation and administration of inhaled NO to maintain these animals alive for at least 8 to 24 hours. Moreover, even this period is too short to assess CDH-associated entities such as late and chronic PHT. Thus, the MCT model, first proposed by Todd and colleagues (Todd et al., 1985), is suitable to recapitulate the new emerging pattern of late and chronic PH seen in CDH survivors and thus to explore if ECFCs induce lung angiogenesis, promote lung growth, and attenuate PHT.

We administered ex vivo expanded ECFCs derived from human cord-blood as an intra-jugular bolus (105 cells/dose) one day (preventive strategy) or one week (rescue strategy) after MCT-administration to newborn immuno-compromised nude rats. Upon assessment two weeks after cell therapy, we observed that ECFC treatment prevented as well as rescued MCT-exposed newborn rats from the development of PHT. We measured the onset and severity of PHT using right ventricular outflow Doppler. As associated markers of PHT, we employed RVH and percent MWT to evaluate cardiac and vascular remodeling, respectively. Associated improvements in alveolar growth and lung compliance were evident in both treatment strategies.

The hypoplastic pulmonary vascular bed characterizing CDH lungs is widely suggested to determine the severity of CDH-associated PHT (Boucherat et al., 2010). From the perspective of our hypothesis, we assumed that promotion of new vessel formation could potentially explain the attenuation of PHT in ECFC treated MCT-rats. In order to examine this, we visualized small to medium sized pulmonary vessels (diameter of 30-100 μm) using barium angiography. Greater mean pulmonary vessel density was measured in MCT-rats two weeks after ECFC administration. However, from these results, we can only speculate on the mechanisms contributing to enhanced vessel formation following ECFC therapy. Direct incorporation of the injected cells leading to formation of blood vessels *de novo* (i.e. ECFC-driven vasculogenesis) and/or the release of proangiogenic mediators (i.e. paracrine stimulation of angiogenesis) may potentially explain new vessel formation. Detailed studies to track the fate of injected ECFCs are necessary to answer this important question. Nevertheless, we attempted to answer a part of this question via therapeutic experiments using the secreted products of the ECFCs, without the cells themselves. We administered CdM from hUCB-ECFCs as daily i.p. injections for two weeks to MCT-exposed newborn rats. In addition to significantly attenuating MCT-induced RVH, ECFC-CdM also preserved alveolar growth and lung compliance. This observation implies a paracrine component underlying the therapeutic effect of hUCB-ECFCs.

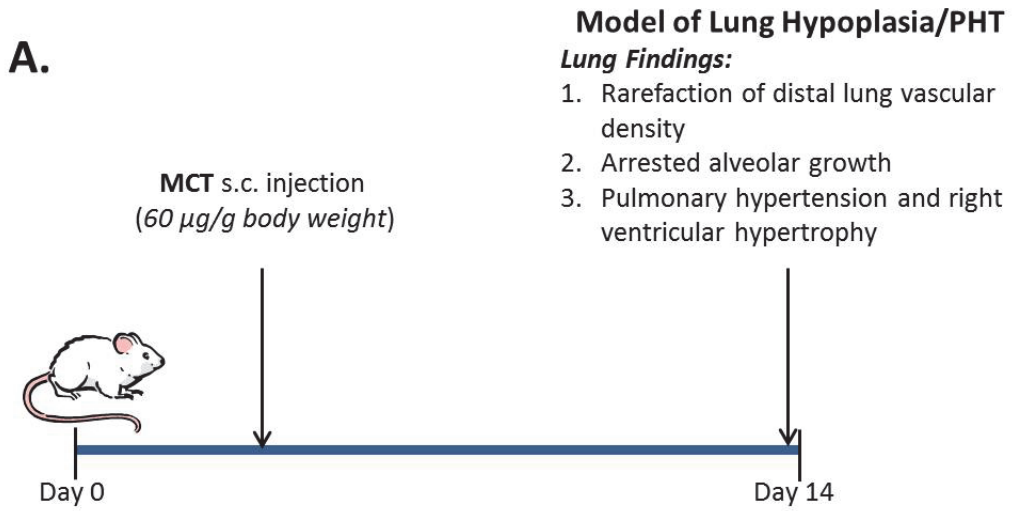
Finally, it is also important to note that a comparable improvement in hemodynamic parameters and vascular remodeling responses have already been reported with bone-marrow (Raoul et al., 2007; Zhao et al., 2005), peripheral

blood (Takahashi et al., 2004) or human cord-blood (Nagaya et al., 2003) derived circulating EPCs on the MCT-induced model of PHT. Considered together, these findings reiterate the notion (Hur et al., 2004) that both early and late-outgrowth progenitors supplement each other in promoting neovasculogenesis. Studies using a cocktail admixture of both cell types tagged with appropriate markers and administered on a suitable therapeutic platform shall validate this hypothesis and more specifically answer some of the existing questions.

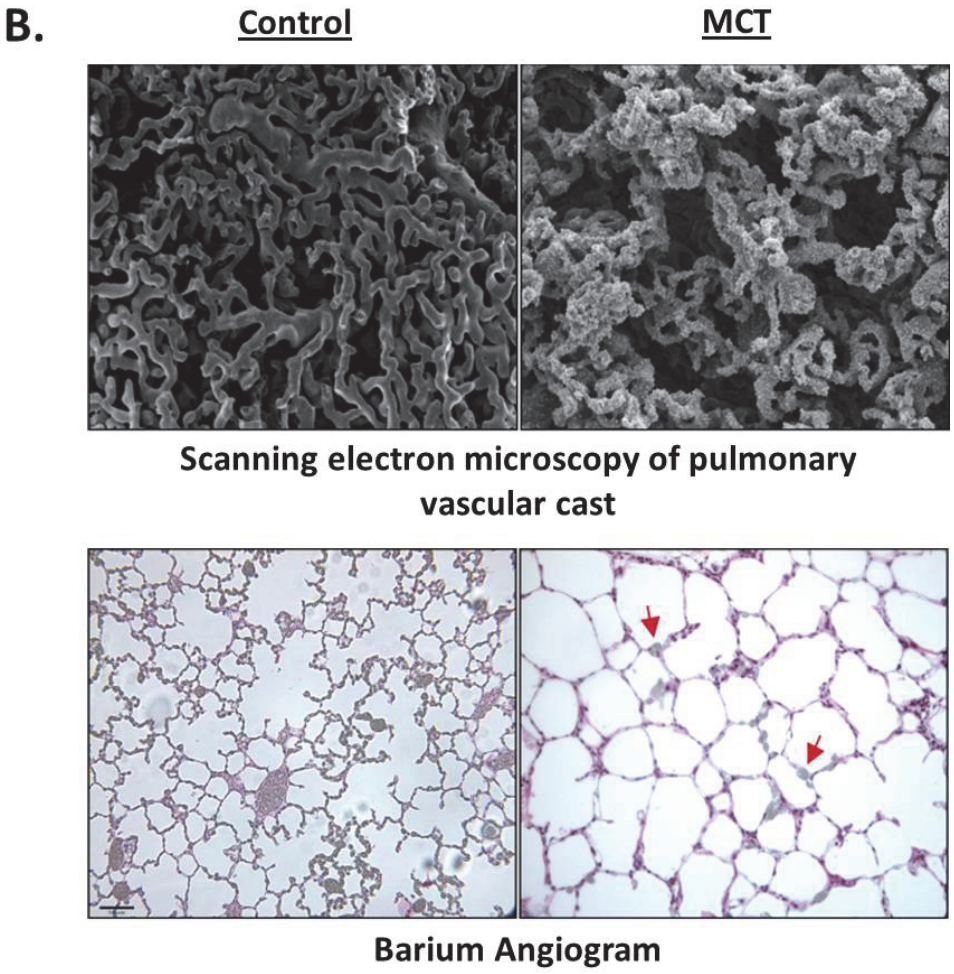
5.5 Conclusion

In conclusion, this is the first study investigating the role of ECFCs in CDH-associated lung hypoplasia and PHT. Here, we isolate ECFCs (the hitherto accepted 'true EPCs') from the growth restricted PHT lungs, assess their functional state, and demonstrate functional impairment. In addition, we also demonstrate the therapeutic potential of hUCB-derived ECFCs to stimulate lung vascular growth and attenuate PHT. These findings shall motivate more detailed assessments of the role of ECFCs in lung vascular development and open the doors for future clinical trials.

Figure 5-1 Monocrotaline induces lung vascular hypoplasia and pulmonary hypertension in newborn rats.

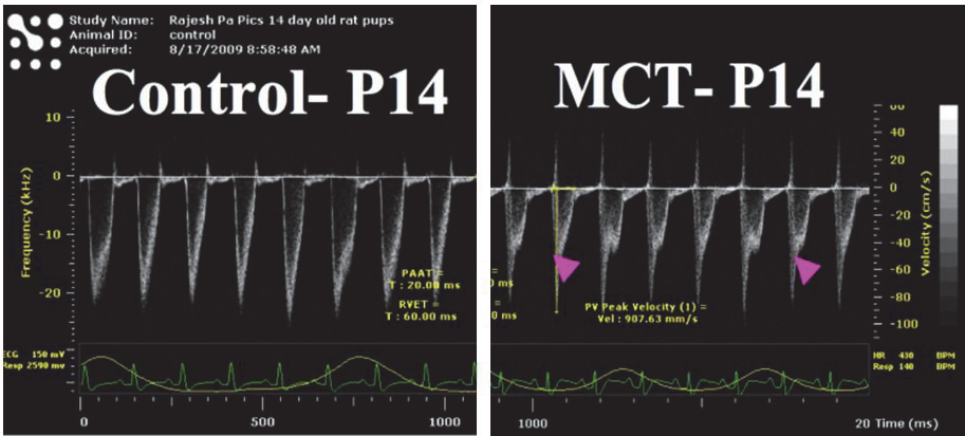


The schematic describes the newborn rat model of monocrotaline (MCT) induced lung hypoplasia and pulmonary hypertension (PHT).



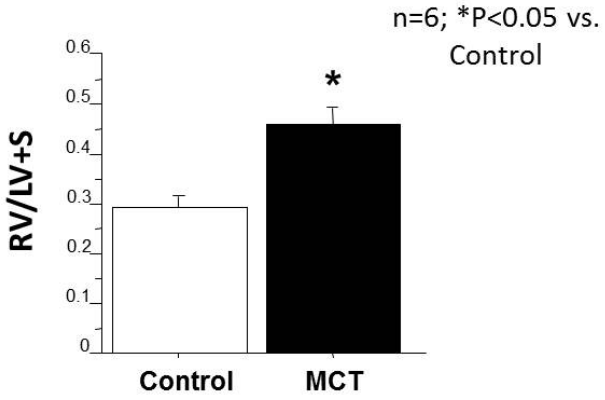
Newborn Sprague-Dawley or Nude rats were administered with a sub-cutaneous injection of MCT (60 μg/g body weight) at postnatal day (PD)-6. Exposure of rat pups to MCT during early postnatal lung development resulted in demonstrable alveolar growth arrest and lung hypoplasia. At PD-14, striking rarefaction of distal lung arterial density was observed and is demonstrated by scanning electron microscopy of pulmonary vascular casts and barium angiogram of lung vasculature.

C.



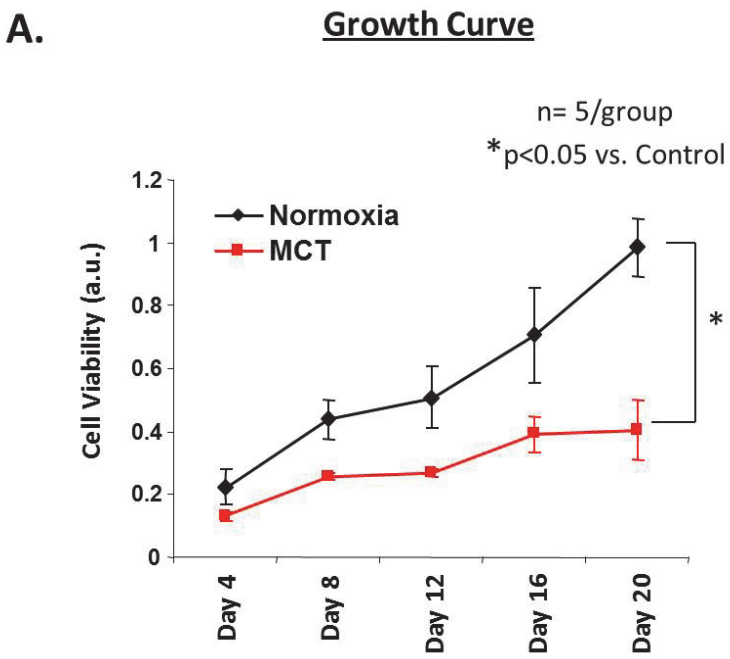
Pulmonary arterial acceleration time (PAT) was significantly decreased in MCT-exposed rats and showed a characteristic notch indicating pulmonary hypertension (pink arrowheads).

D.

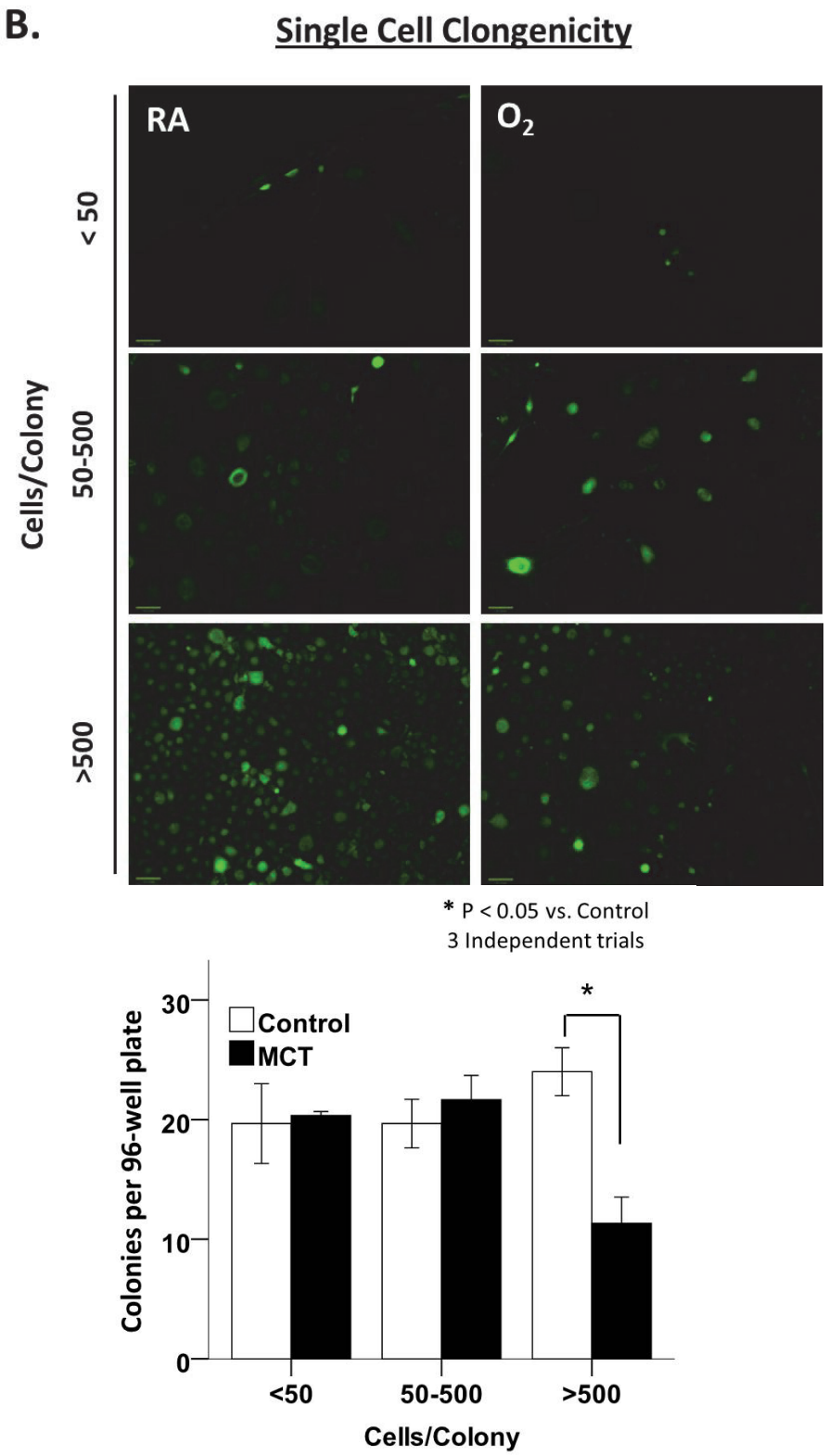


The pulmonary outflow Doppler findings were confirmed by assessing right ventricular hypertrophy (RVH) via measurements of right ventricle/left ventricle plus septum (RV/LV-S) ratio (Fulton Index). MCT-injected rats had significant RVH as indicated by the increase in RV/LV-S ratio compared to controls. Results represent the mean ± standard error of mean (SEM) of 6 animals per group.

Figure 5-2 Cell survival, network forming and colony forming potentials of PV-ECFCs are impaired in MCT-administered newborn rats.

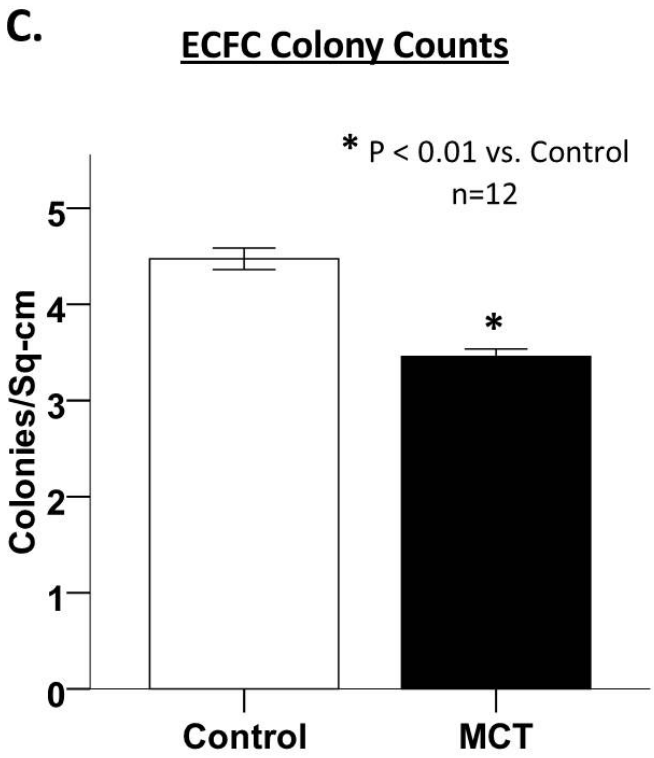


PV-ECFCs were isolated from the MCT-administered and control rats on PD-14 and their proliferative, endothelial network forming and single-cell clonogenic potentials were assessed *in vitro*. Rat PV-ECFCs form the control and MCT-admininstered animals were plated at equal cell densities and cultured for 3 weeks under identical culture conditions. Their viability was recorded using MTT assay performed at the specified time-points. The survival curve of PV-ECFCs from the MCT group was significantly flattened compared to age-matched controls indicating decreased cell viability in culture. Results represent the mean ± SEM of 5 independent trials.



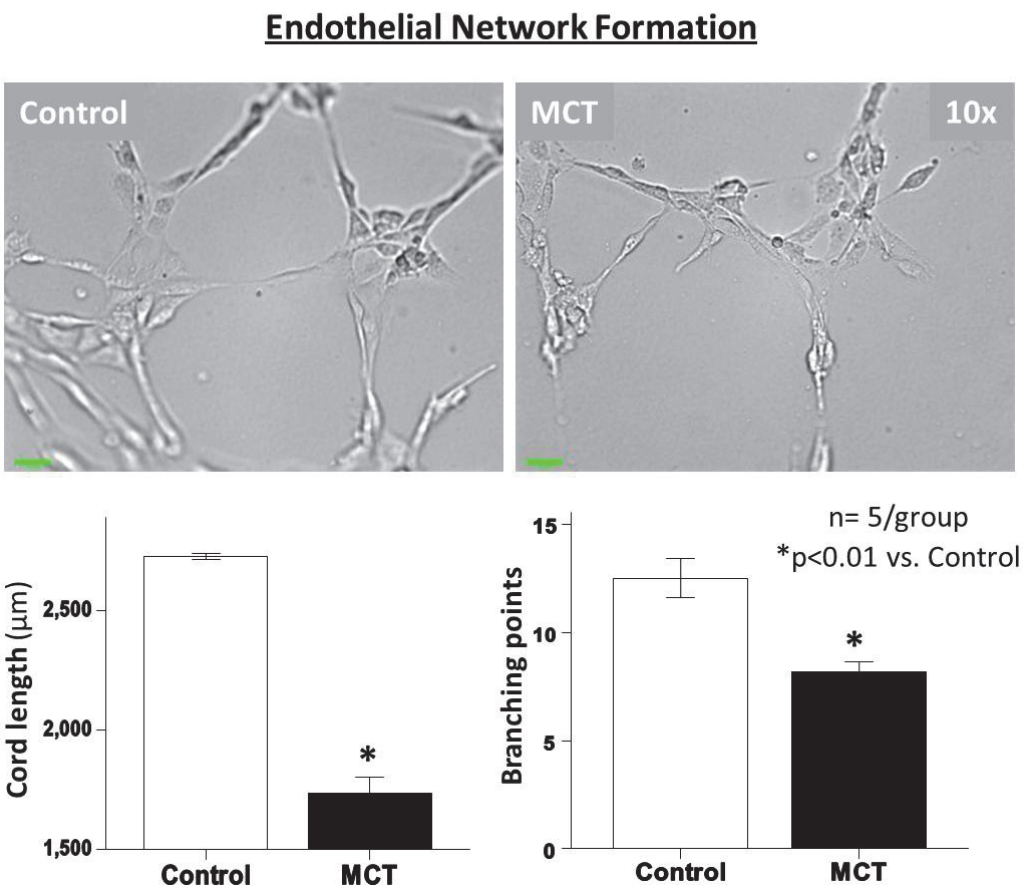
(Figure legend overleaf)

Clonogenic potential of single plated rat PV-ECFCs was assessed by measuring the percentage of single ECFCs capable of generating colonies after 14 days in culture. A significantly lesser percentage of ECFCs from the MCT-administered group were capable of generating colonies with 500 or more cells compared to controls. PV-ECFCs were transduced with enhanced green fluorescence protein (eGFP) for efficient colony detection and demonstration. Results represent the mean ± SEM of 3 independent trials.



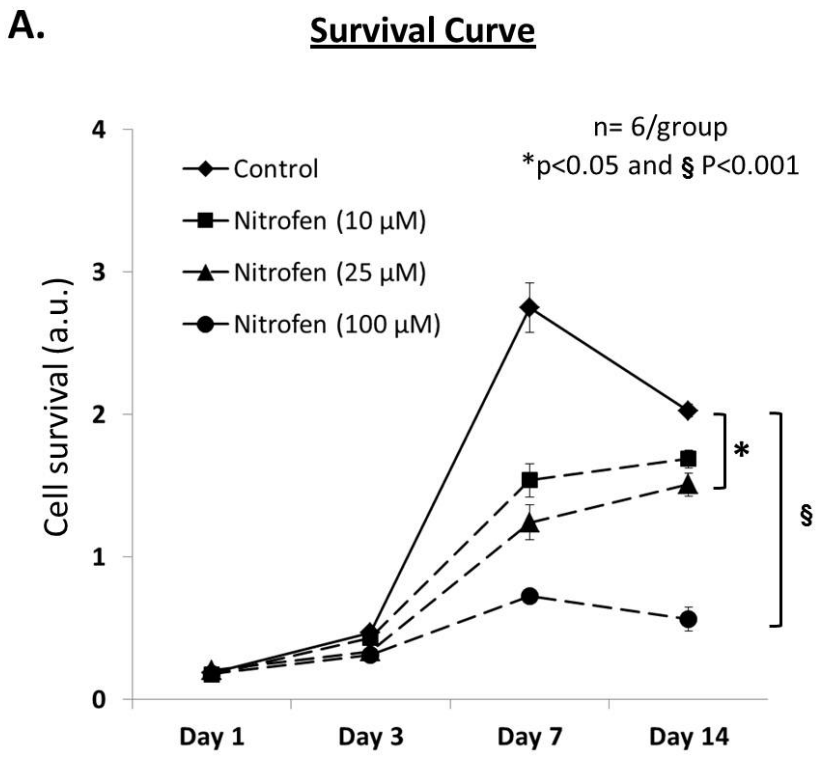
Number of late outgrowth ECFC colonies formed from every 500 CD31+ lung cells plated per sq-cm was evaluated for MCT-exposed and control groups. MCT lungs yielded fewer late outgrowth ECFC colonies per 500 plated CD31+ lung cells than controls. Results represent the mean ± SEM of 12 independent trials.

D.

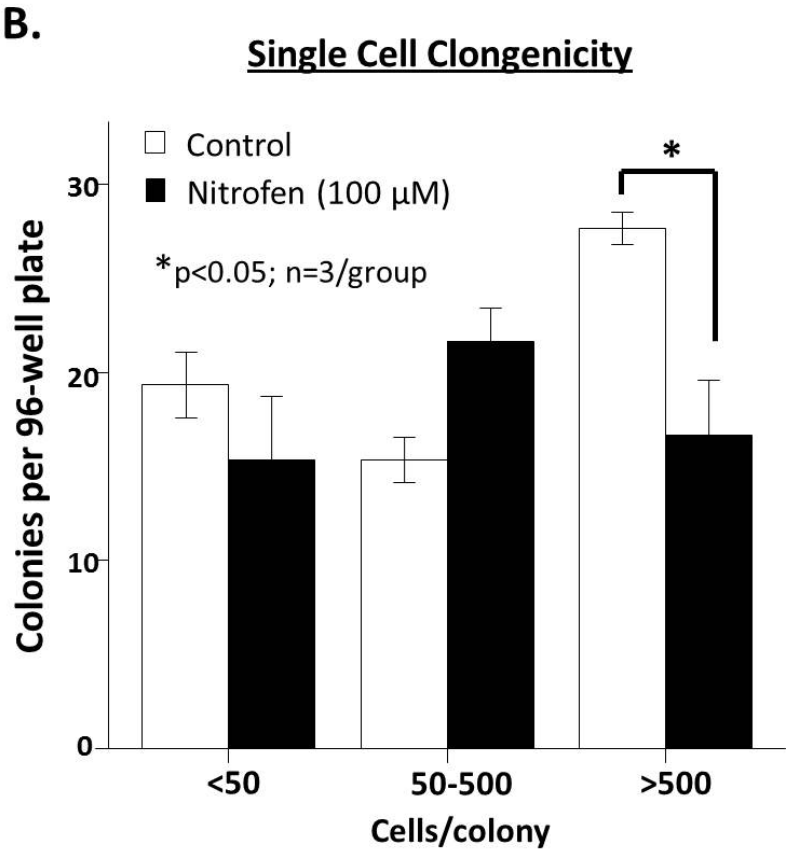


The ability of PV-ECFC to form cord-like endothelial networks was assessed by plating cells from both groups in Matrigel. Quantitative assessment of the tube-like structures revealed a significant decrease in total cord length and number of branching points in the MCT group, compared to controls. Results represent the mean ± SEM of 5 independent trials.

Figure 5-3 Nitrofen impairs cell survival and colony forming potentials of PV-ECFCs.

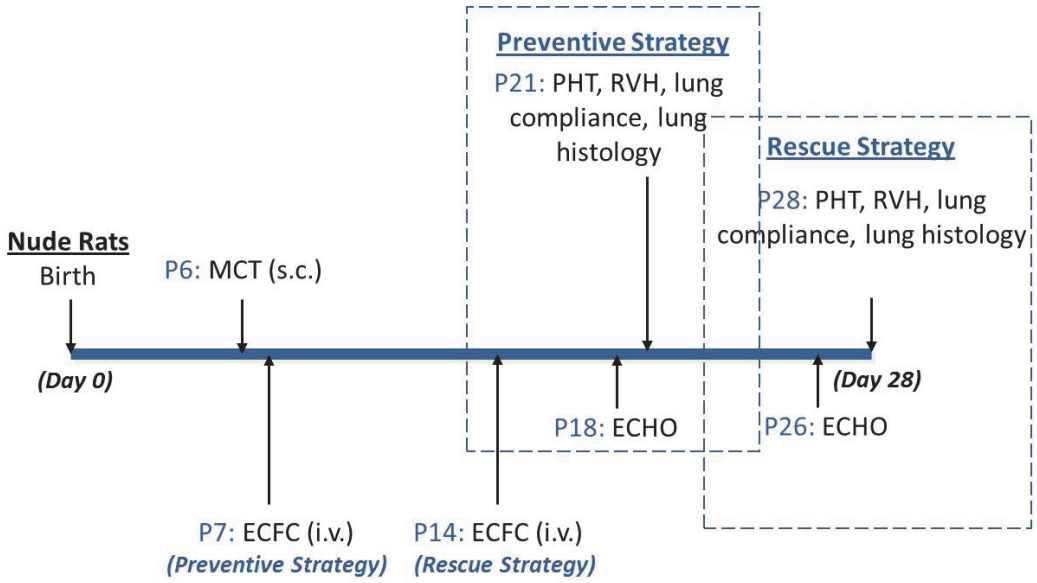


Representative growth curve of PV-ECFCs derived from healthy PD-14 rats cultured for two weeks with increasing concentrations (10, 25 & 100 µM) of congenital diaphragmatic hernia (CDH) inducing teratogen, nitrofen. Nitrofen exposure significantly decreases ECFC survival and growth, observed in a dose dependent manner. Results represent the mean ± SEM of 6 independent trials.



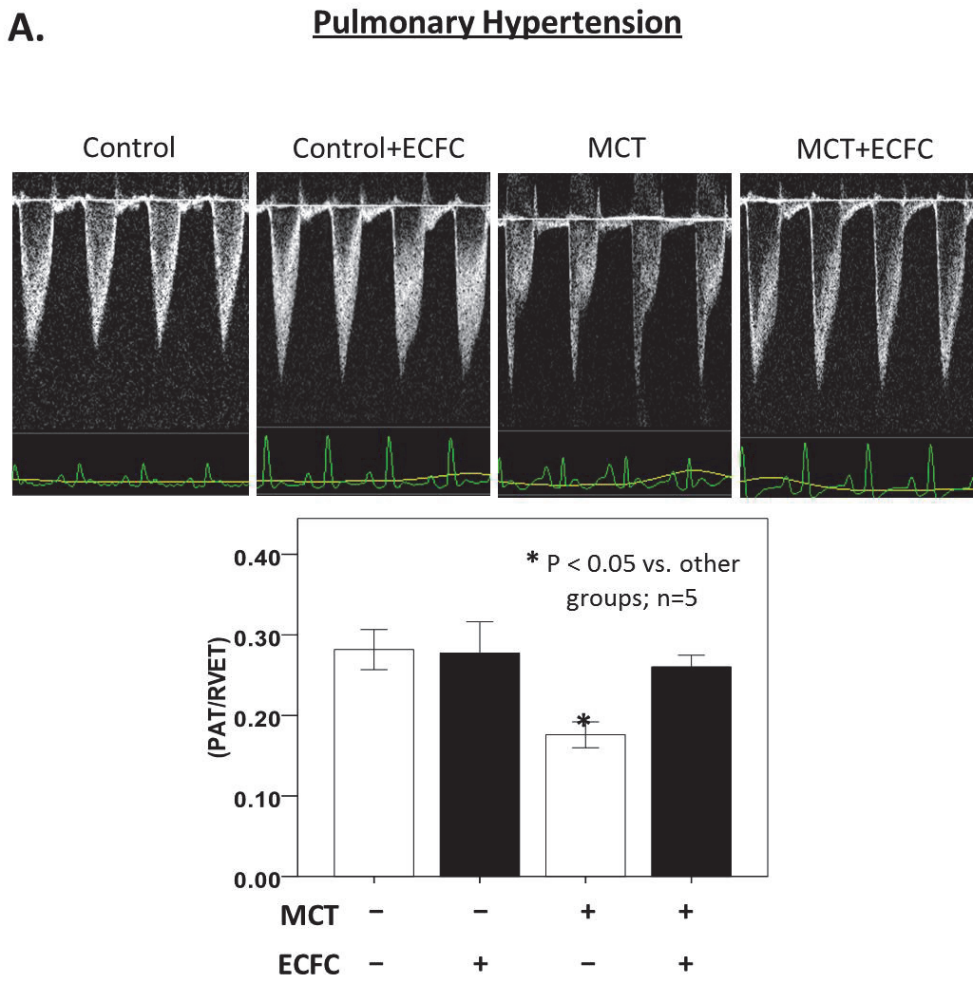
Exposure to 100 µM nitrofen also significantly compromised the ability of single plated PV-ECFCs to generate colonies with 500 or more cells upon maintenance in culture over a 14 day time period. Results represent the mean ± SEM of 3 independent trials.

Figure 5-4 Therapeutic effect of cord blood ECFCs was assessed in the MCT model using preventive and rescue strategies.



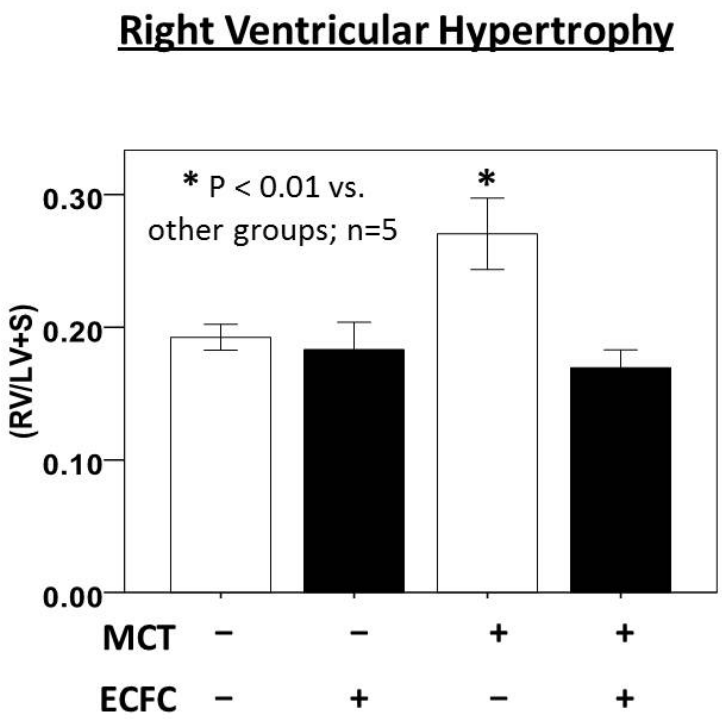
The schematic describes the strategies employed to test the therapeutic effect of human umbilical cord blood (hUCB)-derived ECFCs in the newborn rat model of MCT induced lung hypoplasia and PHT. The rat pups were injected with MCT subcutaneously (60 μg/g body weight) at postnatal day (PD)-6 followed by intra-jugular injections of human umbilical cord blood-derived ECFCs (10^5 cells/animal) on the next day (preventive strategy) or on PD-14 (rescue strategy) after the establishment of alveolar growth arrest. The animals treated by the preventive strategy were harvested around PD-21 and assessed for lung compliance, lung alveolar histology, PHT and RVH. The same assessments were made around PD-28 for the animals managed with the rescue strategy.

Figure 5-5 ECFCs prevent features of pulmonary hypertension in MCT-administered newborn rats.



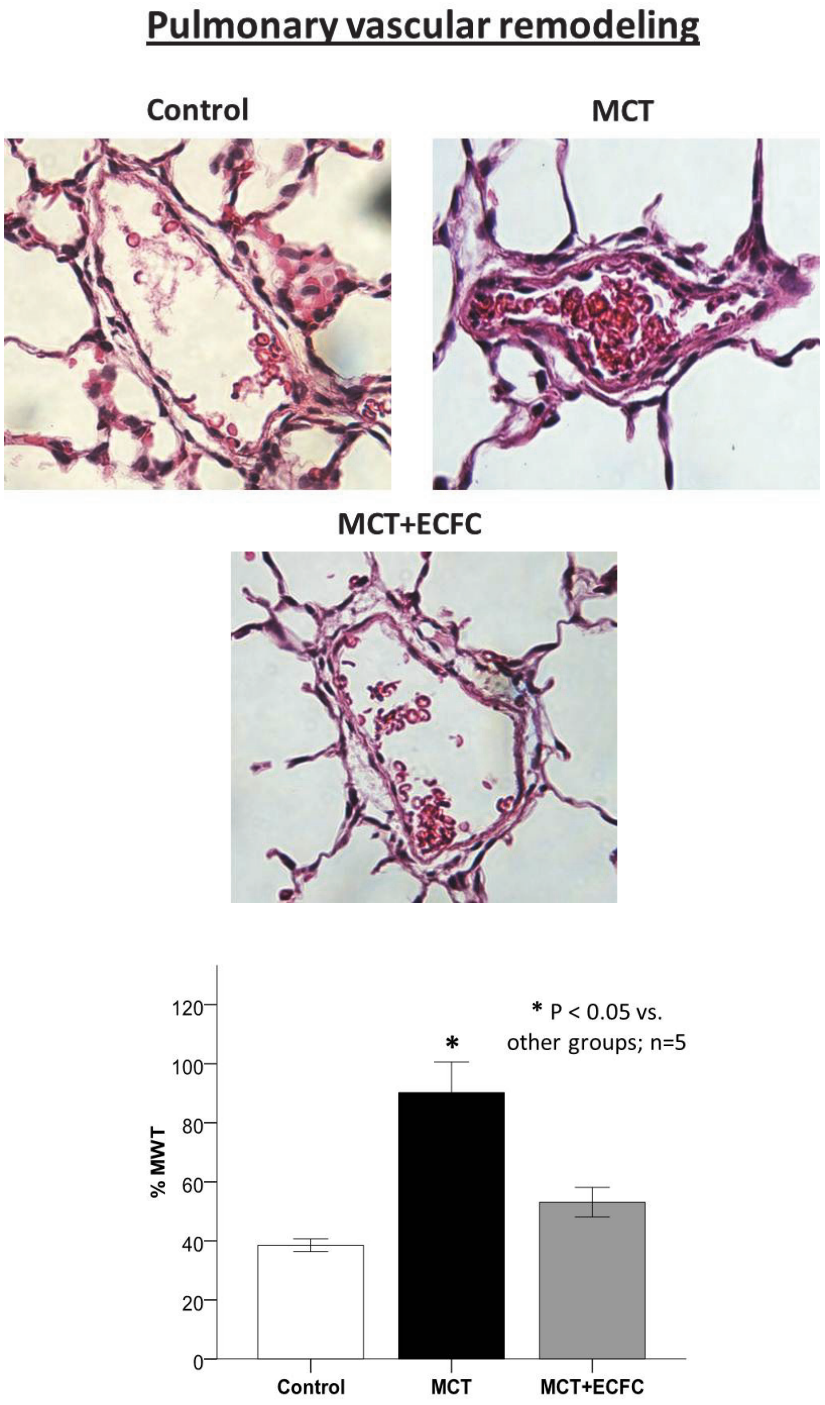
Intra-jugular hUCB-ECFC administration prevented the development of pulmonary hypertension and its associated right ventricular hypertrophy in newborn rats administered with MCT. Pulmonary hypertension was assessed using pulsed-wave Doppler to record the pulmonary arterial acceleration time (PAT). The significant decrease in PAT observed in MCT-injected rats was maintained almost at control levels in ECFC treated animals. Results represent the mean ± SEM of 5 animals/group.

B.



PAT values are expressed as their ratio to right ventricular ejection time (RVET) to normalize for the individual differences in heart rate among subjects. The pulmonary outflow Doppler findings were confirmed by assessing right ventricular hypertrophy (RVH) via measurements of right ventricle/left ventricle plus septum (RV/LV+S) ratio (Fulton Index). The marked RVH observed in the untreated MCT group was demonstrably averted in ECFC treated animals. Results represent the mean ± SEM of 5 animals/group.

Figure 5-6 ECFC therapy averts pulmonary vascular remodeling in MCT-exposed rats.



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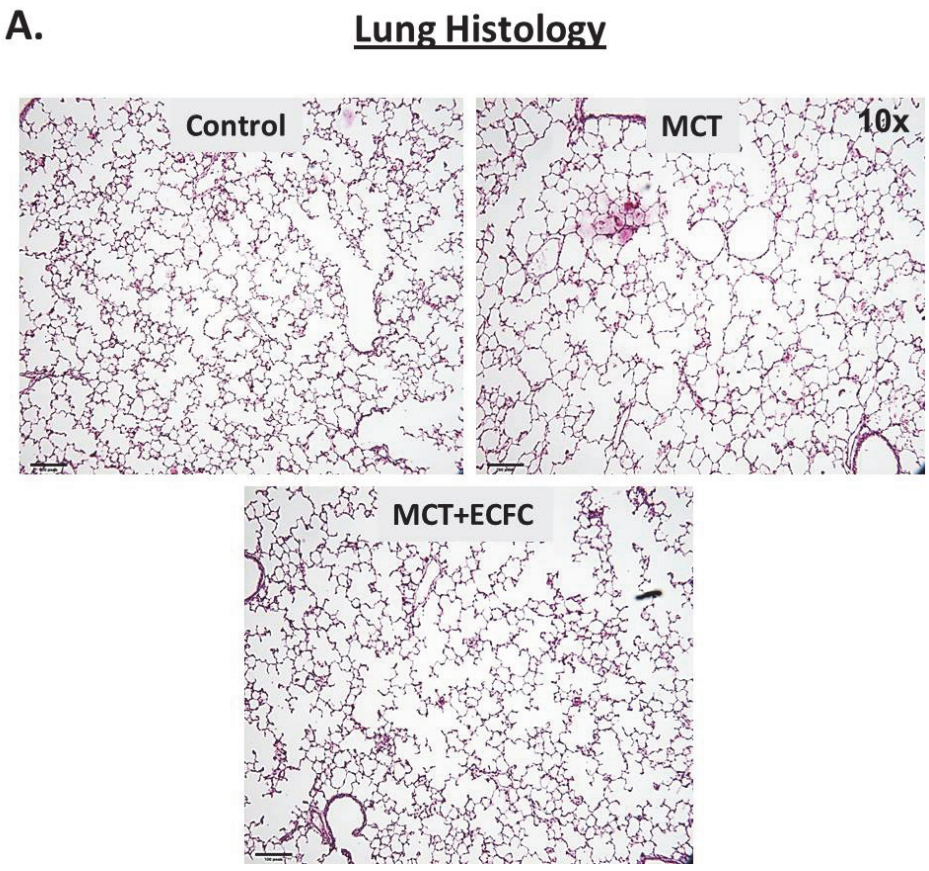
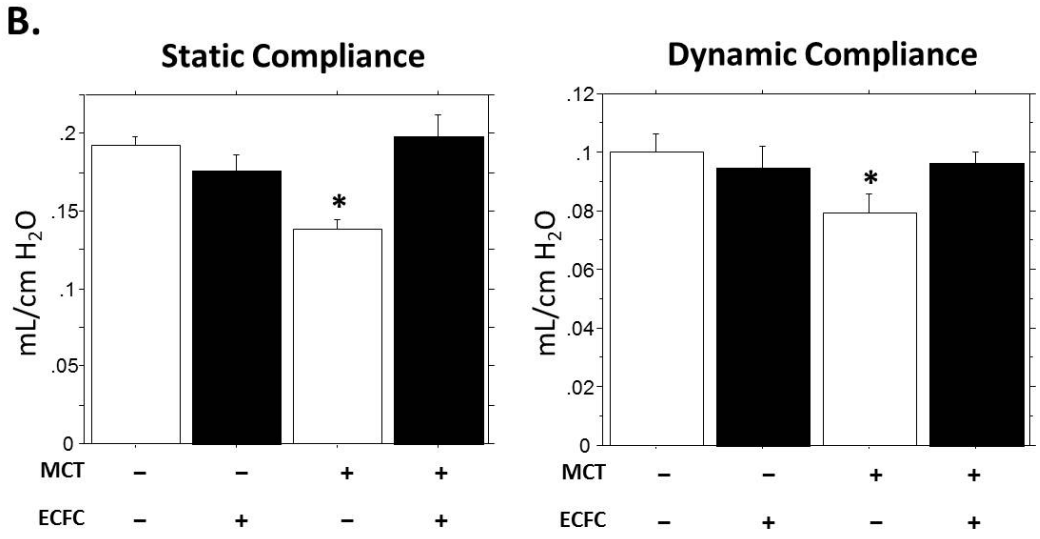
The representative picture of medium sized pulmonary arteries (diameter of 30-100 μm) displaying thickened medial arterial wall in MCT-administered rat lungs as compared with age matched controls. ECFC treatment prevented pulmonary vascular remodeling and preserved % medial wall thickness (% MWT) as compared with untreated MCT-exposed rats. Results represent the mean ± SEM of 5 animals/group.

Figure 5-7 ECFC therapy preserves alveolar growth and lung compliance in MCT-administered newborn rats.



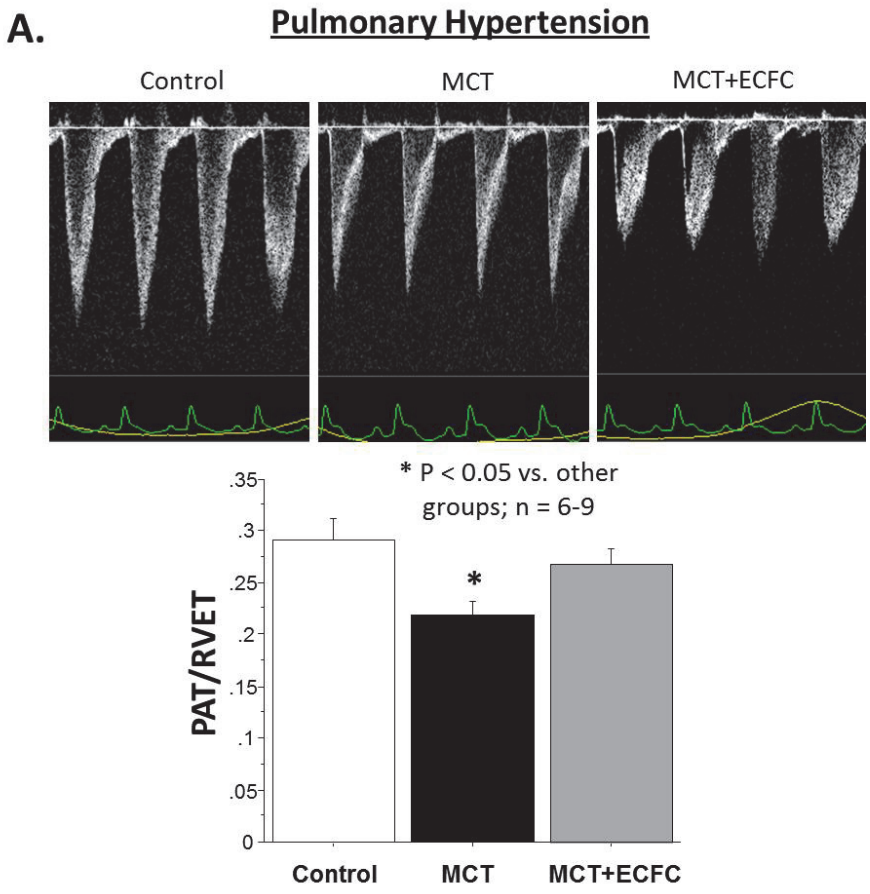
Intra-jugular hUCB-ECFC administration preserved alveolar growth and mechanical properties of lungs in MCT-injected rat pups. Representative H&E–stained lung sections depict larger and fewer alveoli in MCT-exposed lungs (PD-21) as compared to lungs from control animals. Lung architecture was demonstrably preserved in MCT rats treated with hUCB-ECFCs. These are representative images of serial lung sections from 5 animals/group



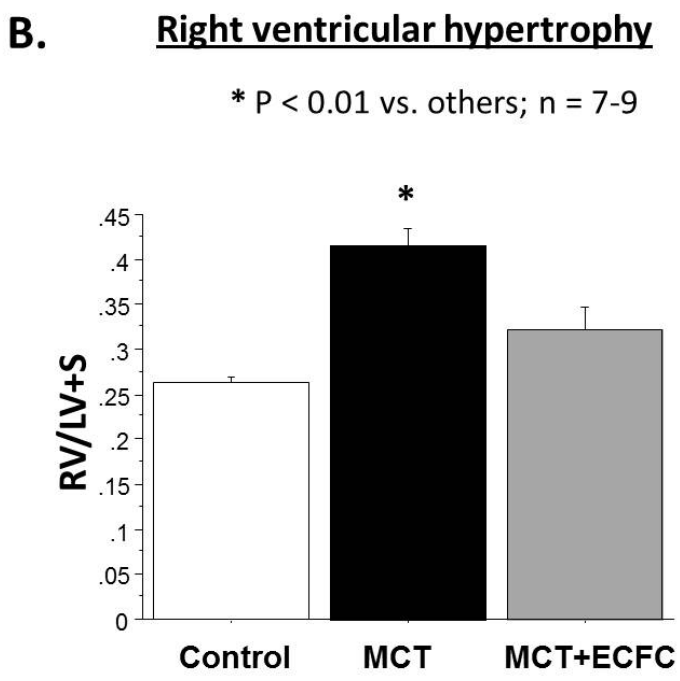
(* P < 0.05 vs. others; n = 4-5)

Invasive lung function testing showed decreased lung compliance in MCT-administered rats, compared to controls. ECFC therapy preserved both static and dynamic compliance parameters closer to control levels. ECFC adminstration in control rats showed no impact on their compliance measurements. Results represent the mean ± SEM of 4-5 animals/group.

Figure 5-8 ECFC therapy reverts pulmonary hypertension and right ventricular remodeling in MCT-administered rats.

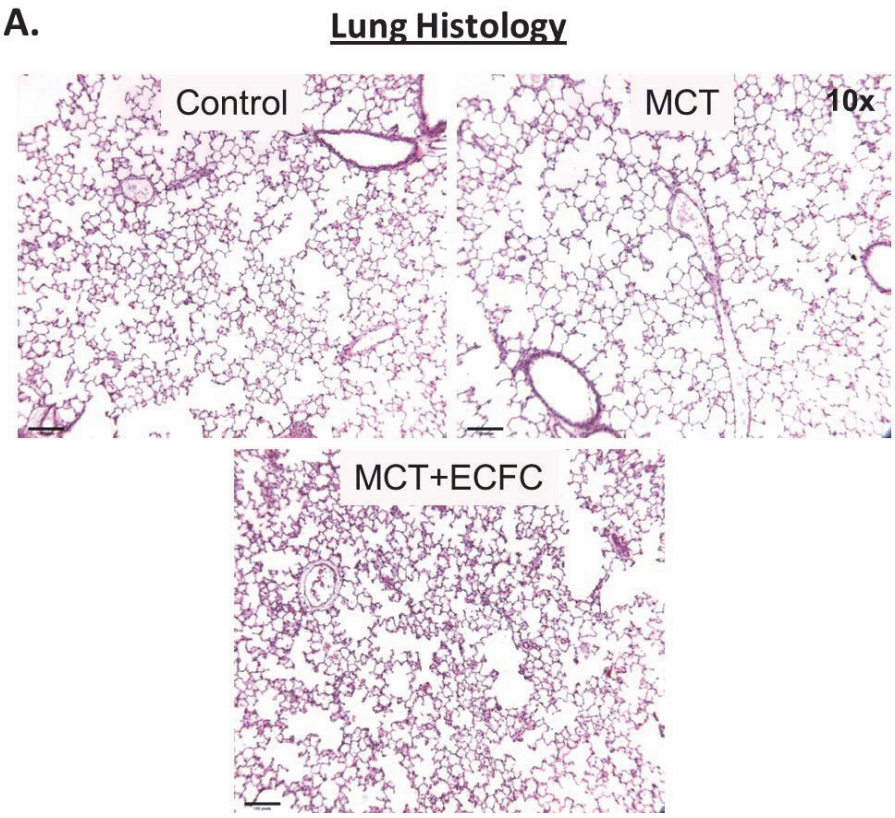


Intra-jugular hUCB-ECFC administration rescued the MCT administered newborn rats from developing pulmonary hypertension and its associated right ventricular hypertrophy. Significant decrease in PAT observed in MCT-injected rats was restored to almost control levels in ECFC treated animals. Results represent the mean ± SEM of 6-9 animals/group.



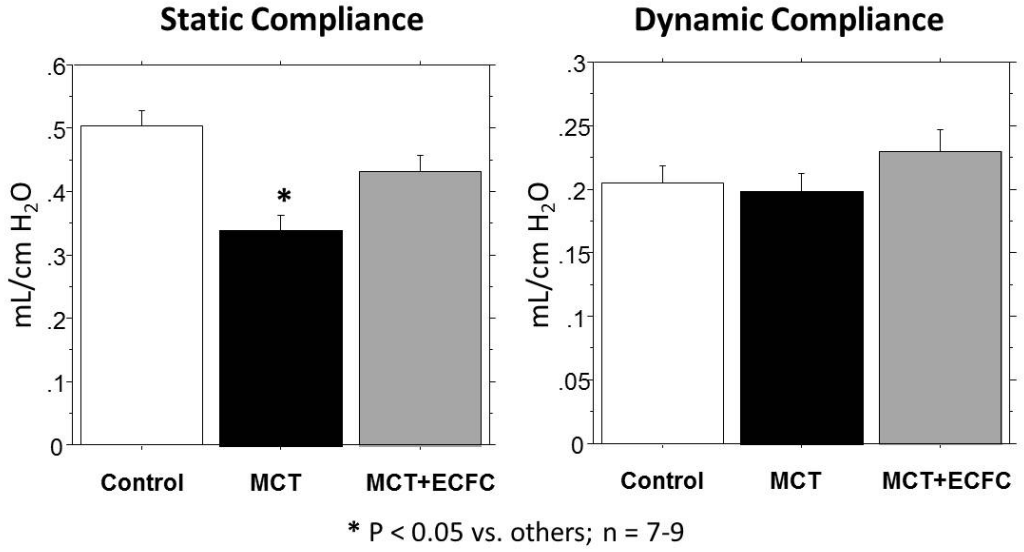
PAT values are expressed as their ratio to right ventricular ejection time (RVET) to normalize for the individual differences in heart rate among subjects. The outflow Doppler findings were supported by the absent or significantly less pronounced increase in RV/LV+S ratio in MCT animals treated with ECFCs. Marked RVH was recorded in the untreated animals of the MCT group. Results represent the mean ± SEM of 7-9 animals/group.

Figure 5-9 ECFC therapy rescues alveolar development and partially restores lung compliance in MCT-administered newborn rats.



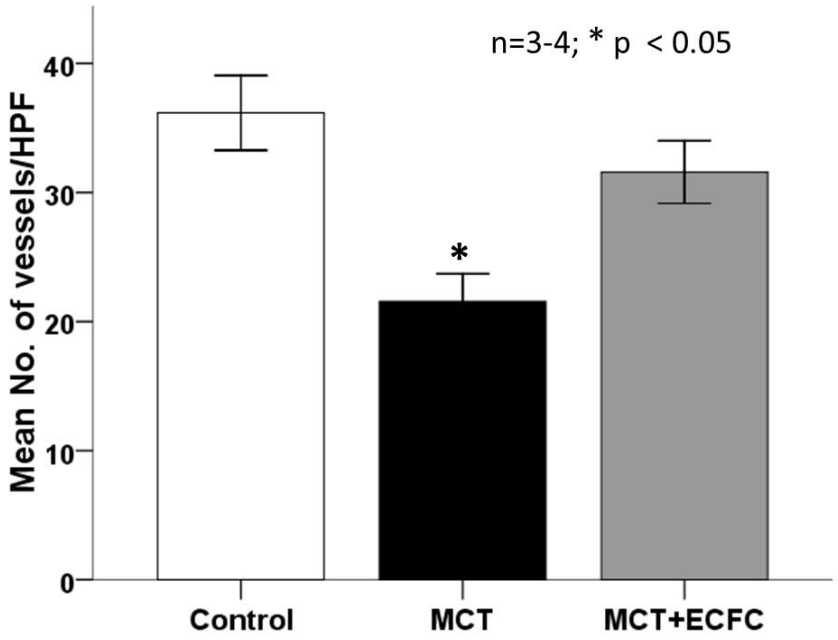
Representative H&E–stained lung sections depict larger and fewer alveoli in MCT-exposed lungs (PD-28) as compared to lungs from age-matched controls. In these animals, intra-jugular ECFC injections on PD-14 rescued alveolar architecture. These are representative images of serial lung sections from 5 animals/group.

B.



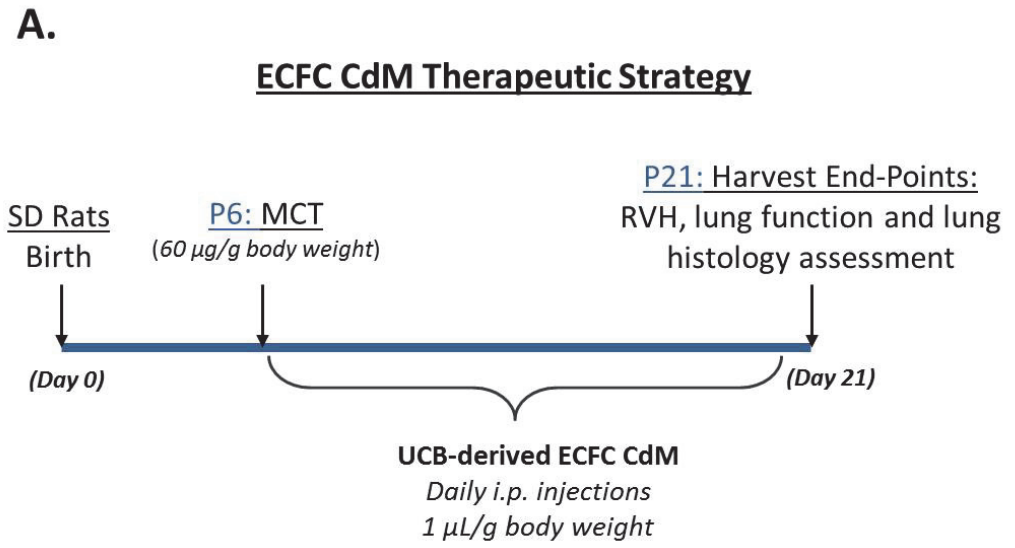
ECFC therapy significantly improved the static compliance parameter of lung mechanics in treated MCT rats. No significant improvement was evident in dynamic lung compliance. Results represent the mean ± SEM of 7-9 animals/group.

Figure 5-10 ECFCs promote pulmonary vessel growth in MCT-exposed rats.

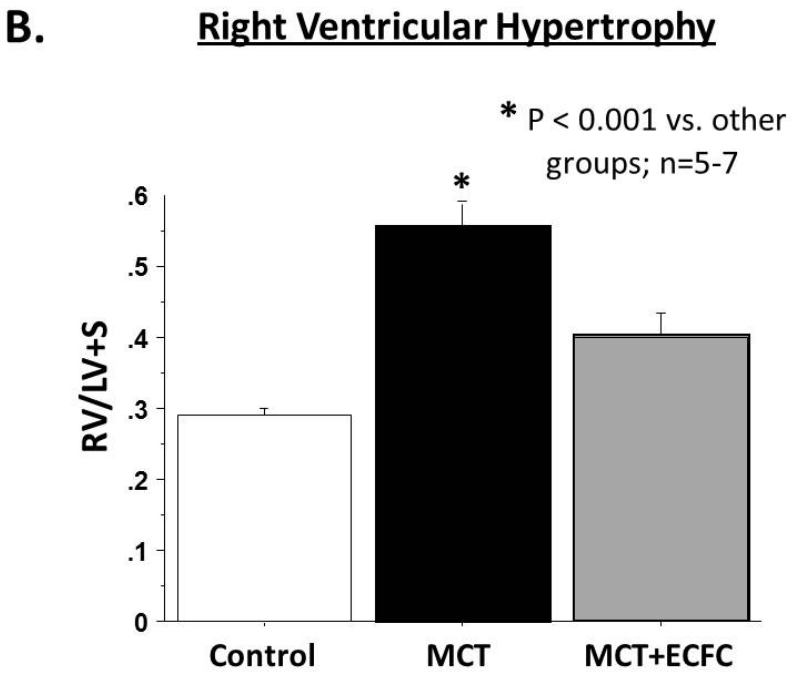


Intra-jugular ECFC administration promoted new vessel formation in the lungs of MCT-exposed newborn rats. At PD 21, barium was infused into the pulmonary arteries of control and. MCT animals and the number of barium-filled small to medium sized (30-100 μm) pulmonary arteries were counted in lung sections. Five sections/lung, and ten high-power fields/section were counted.

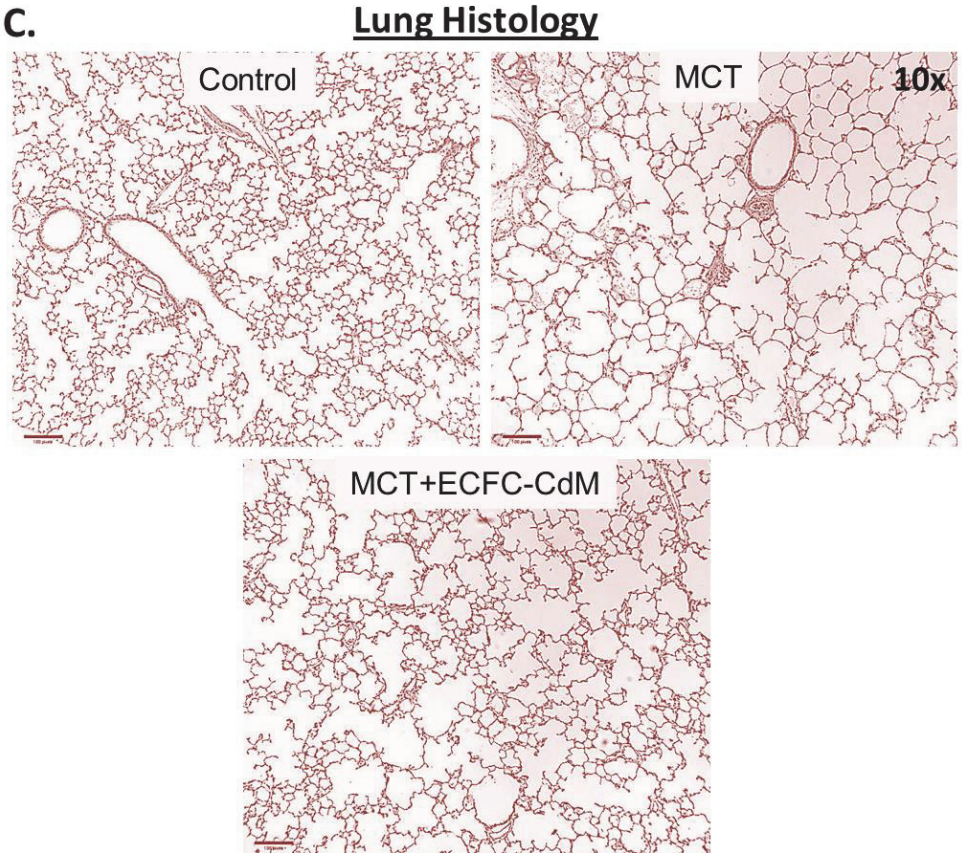
Figure 5-11 ECFC-conditioned media prevents pulmonary hypertension and maintains alveolar growth in MCT-exposed newborn rats.



The schematic describes the strategy employed to treat MCT-exposed rats with conditioned media (CdM) of hUCB-ECFCs.

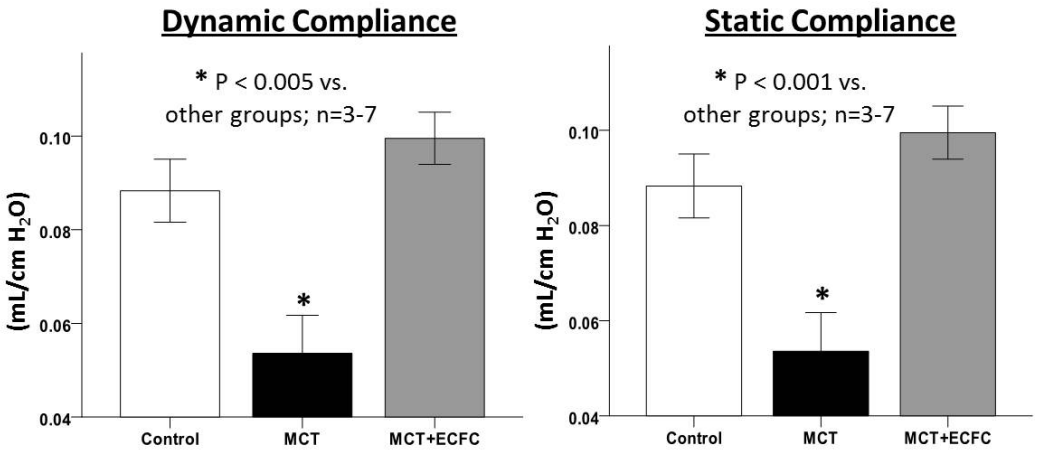


ECFC-CdM was adminstered as daily intra-peritoneal (i.p.) injections (1 μL/g body weight) from PD-6 to PD-21. Daily i.p. injections of ECFC-CdM significantly attenuated right ventricular hypertrophy associated with MCT-exposure. Results represent the mean ± SEM of 5-7 animals/group.



Representative H&E–stained lung sections show demonstrable prevention of alveolar growth arrest in CdM treated animals. These are representative images of serial lung sections from 5 animals/group.

D.



Daily i.p. injections of ECFC CdM also preserved lung compliance in the MCT exposed rats. Results represent the mean ± SEM of 3-7 animals/group.

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Chapter Six: **Isolation of endothelial colony forming cells from human and rat lungs**

Contributions:

The majority of the experimental work described in this paper was performed by ARSA, with technical support from our collaborators in Dr. Yoder's Lab, Indiana University School of Medicine, Indianapolis. The manuscript was written by ARSA and edited by BT.

6.1 Introduction

From either the developmental, anatomical or physiological perspective, vasculature is a "ubiquitous" organ exhibiting a remarkably dynamic capacity for growth and repair. The role of vasculature in the development, lifelong repair and maintenance of the lung, just as with any other organ, is unquestionably paramount. It is also true that most protracted lung ailments beginning from early postnatal life until late adulthood carry an underlying vascular abnormality (Sluiter et al., 2011; Thebaud and Abman, 2007; Voelkel et al., 2007). A closer understanding of this underlying vascular disease component shall reorient treatment strategies towards restoring vascular homoeostasis in the lung. Such an approach either independently or in conjunction with other existing treatments shall provide more effective therapeutic avenues for several debilitating chronic lung diseases (Voelkel et al., 2007).

Ever since the description of circulating endothelial progenitor cells (EPCs) by Asahara et al. (Asahara et al., 1997), postnatal vasculogenesis has been purported as an important mechanism contributing to new blood vessel formation (Asahara, 2007; Khoo et al., 2008). This landmark discovery triggered extensive research attempting to identify the different cell types that can be classified as EPCs and more specifically to correlate their number and function to several diseases (Kirton and Xu, 2010; Steinmetz et al., 2010), including major lung disorders (Borghesi et al., 2010; Huertas and Palange, 2011). Among the different described cell types with "EPC phenotype", endothelial colony forming cells (ECFCs), a population of late-outgrowth EPCs, have received widespread

attention as lineage-specific 'true EPCs' participating in neovasculogenesis (Ingram et al., 2004; Steinmetz et al., 2010; Yoder, 2010). ECFCs reside throughout the vascular endothelium contributing to generalized vascular integrity (Ingram et al., 2005) and are mobilized into a circulating pool of endothelial progenitors, potentially involved in neovasculogenesis via tissue recruitment and homing (Prater et al., 2007). Given these impressive characteristics, it is perhaps to be expected that ECFCs contribute to new vessel formation during lung organogenesis as well as support lifelong lung repair and maintenance. These features also make ECFCs likely candidates underlying the disease processes of several childhood and adult lung diseases. In a recent set of studies, we have shown that ECFCs exist in the developing mammalian lung (see chapter 4). We have also demonstrated that lung ECFCs are functionally impaired in neonatal oxygen-induced lung injury and experimental monocrotaline-induced lung hypoplasia and pulmonary hypertension (see chapters 4 & 5). Therefore, studying the functional characteristics of tissue resident ECFCs shall widen our understanding chronic lungs diseases, for which efficient therapies still remain elusive.

In order to study the survival capacity and other functional characteristics of pulmonary vascular (PV)-ECFCs, a method for the isolation and culture of these cells from rat and human lung samples was established and standardized in our laboratory. This technique takes advantage of the existing protocol for isolating circulating ECFCs from umbilical cord blood or peripheral blood mononuclear cells (Mead et al., 2008) and has been extensively revised and

optimized for successful isolation of resident ECFCs from a solid organ such as the lung. Following enzymatic disintegration of rat or human lung samples, CD31 expressing cells are positively selected out using magnetic-activated cell sorting (MACS) and plated in endothelial-specific growth conditions. Late-outgrowth ECFC colonies that arise after 1-2 weeks in culture are carefully isolated and expanded to yield pure ECFC cultures. The phenotype of ECFCs is confirmed by (i) classical 'cobblestone' morphology of cultured monolayers, (ii) assessment of endothelial cell characteristics via acetylated low-density lipoprotein uptake and Ulex lectin binding, (iii) tube-like network formation in Matrigel (iv) endothelial specific surface antigen expression (v) assessment of their ability to contribute to *de novo* vessel formation in an *in vivo* murine implant model (Ingram et al., 2004). A comprehensive overview of this method is provided in Figure. 6-1. In addition to providing a detailed description of this newly established technique, this chapter also provides a troubleshooting guide for the routine issues that might arise during the isolation process. Isolation and culture of lung resident ECFCs will allow assessment of the functional state of these cells in experimental and clinical lung diseases thereby providing insights into their pathophysiology.

6.2 Materials

6.2.1 Reagents

- DMEM, high glucose 1x (GIBCO-Invitrogen, cat. no. 11965)
- Antibiotic-antimycotic: Penicillin (10 000 U/mL)/streptomycin (10 000 µg/mL)/amphotericin (25 µg/mL) (PSA [100x]) (Invitrogen, cat. no. 15240)

- Human lung tissue sample (e.g., the authors typically use human embryonic lung samples of 15-18 week gestational age, stored in DMEM with 2% antibiotic-antimycotic)
- Dulbecco's phosphate buffered saline (PBS), without calcium and magnesium, pH 7.2 (Sigma, cat. no. D8537)
- Trypan blue solution 0.4% (GIBCO-Invitrogen, cat. no. 15250-061)
- Endothelial basal medium (EBM)-2 (Lonza, cat. no. cc-3156)
- EGM-2 growth factor supplement singlequots (Lonza, cat. no. cc-4176)
- Trypsin-EDTA (0.25%) (Invitrogen, cat. no. 25200)
- Collagen Type I solution (BD Biosciences, cat. no. 354236)
- Fetal bovine serum (FBS; Sigma Life Science, cat. no. F1051-100 mL)
- Collagenase/dispase (Roche Diagnostics, cat. no. 11097 113001)
- Streptavidin tagged M-280 dynabeads (Dynal, cat. no. 112.05D)
- Biotinylatyed anti-CD31 antibody (human- Abcam, cat. no. ab7385; rat- BD Pharmingen, cat. no. 555026)
- Bovine serum albumin (Sigma, cat. no. A7906)
- Glacial acetic acid (17.4 N) (Fisher Scientific, cat. no. 505216)
- 70% ethanol
- Vacuum grease, sterilized (Dow Corning, cat. no. 1658832)
- Dimethyl sulphoxide (DMSO), sterile filtered (Sigma, cat. no. D2650)

6.2.2 Equipment

- 6-, 24- and 96-well plates (BD Biosciences Falcon)
- 25 and 75 cm^2 vented tissue culture flasks (BD Biosciences Falcon)
- 15 mL and 50 mL conical centrifuge tubes (BD Biosciences Falcon)
- 10 cm petri dish, sterile (Coning, cat. no. 430167)
- 1 mL, 10 mL syringes, sterile (BD Biosciences, cat. nos. 309602 & 301604)
- Blades, sterile & disposable
- Pasteur pipettes (VWR, 14673-043), sterilized
- Round bottomed polystyrene culture tubes (12 mm x 75 mm H) (BD Biosciences Falcon, cat. no. T405-3)
- Cryovials (Nunc, cat. no. 375418)
- Anatomical scissors, tweezers

- Cell strainers, nylon (Fisher Scientific; cat. no. 22363547 [40 μm], 22363548 [70 μm])
- 0.22 μm vacuum filtration system (Millipore, cat. no. SCGPU05RE)
- Neubauer chamber (Reichert, Buffalo, N.Y.)
- Magnet (Dynal MPC-STM/MPC-LTM or DynaMagTM -2/-15)
- Assorted pipettes (2-20 μL, 20-200 μL, 100-1000 μL) with sterile, disposable tips
- Centrifuge
- 37^{o}C cell culture incubator
- 37^{o}C waterbath
- Inverted microscope
- Fine tipped marker
- Aluminium foil
- Glass dish
- Cloning cylinders, sterile (Fisher Scientific, cat. no. 07-907-10)

6.2.3 Reagent Setup

MACS buffer

Dulbecco's PBS is dissolved with 0.1% (w/v) bovine serum albumin. Can be stored up to 2 weeks at 4^{o}C

Serum-free 'basal' DMEM (basal-DMEM)

DMEM + 1% (v/v) PSA

10% FBS 'complete' DMEM (complete-DMEM)

DMEM + 10% (v/v) fetal bovine serum + 1% (v/v) PSA

Complete EGM-2 (cEGM-2)

EBM-2 is supplemented with all components of EGM-2 growth factor bullet-kit except human epidermal growth factor (hEGF), 10% (v/v) fetal bovine serum and 1% (v/v) PSA. Stored up to 1 month at 4^{o}C.

Collagenase/dispase digestive solution

5X stock solution: 5 mg of lyophilized collagenase/dispase (0.1 U collagenase & 0.8 U dipase/mg lyophilizate) dissolved per mL double distilled (dd)-water. Stored at -20^{o}C up to one month. Working dilution: 5X stock diluted 5 times with Dulbecco's PBS. Critical: Prepare on the day of the experiment.

Collagen type I solution

0.575 mL of glacial acetic acid (17.4 N) is diluted in 495 mL dd-water (final concentration, 0.02 N) and sterile filtered with 0.22 μm vacuum filtration system. Collagen type I stock is diluted with 0.02 N acetic acid to yield 50 μg/mL working dilution. The volume of collagen used varies depending on the supplied collagen stock concentration. Stored up to 1 week at 4^{o}C.

Sterile vacuum grease

A small amount of vacuum grease is spread into a thin layer in an autoclavable glass dish. After setting the lid, the glass dish is wrapped in aluminium foil, autoclaved and cooled completely.

6.3 Procedure

Critical Step: Work in strictly aseptic conditions (preferably under the cell culture hood) at all times

6.3.1 Preparation of reagents

1. Prepare all reagents described above and store them at 4°C (except trypan blue and ethanol) until use.
2. Prepare two to three 15 or 50 mL centrifuge tubes with fresh DMEM + 1% (v/v) antibiotic/anti-fungal solution and store them over ice (4°C) ready to use.
3. Preheat the incubator to 37°C.

6.3.2 Coating of cell culture surfaces with collagen type I

4. Add 1 mL of the working dilution (50 μg/mL) of collagen type I solution on each well of a six well tissue culture-treated plate (use 300 μL/well for 24 well plates, 2-3 mL/25-cm^2 flask and 5-6 mL/75-cm^2 flask).
5. Incubate 90 min to overnight at 37°C.

Caution: The next 2 steps are done right before plating the well plate with cells.

6. Remove the collagen type I solution and wash the surface twice with PBS (use 500 μL/well of PBS for 24 well plates, 5 mL/25-cm^2 flask and 10 mL/75-cm^2 flask).
7. Use immediately for cell culture.

6.3.3 Preparation of anti-CD31 tagged magnetic beads

8. Take 1 mg (100 µL of supplied suspension) of streptavidin tagged M-280 dynabeads in a 1.8 mL cryovial.
9. Add 500 µL MACS buffer and mix well.
10. Place the cryovial in the magnet for 1 min and pipette off supernatant completely.
11. Remove the cryovial from the magnet and resuspend the washed dynabeads with 500 µL MACS buffer.
12. Repeat steps 10 and 11 two more times
13. At the end of the third wash, resuspend the dynabeads in MACS buffer and add 10 µg of biotinylated anti-CD31 antibody while mixing, to reach an overall volume of 100 µL

Critical Step: The volume of antibody solution containing 10 µg of antibody varies depending on the supplied concentration. Add appropriate volume of antibody solution first and then make it up to 100 µL by adding MACS buffer.

14. Incubate for 30 min at 18-25^{o}C (RT) with gentle tilting and rotation.
15. Add 500 µL MACS buffer and pipette up and down several times.
16. Wash dynabeads three times as described in steps 10 and 11.
17. Resuspend dynabeads in 100 µL MACS buffer. Leave it on ice (4^{o}C) until use.

Critical Step: Anti-CD31 tagged magnetic beads can be prepared as described above, 1 day before the experiment and stored at 4^{o}C until use. This reduces effort on the day of the experiment, especially if performing this protocol for the first time.

6.3.4 Preparation of lung samples

18. Collect fetal or adult biopsied/autopsied lung samples in basal DMEM and immediately store on ice until use.

Alternative procedure: In case of isolating ECFC from rat lungs, follow steps 19-22.

19. Sacrifice the rat using sodium phenobarbitol (65 mg/kg, i.p.).
20. Wet the chest of the rat with 70% ethanol and open the chest of the rat with anatomical scissors.
21. Remove whole lungs of the rat with anatomical scissors and tweezers.
22. Transfer the lungs into 15 or 50 mL centrifuge tubes with basal DMEM (prepared in step 2) and immediately store on ice.

Caution: The lung samples procured by the above steps should ideally be processed for ECFC isolation, the same day. If the samples are shipped from a center elsewhere, lungs can be stored at 4^oC in basal DMEM for no longer than 24 hrs since removal from the body. Samples should not be allowed to freeze at any time during shipment.

6.3.5 Isolation of Total Lung Cells

23. Transfer lungs from the centrifuge tubes into a Petri dish.
24. Wash whole lungs with serum-free basal DMEM
25. Dissect out the peripheral rims of all lung lobes
26. Add a few drops of basal-DMEM and chop the lungs using sterile, disposable blades to very small pieces (approximately 1-2 mm^2) that could be drawn into a 5 mL pipette.

27. Transfer the pieces into a 15 mL centrifuge tube containing 1x working dilution of collagenase/dispase digestive solution (7-10 mL per 0.5 g wet tissue).
28. Digest the lung at 37^{o}C for 45 min under continuous horizontal shaking (300 r.p.m.).
29. Quench the lung-digestive solution mixture with equal volume of complete-DMEM
30. Draw the mixture up and down several times using a 5 mL pipette.
31. Place 70 μm cell strainer on a 50 mL tube
32. Under sterile conditions, strain the lung digest through the 70 mm cell strainers by releasing the lung digest on the cell strainer using a 5 mL pipette.

Caution: Slowly release the lung digest on the strainer to avoid overflowing of digested suspension. More than one 70 mm cell strainers may be needed at this step.

33. Wash the cell strainer with 2-3 mL of complete-DMEM
34. Re-strain the cell suspension through a 40 μm cell strainer into a 50 mL centrifuge tube
35. Centrifuge the 50 mL tube at 300 g at 4^{o}C for 10 min.
36. Decant the supernatant and resuspend the cell pellet in 10 mL of complete-DMEM.
37. Centrifuge the 50 mL tube at 300 g at 4^{o}C for 10 min.

38. Decant the supernatant and resuspend the cell pellet in 5 mL of MACS buffer.

6.3.6 Magnetic selection of CD31+ cells

39. Determine cell number of each sample of total lung cells using a Neubauer chamber. Prepare 1:10 and 1:100 dilutions of each sample in addition to the undiluted suspension. Mix 10 µL of these dilutions in a 1:1 ratio with trypan blue.
40. Centrifuge the cell suspension at 300 g for 10 min.
41. Decant the supernatant and resuspend the cell pellet in 1 mL of MACS buffer per 10^7 total cells.
42. Add 25 µL dynabeads per 10^7 total cells.

Caution: The volumes used above for magnetic selection are for up to 107 total cells. When working with fewer than 10^7 cells, use the same volumes as indicated. If a sample contains more than 10^7 cells, scale up all reagent and total volumes accordingly.

43. Incubate for 30 min at 2-8^{o}C with gentle tilting and rotation.

Critical Step: Use a mixer that provides tilting and rotation of the tubes to ensure dynabeads do not settle at the bottom of the tube. Strictly adhere to the incubation time and temperature to reduce phagocytic activity and other metabolic processes and to avoid lower purity or viability of the isolated cells.

44. Double the cell suspension volume with MACS buffer to limit trapping of unbound cells.
45. Place the tube in a magnet in aseptic conditions for 2 min.

46. Discard the supernatant and gently wash the bead-bound cells with 1 mL MACS buffer.
47. Place the tube in the magnet for 1 min and discard the supernatant.
48. Repeat steps 46 and 47 four times. At the end, centrifuge the cell suspension at 300 g for 10 min. Aspirate the supernatant.
49. Resuspend cells in cEGM-2 to obtain a suspension of 7.5x104 to 1.0x105 cells/mL.

6.3.7 Plating of CD31+ cells in culture

50. Pipette 2 mL (approx. 4000-5000 cells) into each well of a 6 well plate precoated with rat tail collagen type I and place in a 37^{o}C, 5% CO_2 humidified incubator.
51. After 24 hr (day 1), slowly remove the medium from the well with a pipette.

Caution: Medium is aspirated slowly at a rate of 1 mL per 4-5 sec. A small quantity of medium is left behind in the well to prevent drying of the plate surface.

52. Slowly add 2 mL of cEGM-2 to the well. Return the plates to the incubator.
53. Refresh the medium daily (as described in steps 51 and 52) until day 7 and every other day thereafter.

Critical Step: ECFC colonies appear between day 4 and day 14 of culture as well circumscribed areas of cobblestone-appearing cells (Figure 6-2A). Individual colonies should be isolated and expanded on day 7 to day 14. Mesenchymal stem cell (MSC)-like contaminating cells appear as dendritic/fibroblast-like cells (Figure 6-4) that can overgrow ECFC. When these contaminating cells are seen, ECFC colony isolation should be done very soon to get a pure subculture of ECFC.

6.3.8 ECFC colony isolation

54. Visualize the ECFC colonies using an inverted microscope and outline the colony boundaries with a fine tipped marker on the underside of the well.
55. Using sterile forceps, pick a cloning cylinder from its packing and dip the bottom surface into autoclaved vacuum grease to acquire a uniform coating.

Critical Step: Always apply the minimum amount of vacuum grease necessary to uniformly coat the bottom surface of the cloning cylinder and form a good seal with the culture plate. Too much grease will interfere with cell collection. Prepare cloning cylinders just prior to use.

56. Aspirate the culture medium and wash the well with 2-3 mL PBS.
57. After aspirating the PBS, set a clonal ring around each colony and press firmly against the plate with forceps.

Caution: Carefully apply vertical pressure to seal the cloning cylinder against the culture plate. Avoid any inadvertent sliding of the cylinder as this can disrupt the ECFC colony.

58. Using a 20-200 µL pipette, add 50-150 µL of warm trypsin/EDTA into each cloning cylinder.

Critical Step: Make sure that sufficient quantity of trypsin/EDTA is added to cover the entire area within the cloning cylinder.

59. Incubate plates up to 5 min until the cells have completely detached.
60. Draw 200 µL of cEGM-2 into a 20-200 µL pipette.

61. When all cells within the cloning cylinder have detached, place the tip of the pipette into the center of the cylinder and pipette up and down several times.
62. Collect the entire volume into a sterile microcentrifuge tube containing equal volume of cEGM-2 and mix well.
63. Wash the area within the cloning cylinder 1-2 times with 200 µL cEGM-2 until all detached cells are collected into the microcentrifuge tube.
64. Seed the cells from each ECFC colony into one well of a 24-well cell culture plate precoated with rat tail type-I collagen in a total volume of 1 mL of cEGM-2 and culture in a 37^{o}C, 5% CO_2 humidified incubator for expansion

Critical Step: If the ECFC colony is small (fewer than 100 cells), cells from 2-3 colonies can be pooled and plated into one well of a 24-well cell culture plate.

6.3.9 Further Purification and Expansion of ECFC

65. When cells in the 24-well plate approach confluence, remove cell culture medium and wash the culture surface with PBS.
66. After removal of PBS, add 100 µL of trypsin/EDTA per well of the 24-well plate and incubate plates up to 5 min until the cells have completely detached.
67. Pipette up and down thoroughly with 500 µL of cEGM-2 to collect the detached cells into a 15 mL centrifuge tube.
68. Wash the well 1-2 times with 500 µL cEGM-2 until all detached cells are collected into the centrifuge tube.

Critical Step: Cells from up to 2-3 confluent wells can be pooled into one centrifuge tube.

69. Make the volume of the cell suspension up to 12-14 mL with cEGM-2 and mix well by pipetting up and down gently.

70. Plate the cells in a 75 cm^2 vented tissue culture flask precoated with rat tail type-I collagen and culture in a 37^oC, 5% CO_2 humidified incubator for expansion.

71. Replace culture medium with fresh cEGM-2 every other day and culture until cells approach 80-90% confluence.

Critical Step: Cultures reach 80-90% confluence in 4-7 days.

72. When the tissue culture flasks have approached 80-90% confluence, carefully examine the cultured cells using inverted microscope to spot areas of MSC-like contaminating cell colonies as opposed to uniform cobblestone-appearing ECFC monolayer.

Critical Step: It is not uncommon to find MSC-like contaminating cell colonies interspersed with the ECFCs in early passages of the culture (Figure 6-4). The contaminating cells appear as described under step 53. The PV-ECFCs can be purified further by performing approximately 3-5 cycles of CD31-positive magnetic selection and subculturing.

73. Remove culture medium from the cell culture flasks and wash the culture surface liberally with PBS.

Critical Step: For a 75 cm^2 tissue culture flask, use 8-10 mL of PBS for the washes.

74. Aspirate PBS and add trypsin/EDTA.

Critical Step: Warm trypsin/EDTA to 37^{o}C prior to use. Use 3 mL trypsin/EDTA per 75 cm^{2} culture flask.

75. Incubate the flask at 37^{o}C until the cells detach completely.

76. Add 8-10 mL of cEGM-2 and collect the cells completely into a 15 mL centrifuge tube.

77. Centrifuge the 15 mL tube at 300 g at 4^{o}C for 5 min.

78. Decant the supernatant and resuspend the cell pellet in 1 mL of MACS buffer.

79. Take up 10 µL of the ECFC suspension and mix with 10 µL trypan blue.

80. Apply 10 µL of this mixture into a Neubauer chamber and count the cells

Critical Step: Usually a confluent 75 cm^{2} tissue culture flask contains 2-4 million ECFCs. Up to 107 cells can be suspended in 1 mL of MACS buffer.

81. Perform a MACS selection of CD31 positive cells following the procedure described in steps 42-49.

Critical Step: This step is to remove out the MSC-like contaminating cells to obtain a pure culture of ECFCs. If contaminating cells are still present upon further passage, repeat MCS selection CD31 positive cells at each passage until a pure ECFC culture is obtained. Usually pure ECFC cultures are obtained after 2-4 cycles of CD31-positive selection and passaging.

6.4 Trobleshooting Guide

(see Table 6-1)

6.5 Time Considerations

Isolation of CD31+ cells from lung samples and their initial plating in culture requires ~4.5 hrs. The approximate timeline for the different steps this involves are as follows:

- Preparation of reagents: 2 hrs
- Remove lungs from rat: 5 min
- Isolation of total lung cells: 1 hr 45 min
- Magnetic selection: 30 min

In case of human lung samples, immediate processing may not be possible in most circumstances. If lung samples are shipped from an external centre, carefully store at 4^{o}C in basal DMEM for not more than 24 hrs. In the authors' experience, ECFC colonies can be isolated following this holding period; however, the number of colonies will decrease as the time between sample collection and processing increases.

Late-outgrowth ECFC colonies usually arise in culture of CD31+ lung cells between days 4 and 14. These colonies can be isolated and expanded in culture in two to three 75-cm^2 flasks in ~2 weeks for human lung samples and ~3 weeks for rat lungs. The expanded cells should be processed through 2-4 cycles of CD31+ selection and passaging to obtain pure cultures. This may take an additional 2-3 weeks. There is variability in number of primary ECFC colonies obtained and their population doubling times between donors for human lungs.

For rats, since routinely lung tissue is pooled from several animals, not much variability is observed.

6.6 Anticipated Results

6.6.1 Initiation and propagation of ECFCs

ECFC colonies appear between 7 and 14 days of culture. For the human lung samples, donor age and disease states may affect the affect the number and time of appearance of ECFC colonies. For rat, the variability depends on the different treatment groups used in the experiment. Routinely, embryonic human lung samples can be expanded to 10^7 cells in 8-10 weeks. Rat lungs, on the other hand, may require a longer time (12-15 weeks) to yield the same number of ECFCs. Both rat and human PV-ECFCs have the potential to be cultured for up to 15 passages before they attain morphologically discernible senescence.

6.6.2 Phenotypic characterization of PV-ECFCs

PV-ECFCs grow as a monolayer with the characteristic 'cobblestone' morphology. They uptake AcLDL, bind Ulex lectin and form capillary tube-like networks when plated on Matrigel (Figure 6-2A). ECFCs uniformly express the endothelial cell-specific surface antigens CD31, CD146, CD105 and CD144, but do not express hematopoietic cell specific surface antigen CD45 or monocyte/macrophage marker CD14 (see Chapter 4 for detailed methods) (Figure 6-2B). In addition, human PV-ECFC are capable of giving rise to clusters (up to 50 cells) or colonies (50-500 cells or more than 500 cells) in 96-well plates where

ECFCs are plated at a seeding density of 1 cell per well. In at least three independent trials by the authors, 6.0 ± 1.0 % and 7.6 ± 2.8 % of single plated human PV-ECFCs formed colonies with 50-500 cells and more than 500 cells, respectively (Figure 6-2C). This characteristic of 'single-cell colony forming potential' is important in distinguishing ECFCs from mature endothelial cells (Ingram et al., 2004).

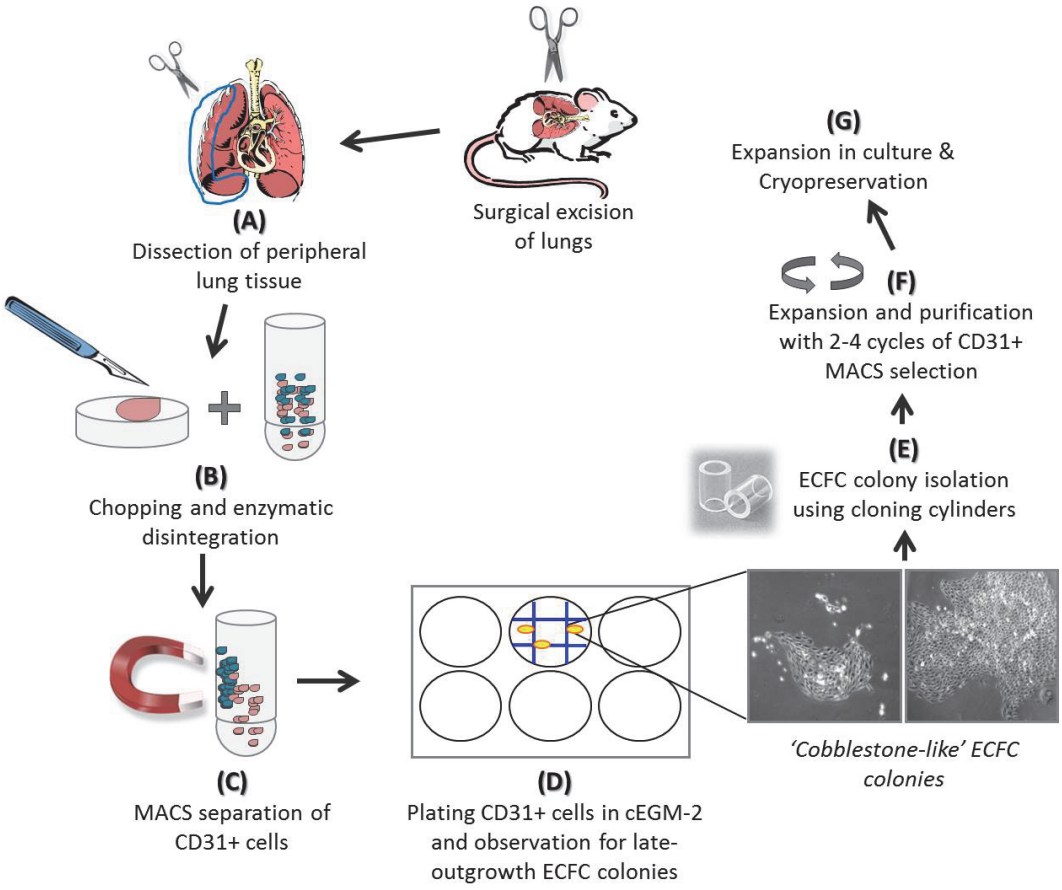
The most stringent means to verify the functionality of ECFCs is to assess their ability to contribute to *de novo* vasculogenesis. In order to assess this property, ECFCs are loaded on collagen-fibronectin matrices and and implanted subcutaneously in NOD/SCID mice. Cellularized collagen-fibronectin implants are excised after 14 days post-implantation and examined for vascularization by immunohistochemical staining. RBC perfused anti-human CD31+ vessels are identified in implants loaded with human PV-ECFCs (Figure 6-3).

Table 6-3 Troubleshooting Guide

Problem	Possible Reason	Solution
Too few viable cells after enzymatic disintegration	Poor storage conditions or prolonged storage of lung samples	For rat lung, it is best to process them immediately after excision. For human lung, if immediate processing is not feasible or if being shipped from an external centre, carefully store at 4^{o}C in basal DMEM for not more than 24 hrs. Samples should never be allowed to freeze anywhere during this storage period.
	Too long or too vigorous shaking during enzymatic digestion step	Do not leave the chopped lung sample for more than 45 min in pre-warmed collagenase/dispase. Do not shake at speeds greater than 300 r.p.m.
Too few viable cells after CD31+ MACS selection	Miscalculation during anti-CD31 tagging of dynabeads	Recheck the amount of anti-CD31 antibody added per unit volume of dynabeads
	Miscalculation during coating of cells with anti-CD31 tagged dynabeads	Recheck the amount of anti-CD31 tagged dynabeads added per 10^7 cells
	Incubation temperature too high or too low	Strictly adhere to the incubation time and temperature specified in the protocol
No ECFC colonies appear	Harsh treatment of plated CD31+ cells	Remove and replace medium very slowly and gently during the first week of culture. Do not use vacuum suction to remove medium.
	Very low CD31+ cell seeding density	Plate at least 3-5 x 10^3 CD31+ cells/well at step 50.
Too many contaminating cells around ECFC colonies	Antibody problem	Antibodies may not be very specific. Use antibodies from standard manufacturers, preferably the same ones used in the protocol. Do not subject antibodies to repeated freeze-thaw cycles and leave it on ice all the time before use.

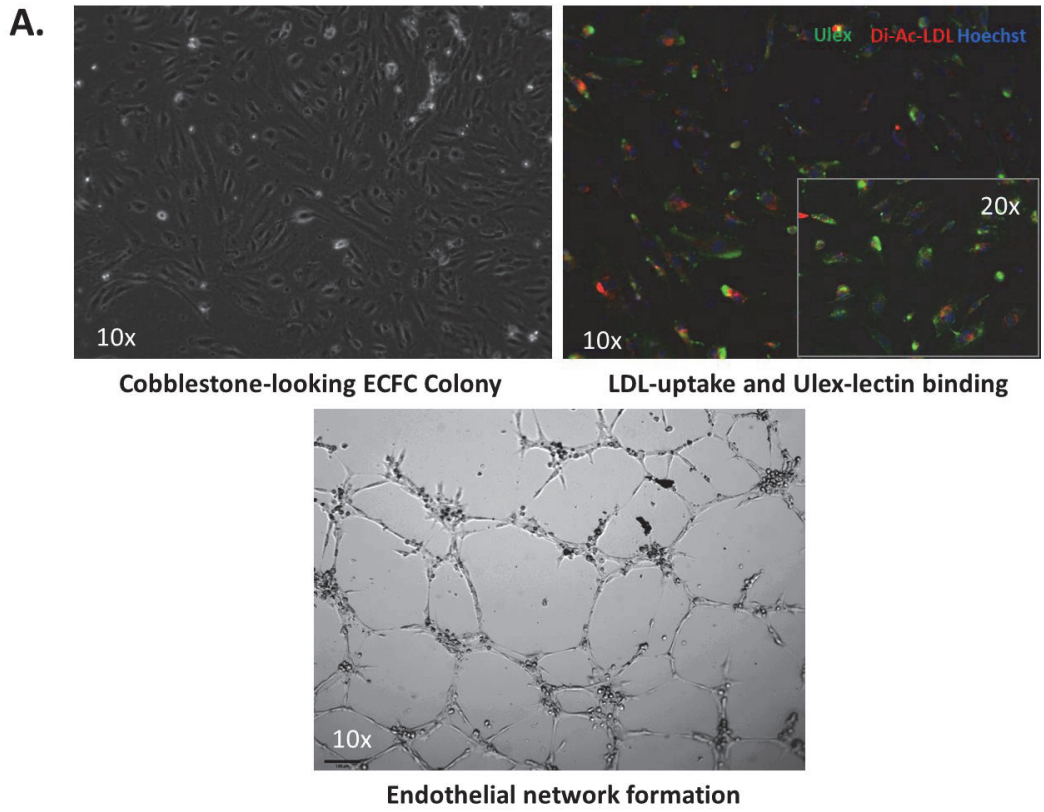
Problem	Possible Reason	Solution
	Ineffective coating of cells with anti-CD31 tagged dynabeads	Strictly adhere to the incubation time and temperature specified in the protocol. High phagocytic activity will result in non-CD31+ cells being selected out in large numbers.
	Improper timing for ECFC colony selection	ECFC colonies generally appear anytime between 4 and 14 days of culture. If allowed unattended for too long after appearance of ECFC colonies, MSC-like contaminating cells will quickly overgrow the cultures.
ECFCs do not attach and grow	Ineffective type I collagen coating of culture surfaces	If plated on uncoated culture surfaces, ECFCs never attach and grow. Use fresh and properly constituted type I collagen solution for coating. If expired collagen stock or improperly constituted working dilutions are used, ECFCs may fail to attach and grow.
ECFCs do not detach	Serum may block trypsin activity	Wash 2-3 times with PBS to remove leftover serum components, prior to adding trypsin.
	Trypsin activity is low	Use fresh, warm trypsin. Freeze trypsin as smaller aliquots such that once thawed, the entire aliquot is used and not exposed to repeated warming and cooling.
ECFCs are not dividing	Cells are senescent	ECFC-derived mature endothelial cells eventually undergo senescence. For best results in functional assays, use ECFCs between passages 5 and 10.

Figure 6-1 Schematic representation of rat lung ECFC isolation.



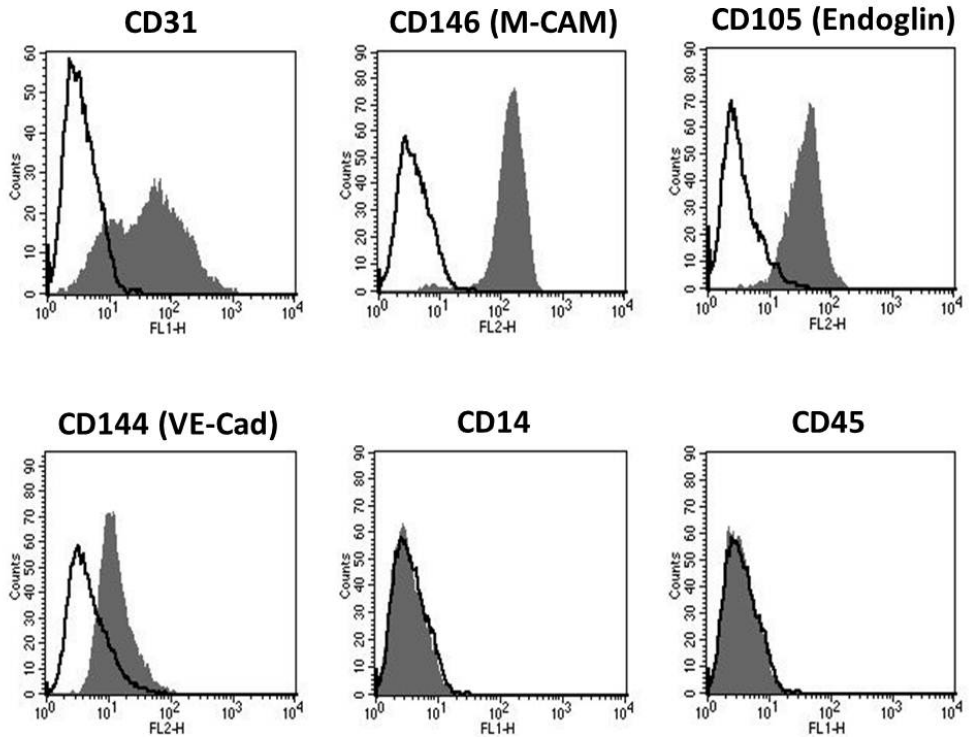
After thoracotomy and careful excision of the rat lung: **(A)** Dissect out the peripheral rims of all lung lobes. **(B)** Chop the lung into small pieces and disintegrate the lung tissue into a single cell suspension by incubating in collagenase/dispase digestive solution. **(C)** Select out CD31+ cells from the total lung cell suspension using magnetic-activated cell sorting (MACS). **(D)** Plate and culture the CD31+ lung cells in type I collagen-coated 6 well plates containing complete-Endothelial Growth Medium-2 (cEGM-2). Late-outgrowth 'cobblestone-like' ECFC colonies appear between 4-14 days. **(E)** Carefully mark and isolate the individual ECFC colonies using cloning cylinders. **(F)** Expand ECFCs by culturing them with cEGM-2 and purify the culture with 2-4 cycles of CD31+ selection and passaging. **(G)** Expand the cells in culture and cryopreserve them for future use.

Figure 6-2 Representative phenotypic analysis of human PV-ECFCs.

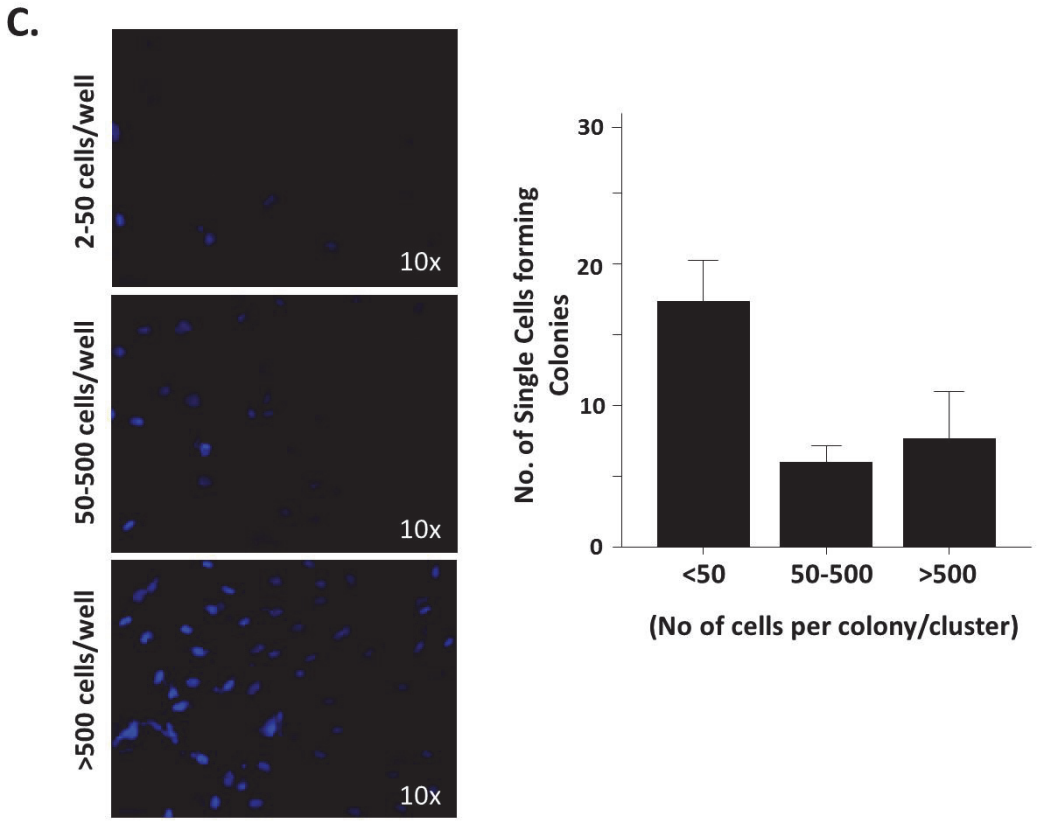


Human PV-ECFCs form cobblestone-like colonies when cultured as a monolayer. Cells are observed by phase contrast microscopy at 10x magnification. Cultured PV-ECFCs demonstrate Dil-acLDL uptake and Ulex europaeus-lectin binding. The cells ingest Dil-AcLDL (red) following 4 hrs of incubation and bind Ulex europaeus-lectin (green) following 1 hr of incubation after fixation. Counterstaining with Heochst 33258 illustrates that all adherent cells are positive for LDL-uptake and Ulex-lectin binding. It was observed that a modest number of stray MSC-like contaminating cells did not take up LDL. But these cells were totally lost following 2-4 cycles of CD31-positive selection and passaging. PV-ECFCs formed tube-like structures when suspended in matrigel and observed by light microscopy

B.

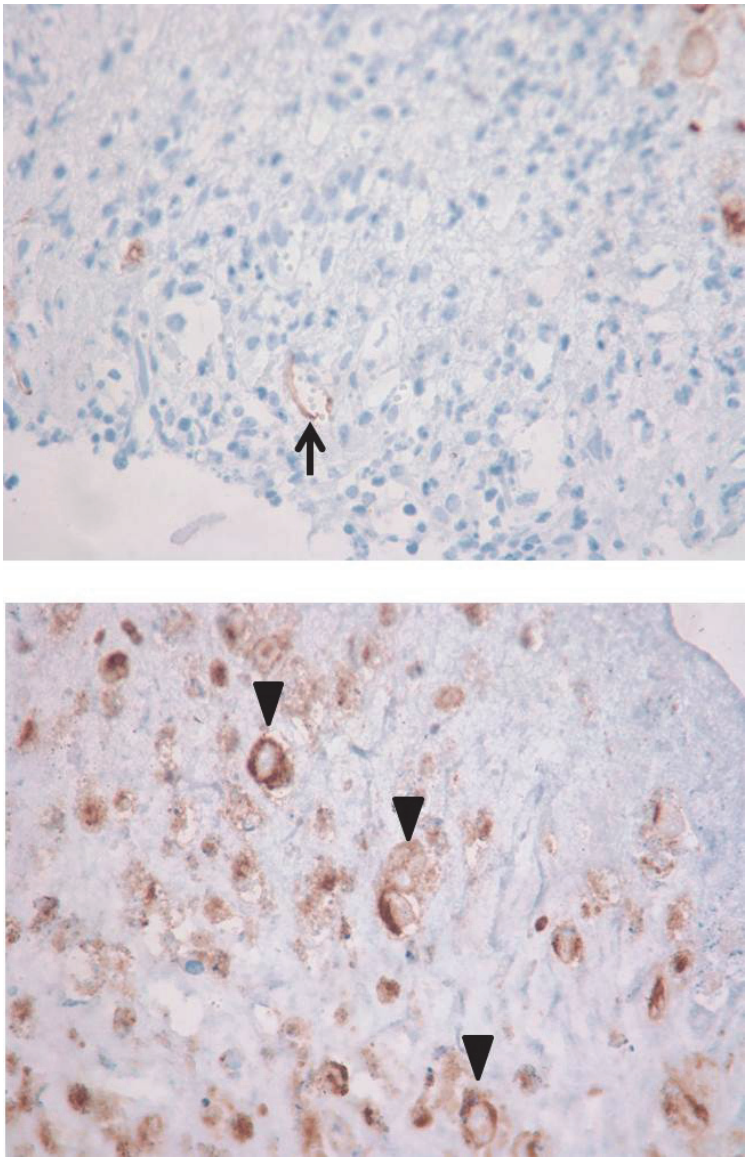


Human PV-ECFCs were positive for endothelial-specific cell surface antigens CD31, CD146 (M-CAM), CD144 (VE-cadherin), CD105 (endoglin) and negative for hematopoietic cell specific CD45 and monocyte/macrophage specific CD14. Filled grey histograms represent antigen staining with negative isotype controls overlaid in white.



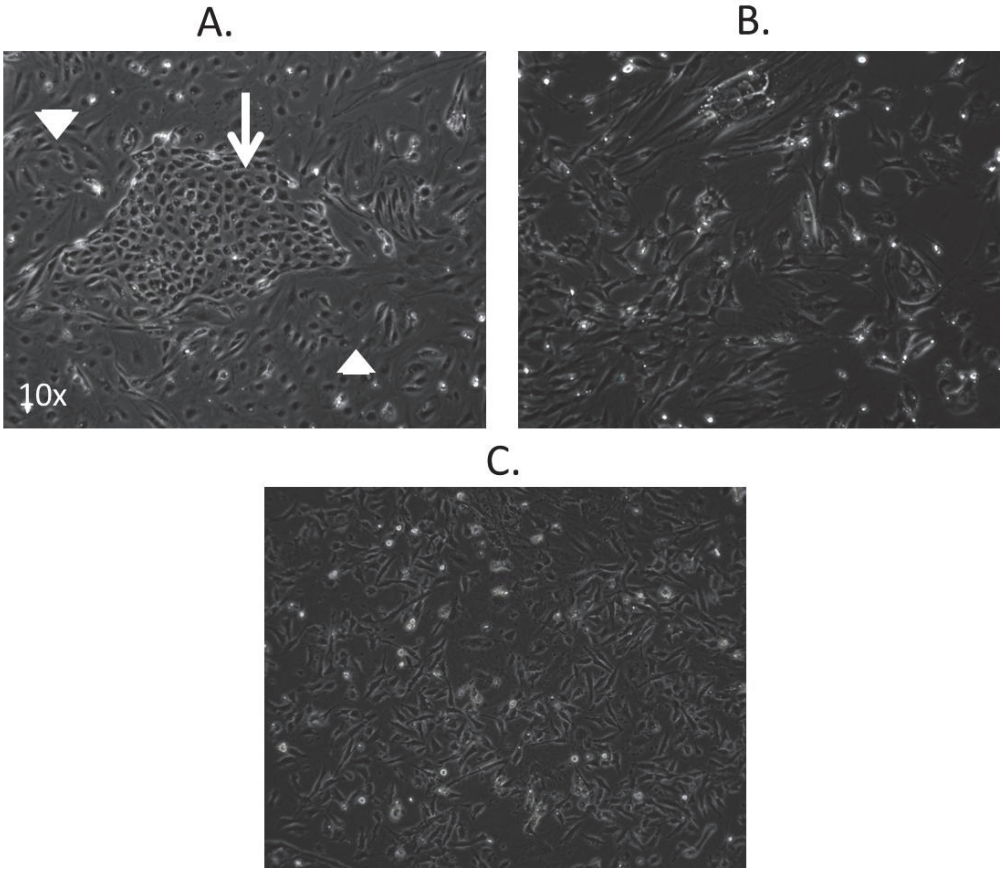
Human PV-ECFC were capable of giving rise to clusters (up to 50 cells) or colonies (50-500 cells or above 500 cells) in 96-well plates where ECFCs were plated at a seeding density of 1 cell per well. Results represent the average ± standard error of mean of 3 independent experiments.

Figure 6-3 Human PV-ECFCs to contribute to *de novo* vasculogenesis.



Human lung ECFCs form blood vessels *de novo* when seeded on fibronectin-collagen plugs (10^6 ECFCs per implant) and implanted subcutaneously into the flanks of NOD/SCID mice. 7 days post implantation, the cellularized implants were excised, paraffin embedded and stained with hematoxylin and eosin (H&E) and anti-human CD31 (brown). Black arrows indicate red blood cell (RBC)-perfused, anti-human CD31+ vessels within the gel implant. The black arrow heads indicate human-CD31+ vessels forming a probable anastomotic network in within the gel implant.

Figure 6-4 Representative photomicrographs of contaminating cells in ECFC culture.



(A) Contaminating cells with mesenchymal stromal cell (MSC)-like or fibroblast-like phenotype (white arrow heads) are seen around a cobblestone-like rat lung ECFC colony (white arrow) using phase contrast microscopy at 10x magnification. **(B)** Contaminating cell cluster with fibroblast-like phenotype observed in passage 2 rat PV-ECFC culture. **(C)** Contaminating cell colony observed in passage 2 human PV-ECFC culture.

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Chapter Seven: **General Summary**

Contributions:

This chapter was written by ARSA and edited by BT.

7.1 Overview

Developmental arrest of the distal lung is the primary pathology characterizing the two major neonatal pulmonary ailments, bronchopulmonary dysplasia (BPD) and congenital diaphragmatic hernia (CDH). The former is a developmental disruption of the distal air-spaces secondary to ventilator and O_2-therapy for acute respiratory failure in preterm neonates (Kinsella et al., 2006). The latter, on the other hand, is a complex congenital malformation characterized by a developmental defect in the diaphragm, followed by herniation of abdominal organs into the thorax. Infants with CDH invariably suffer pulmonary dysgenesis, which is marked by clinically manifest pulmonary hypoplasia with varying degrees of pulmonary hypertension (PHT) (Abman, 2007). A deeper understanding of the mechanisms regulating alveolar development is critical to formulate effective management strategies for these debilitating disorders of lung development. In fact the essential objective of the research presented in this thesis is to broaden the limits of our current understanding of lung development, injury-repair and regeneration. In order to address this objective, we used two established models of arrested lung growth, simulating the major pathophysiological elements of BPD and CDH. The first one is the oxygen-induced neonatal rodent model of lung injury. Housing newborn mice or rats in hyperoxia (85% to 95% O_2) during their early postnatal life results in alveolar simplification and vascular rarefaction, strikingly reminiscent of human BPD. The other one is the monocrotaline (MCT)-induced newborn rat model of lung

hypoplasia and PHT. This model dependably mimics the hallmarks of CDH-associated pulmonary dysgenesis such as alveolar hypoplasia, lung vascular paucity and PHT. The outcomes and interpretation of our experiments employing the two animal models have been elaborately presented in the preceding chapters. In brief, firstly, we show that activation of the prosurvival factor Akt is important for normal lung development. We also demonstrate the putative therapeutic application of this prosurvival pathway in preventing alveolar epithelial damage and preserving lung development in newborn rats subject to hyperoxic challenge. Secondly, our studies are one of the first ones to establish the presence of endothelial colony forming cells (ECFCs) in the developing lung. ECFCs are currently the accepted 'true' progenitor cells of the vascular endothelium (Kirton and Xu, 2010; Yoder et al., 2007). Thirdly, we show that the functional capacity of these pulmonary vascular (PV)-ECFCs is impaired in lungs growth-arrested by hyperoxia or MCT. Finally, this gave us the rationale to explore and demonstrate the therapeutic potential of human umbilical cord blood (hUCB)-derived ECFCs in experimental BPD and CDH-associated lung hypoplasia/PHT. The key findings described in this thesis are as follows:

I. Control of excessive apoptosis in alveolar epithelial cells, via stimulating prosurvival Akt pathway, prevents arrested alveolar development.

a. Inhibition of Akt phosphorylation using wortmannin (pharmacological upstream inhibitor of Akt) arrests normal alveolarization.

b. Oxygen-induced irreversible arrest in alveolarization is associated with decreased Akt activation in the lungs.

c. Akt activation decreases apoptosis and improves cell survival in alveolar type 2 (AT2) pneumocytes exposed to hyperoxia (95% O_2) *in vitro*.

d. Akt overexpression via intratracheal Akt gene therapy preserves alveolar architecture and prevents pulmonary vascular remodeling in hyeroxia-exposed newborn rats

II. ECFCs exist in the distal vasculature of the developing mammalian (rat and human) lung

III. Rat PV-ECFCs are functionally disrupted in experimental BPD and CDH-associated lung hypoplasia

a. ECFC functionality, defined by their- (i) survival and viability in culture, (ii) capacity to generate colonies from plated single cells, and (iii) ability to form tube-like networks in Matrigel, is impaired in lungs growth-arrested by hyperoxia or monocrotaline (MCT) exposure

b. *In vitro* exposure to CDH-inducing teratogen, nitrofen, disrupts growth and colony forming capacity of rat PV-ECFCs

IV. Therapy with hUCB-ECFCs preserves normal alveolar development in experimental BPD and CDH-associated lung hypoplasia

a. Therapeutic administration of hUCB-ECFCs preserves alveolar growth and prevents PHT via maintaining normal lung vascularity in lungs exposed to hyperoxia or MCT-mediated injury

b. ECFCs secrete paracrine factors that prevent impaired alveolar growth and sustain lung vascularity in experimental models of hyperoxia or MCT-induced lung injury

c. Therapeutic benefit of ECFC treatment persists for up to 10 months in rats exposed to postnatal hyperoxia, without evidence of serious adverse effects.

The insights derived from the data presented in the previous chapters are integrated and briefly discussed here, under the following sub-sections: (i) Apoptosis regulation and normal alveolar development, (ii) ECFC-driven Lung vascular development and alveolar homeostasis and, (iii) Newer therapeutic possibilities with ECFCs for lung repair. Under each sub-section, suggestions are made for pertinent future studies.

7.2 Apoptosis regulation and normal alveolar development

The presence of architecturally and functionally intact alveoli is critical for maintaining physiological gas exchange activity in the lungs. Conditions marked by defective alveolar development (e.g. BPD and CDH) or pathological destruction of existing alveoli (e.g. pulmonary emphysema) progressively result in

respiratory insufficiency. A closer understanding of the alveolar developmental and maintenance programs is crucial to effectively tackle these ailments. Apoptotic cell death is one such fundamental cellular program contributing to organogenesis, remodeling and homeostasis of the lung (Del Riccio et al., 2004). Evidence indicates that apoptosis of AT2 cells constitutes an integral mechanism in the transformation of air-filled distal lung saccules into functional alveoli (Scavo et al., 1998; Schittny et al., 1998). However, when apoptosis surpasses physiologic levels as seen in hyperoxic injury, for instance (Pagano and Barazzone-Argiroffo, 2003), it may become a potential culprit precipitating alveolar destruction. Excessive apoptosis induced in the developing alveoli by means other than hyperoxia has also been shown to produce BPD like changes in the lung (De Paepe et al., 2008). Therefore, by analogy, we hypothesized that controlling excessive apoptosis in the alveolar epithelium via stimulating intracellular prosurvival mediators would prevent arrested alveolar development.

As a proof of concept we examined the effect of wortmannin, a pharmacological inhibitor of the prosurvival phosphatidyl inositol-3-kinase (PI3K)/Akt pathway, on newborn rats. Postnatal Akt inhibition during the critical "alveolar stage" of lung development resulted in disrupted alveolarization, with a pattern of alveolar simplification that was reminiscent of BPD. Conversely, in oxygen-induced experimental BPD in newborn rats, alveolar simplification was associated with a decreased activation of lung Akt. Corresponding with the studies by De Paepe and collegues (De Paepe et al., 2008), our data imply a

potential role of the prosurvival PI3K/Akt pathway in determining the ability of alveolar pneumocytes to resist hyperoxic challenge. Directed by these observations as well as implied by earlier reports (Bao et al., 2005; Lu et al., 2001), we proceeded to determine the possible therapeutic benefits of activating PI3K/Akt pathway in the lung. Rat lung epithelial cells cultured in hyperoxia (95% O_2) showed decreased apoptosis and improved cell survival following forced overexpression of active Akt by adenovirus-mediated gene transfer. In vivo, adenovirus-mediated Akt gene transfer preserved alveolar architecture in newborn rats housed in hyperoxia. Our results, thereby, establish a framework to further clarify the role of PI3K/Akt-mediated inhibition of apoptosis in the human lung epithelium. We speculate that the manipulation of apoptotic pathways may have therapeutic potential to prevent lung injury and preserve normal lung growth.

Future directions:

I. Apoptosis inhibition using growth factors such as VEGF (Gerber, 1998), IGF-1 (Kulik, 1997), KGF (Ray, 2005) and hepatocyte growth factor (Xiao, 2001) involves activation of PI3K/Akt signaling. Changes in Akt signaling in the presence of these growth factors might confer clinically significant therapeutic benefit in experimental models of BPD. A systematic understanding of these mechanisms will allow development of clinically viable approaches for therapeutic Akt activation.

II. It sounds logical to think that a treatment strategy based on Akt activation involves the potential harm of inhibiting physiological apoptosis and inducing proliferation in cells other than alveolar pneumocytes, with possible counterproductive outcomes (Pierro and Thebaud, 2010). A possible way to circumvent these adverse effects would involve exploring targeted gene-delivery approaches to protect AT2 cells.

III. Activation of the Akt kinase orchestrates a number of signaling pathways that apparently promote the vasculogenic potential of endothelial progenitor cells (EPCs) (Ma and Han, 2005; Shiojima and Walsh, 2002). Bridging this knowledge with the observations of impaired ECFC function in experimental BPD and CDH-associated lung hypoplasia (chapter 4 and 5), shall offer newer therapeutic opportunities. Akt mediated potentiation of functionally impaired PV-ECFCs will be a worthwhile future research direction.

7.3 Lung vascular development and alveolar homeostasis

Originally, it was believed that during lung organogenesis, neovasculature passively follows the developing airways (Hall et al., 2000). However, this notion became challenged by reports demonstrating the interdependence between alveolar growth and new vessel formation in the distal lung (Abman, 2001). Secondary abnormalities occur in one of the processes when the other is primarily affected. Indeed, it is increasingly realized that the vascular rarefaction associated

with impaired lung development in BPD and CDH goes well beyond being a consistent structural attribute into representing a significant underlying contributor to their pathogenesis (Bourbon et al., 2005; Hislop, 2005). On the other hand, strategies promoting lung vascularization have preserved alveolar growth in various models of impaired lung development (Thebaud and Abman, 2007). For instance, we have shown that the proangiogenic factor VEGF in combination with angiopoietin-1 preserves lung alveolarization via promoting angiogenesis, in the rat model of oxygen-induced BPD (Thebaud et al., 2005). If angiogenic growth factors and adequate lung vascularization contribute to lung integrity, then vascular progenitor cells (termed "endothelial progenitor cells" or EPCs) are appealing candidate cells likely to be involved in the same mechanisms. Quite recently, strategies involving mobilization of endogenous EPCs from the bone marrow (Ishizawa et al., 2004) or supplementation with exogenous EPCs (Balasubramaniam et al., 2010; Takahashi et al., 2004; Zhao et al., 2005) have proved successful in treating experimentally induced lung injury. However, one major limitation that has not been completely addressed in these studies is the lack adequate techniques to unambiguously identify and define cell types grouped as EPCs (Timmermans et al., 2009). Nevertheless, among the various populations of EPCs described so far (Kirton and Xu, 2010), ECFCs demonstrate a phenotype indistinguishable from cultured endothelial cells. In addition, by virtue of their high potential for proliferation, capacity for self-renewal, and ability to form blood vessels *de novo*, ECFCs qualify as 'true progenitors' as opposed to differentiated endothelial cells (Ingram et al., 2004;

Yoder et al., 2007). More importantly, these 'late outgrowth' cells do not express hematopietic/myeloid markers such as CD14, CD45 and CD115 nor do they display phagocytic activity (Yoder et al., 2007). These evidences suggest that ECFCs could be the primary progenitor population participating in neovasculogenesis and in turn makes them putative candidates contributing to the pathogenesis of diseases involving impaired vascular growth, such as BPD and CDH. Hence, examining the role of ECFCs in these diseases is an important research direction in the quest for evolving newer lung management strategies.

For addressing this, we framed a two-step hypothesis. First, we suggested that ECFCs exist in the vasculature of the developing lung. Second, we supposed that the functional impairment of ECFCs, at least in part, underlies the pathogenesis of arrested alveolar development. Working on the first part of the hypothesis, we developed a procedure to isolate PV-ECFCs from enzymatically disintegrated rat and human peripheral lung tissue. The fundamental principle of our isolation procedure is derived from the published protocol for recovering circulating ECFCs from peripheral blood (Mead et al., 2008). The PV-ECFCs isolated by this novel procedure displayed all properties that have been described earlier (Ingram et al., 2005; Mead et al., 2008) to characterize circulating ECFCs (Chapters 4 and 6). Their essential endothelial phenotype was demonstrated by acetylated-LDL uptake, Ulex-lectin binding and formation of tube-like networks in Matrigel. They expressed endothelial-specific surface markers (CD31, VEGFR2 and vWF) and were negative for CD14 and CD45 expression, indicating

their non-myeloid/non-hematopoietic origin. In addition, ~25% or more of the isolated PV-ECFCs were able to generate colonies with 500 or more daughter cells, when plated as single cells. It was also possible to serially replate cells from those colonies at a single-cell seeding density to obtain secondary and tertiary generations of ECFC colonies with comparable cell numbers. These data validate the ECFC-phenotype of the isolated cells and thereby demonstrate that ECFCs exist in the distal vasculature of developing human and rat lungs. Advancing into the next part of the hypothesis, we examined the functional capacity of these vascular ECFCs derived from developmentally arrested lungs of rats with experimental BPD or lung hypoplasia (modeling CDH-associated pulmonary dysgenesis). The results of these experiments presented in chapters 4 and 5 indicate that the *in vitro* survival, colony forming and vessel forming potentials of PV-ECFCs were impaired in both disease models studied. The findings clearly suggest that impaired ECFC functionality could, at least in part, underlie arrested alveolar growth in BPD and CDH-associated lung hypoplasia. They also provide the rationale to explore therapeutic ECFC supplementation as a potentially effective treatment in these disease models.

Future directions:

As a natural consequence of these crucial findings on the existence and functional status of ECFCs in the developing lung, several important questions arise. These questions invite several new study directions. Some of the prominent ones are as follows:

I. The findings presented in chapters 4 and 5 imply the possible involvement of disrupted signalling between the ECFCs and alveolar epithelial cells in the pathogenesis of arrested alveolar growth. Exploring these interactions may usher in newer remedies for altered alveolar development. One way of studying this is to perform crossover experiments, co-culturing AT2 cells and PV-ECFCs from control vs. growth-arrested lungs and vice versa. Similar crossover studies can also be performed by exposing AT2 cells to conditioned media (CdM) of ECFCs derived each group and vice versa.

II. One prominent limitation observed while studying ECFCs in the CDH-associated lung dysgenesis model was the inability to successfully isolate PV-ECFCs from the lungs of term rat foetuses. In addition, even the ECFCs isolated from PD-14 lungs (chapters 4 and 5) were already in culture for over 6-8 weeks (passage 4 or more) when used for functional assays. This is basically due to the multiple steps involved in the current protocol employed to isolate lung ECFCs (chapter 6). Developing more effective ECFC identification and isolation strategies will aid studying them during earlier stages of lung development and in conditions closer to their natural milieu. In addition, this will offer the framework to test the efficacy of promising antenatal treatment strategies (Luong et al., 2011).

III. Pulmonary vascular remodeling is an important pathological feature of PHT. Studies indicate that pulmonary arterial smooth muscle cells undergo uncontrolled proliferation and show drastic phenotypic alterations (Sakao et al., 2010). Altered interactions between the proximately positioned PV-

ECFCs and pulmonary arterial smooth muscle cells could be a putative mechanism determining these changes. Examining this hypothesis may offer newer insights into the disease process of PHT.

7.4 Therapeutic possibilities with ECFCs for lung repair

The outcomes elaborated in chapters 4 and 5 and recapitulated in the previous section lend sound credence to the hypothesis that functionally deprived vascular ECFCs contribute to arrested lung growth. One logical implication of these data is the possibility of restoring normal development in growth-arrested lungs via therapeutic supplementation with ex vivo expanded healthy ECFCs. In fact, the stupendous growth in stem cell research has opened up the possibility of therapeutic replenishment of endogenous stem/progenitor cells with *in vitro* propagated cells derived from exogenous sources (Alphonse and Thebaud, 2011; Weiss et al., 2008). More importantly, promising therapeutic outcomes using 'early outgrowth' EPCs have already been demonstrated in models of lung injury (Fadini et al., 2007; Huertas and Palange, 2011). We, therefore, speculated that therapeutic administration of hUCB-ECFCs shall rescue the developing lung from arrested alveolar growth. In parallel, since a growing body of research suggests that the beneficial effects of therapy with stem cells, including EPCs, are primarily accounted for cell-derived paracrine mediators (Lee et al., 2011; Prockop, 2007; Yang et al., 2010), we tested the lung regenerative potential of CdM obtained from hUCB-ECFC. ECFC therapy, with either whole cells or their

CdM, restored alveolar development and attenuated PHT in newborn mice exposed to hyperoxic damage. Similar therapeutic improvement was also observed in newborn rats subject to MCT mediated lung hypoplasia/PHT. When ECFC-treated BPD mice were followed up for 10 months (long-term studies), we observed persistent therapeutic benefit with no detectable adverse reactions. Together, these data suggest that therapeutic supplementation with ECFCs represents a new and safe cell based therapy for lung diseases characterized by or associated with disrupted alveolar development.

Future directions:

I. The current notion on postnatal vasculogenesis is: (a) 'late outgrowth' ECFCs generate the endothelial lining and get incorporated into the intima of new blood vessels; (b) 'early outgrowth' EPCs, on the other hand, putatively play an important role in vascular repair/neovasculogenesis of ischemic tissues via paracrine secretion of angiogenic cytokines (Hur et al., 2004). Consistent with this supposition, results very similar to our studies have been reported by other groups treating experimental BPD and PHT with bone marrow or peripheral blood derived 'early outgrowth' EPCs (Borghesi et al., 2010; Toshner and Morrell, 2010). In this backdrop, it would be appropriate to investigate the degree of engraftment and actual incorporation of the administered ECFCs into the newly derived blood vessels in the lungs of treated animals.

II. In addition, observations on the capacity of ECFCs to exhibit a paracrine therapeutic effect (chapters 4 and 5) invites an interesting new direction in ECFC research. It might be rewarding to characterize the CdM of ECFCs with an outlook of identifying potential growth factors or small molecules that faithfully reproduce the therapeutic benefits.

III. More importantly, assessing the contents and functional effects of ECFC CdM will throw more light into the speculated paracrine interactions between ECFCs and alveolar epithelial cells.

7.5 Concluding Comment

The central pathophysiology of the two major neonatal health problems, BPD and CDH, derives from arrested alveolar development. An understanding of the mechanisms that regulate alveolar development is critical in developing efficient treatment strategies. The research described in this thesis adds newer insights into two important mechanisms contributing to alveolar growth impairment: (a) Uncontrolled apoptosis of alveolar epithelial cells and (b) Disrupted ECFC-driven neovasculogenesis in the developing lung. It also suggests opportunities for better therapies via activation of intracellular prosurvival factors in the lung epithelium or therapeutic supplementation with ECFCs from a clinically relevant exogenous source. In this attempt, our studies have inevitably generated newer questions and hypotheses to seek answers for. A good part of those ideas are currently being studied in Dr. Thebaud's laboratory.

Finding answers to those questions will eventually culminate in effective treatment strategies, improved survival and better quality of life in the unfortunate newborn victims of BPD and CDH.

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