University of Alberta

Endothelial Colony Forming Cells: Role in Bronchopulmonary Dysplasia and evaluation of new therapeutic strategies

by

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Dedication

I dedicate this thesis to my parents Nasiruddin and Khadija Bana and to my sister Salima and brother Farhad for always being by my side

ABSTRACT

Bronchopulmonary dysplasia (BPD), a chronic lung disease of prematurity, results in alveolar simplification and respiratory distress in the newborn. Vascular component is implicated. Lung damage involves a deficiency in the number and function of progenitor cells.

We hypothesize that endothelial colony forming cells (ECFCs) exist in the developing human lung, are impaired in hyperoxia and secrete exosomes.

ECFCs from human fetal lung expressed CD31, CD105, CD144, CD146 and were negative for CD14 and CD45. In hyperoxic conditions, cord formation and clonogenic potential was impaired. Mesenchymal stem cell conditioned media (MSC CdM) improved clonogenic potential. Exosomes were isolated from human cord blood derived ECFC CdM and characterized using electron microscopy and protein expression.

This study provides novel finding that ECFCs exist in human fetal lung and their function is impaired in hyperoxia. They may exert their effect by exosomes. This provides a rationale for use of exogenous stem cells in BPD.

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List of Abbreviations

Abbreviation	Definition
ANOVA	Analysis of variance
BPD	Bronchopulmonary dysplasia
BSA	Bovine serum albumin
CAC	Circulating angiogenic cells
CdM	Conditioned medium
CFU	Colony forming unit
DMEM	Dulbecco's Modified Eagle Medium
Dil-Ac-LDL	Acetylated low density lipoprotein labeled with Dil
EBM	Endothelial basal medium
ECFC	Endothelial colony forming cells
FBS	Fetal bovine serum
cEGM2	Complete endothelial growth medium 2
EPC	Endothelial progenitor cell
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
iPS cells	Induced pluripotent stem cells
MNC	Mononuclear cell
MSC	Mesenchymal stem cell
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffer solution
PEEP	Positive end-expiratory pressure
PSF	Penicillin/streptomycin and amphotericin B
RNA	Ribonucleic acid
UEA-1	Ulex Europaeus Agglutinin-1

VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Chapter 1

Introduction

Note:

Portion of this chapter has been published as a review article in *Journal of Clinical and Experimental Cardiology*. **The coauthors of this paper are Dr. Bernard Thebaud, Dr. Duncan Stewart and Shirley Mei.**

1.1. Bronchopulmonary Dysplasia (BPD) – a chronic lung disease of prematurity

BPD was first described in 1967 as a chronic lung disease that occurred in preterm infants who had been mechanically ventilated for respiratory distress syndrome [1]. The features of this "old BPD" included mucosal metaplasia of the airways, emphysema and interstitial fibrosis. Clinically, BPD was defined as a disorder occurring in infants who received mechanical ventilation in their first postnatal week of life for at least three days, including the need for supplemental oxygen at 28 postnatal day [2]. Over the years, as a result of advancement in therapeutic strategies and neonatal intensive care, survival of extremely premature infants has become possible. Therefore, BPD was characterized as an increase in alveolar disorders rather than airway injury [3,4]. This led to the definition of "new BPD" in which the prominent feature is the impairment of alveolar formation with fewer and poorly vascularized alveoli and a reduction in gas exchange surface area [5,6].

1.2. Etiology and pathogenesis

BPD is a multifactorial disease. Pathologically, it is marked by simplified alveoli with both alveolar hypoplasia and decreased acinar complexity. The capillaries are decreased in number and are dysmorphic. There is minimal fibrosis with variable interstitial fibroproliferation. There are less severe arterial and arteriolar vascular lesions with variable airway smooth muscle hyperplasia. Also, as a

consequence of oxidant or other injury to type 2 cells in the lungs, there may be abnormal surfactant metabolism [6,7].

1.2.1. *Prematurity*

One of the primary determinants of the risk of developing BPD is gestational age of less than 37 weeks [8]. The lung is most susceptible to damage if birth occurs during the saccular stage of the development, between 23 to 32 weeks of gestation. The airway supporting structure is poorly developed in addition to deficiency of surfactant, decreased compliance and underdeveloped antioxidant mechanisms [9]. Also, in some premature infants, there is fetal growth restriction which is also an independent risk factor for BPD [10].

1.2.2. *Hyperoxia*

The effect of hyperoxia on the lungs has been studied in neonatal rodents and found to be similar to immature human lungs. Hyperoxia exposure induces inflammatory responses in the lungs that disrupt alveolarization leading to inflammation, alveolar simplification and vascular remodeling [11,12]. Since it is known that reactive oxygen species mediate oxygen toxicity, antioxidant therapy is thought to be a beneficial preventive or treatment option in BPD [13,14].

1.2.3. *Mechanical ventilation*

Prolonged mechanical ventilation in preterm lambs has been shown to disrupt lung development and produce histological changes similar to that of preterm

infants with BPD [15]. An angiogenic shift takes place in ventilated preterm lungs with a down-regulation of proangiogenic genes. This may lead to deficient alveolarization that marks BPD [16]. High tidal volume[17], insufficient positive end-expiratory pressures (PEEP)[18] and high fractions of inspired oxygen (FiO_2)[19] are all implicated in oxidative stress and injury caused by mechanical ventilation.

1.2.4. *Infection*

Antenatal infection such as chorioamnionitis is thought to cause BPD. Amniotic fluid of infants who subsequently develop BPD have increased levels of proinflammatory cytokines [20]. In addition, infection with Ureaplasma urealyticum causes impaired lung development due to dysregulated inflammatory responses which results in BPD [21]. However, a meta-analysis showed that statistically chorioamnionitis cannot be considered an independent risk factor for BPD [22].

1.3. Current treatment

Since BPD has a multifactorial etiology, there is no single treatment that has been found to have a beneficial effect. Multiple therapies in combination are a mainstay for the treatment of BPD. Even then, the rate of BPD has failed to decrease over the years. The current treatment for BPD includes mechanical ventilation, oxygen therapy, antenatal and postnatal corticosteroids, surfactant, caffeine and vitamin A [7,23]. However, other areas, particularly cell based

therapies, including stem cell therapy, needs to be explored as a therapeutic strategy for prevention and treatment of BPD. Our research focuses on studying the role of stem cells in lung injury and repair with a focus on BPD and evaluating the use of stem/progenitor cells as therapy. The next section highlights stem cells in general.

1.4. Definition and Types of Stem Cells

Stem cells are cells that have the capacity of self renewal and the ability to undergo differentiation into multiple phenotypes [24,25]. Therefore, they are known to play an important role in organogenesis, regeneration and tissue repair and maintenance. Stem cells have the capacity to produce one or more lineages. Depending on this differentiation potency of stem cells, they can be classified as totipotent (differentiate into all cell types e.g. zygote), pluripotent (differentiate into cells from all three germ layers), multipotent (capable of producing more than one cell lineage) or unipotent (differentiate into one cell type).

1.4.1. *Stem cells can also be classified according to their tissue of origin* [26]

Embryonic Stem Cell [27]**:** They are derived from the blastocyst of an embryo from *in vitro* fertilization. Under appropriate conditions, ESCs are pluripotent and can differentiate into specialized somatic cells. ESCs have garnered a lot of controversy because of the ethical issues regarding the destruction of a human embryo. Some of the ethical and technical limitations of ESCs may be overcome by the recent advent of induced pluripotent stem cells (see below).

Somatic Stem and Progenitor Cells: These cells can be isolated from adult human tissues as well as from cord blood. They are not as potent as ESCs and they have increasing degrees of fate restriction. These cells include amongst others, mesenchymal stem/stromal cells (MSCs) and endothelial progenitor cells (EPCs).

Induced Pluripotent Stem Cell (iPS): One of the most transformative contributions to the field of stem cell biology in the last decade is the engineering of pluripotency into somatic cells by the ectopic expression of transcription factors linked to pluripotency. Dr. Yamanaka's group was the first to reprogram mouse [28] and then human [29] fibroblasts through retroviral transduction by screening a panel of 24 transcription factors that are highly expressed in ES cells. This cadre of genes was progressively reduced to four that encode the transcription factors octamer 3/4 (Oct4), SRY box–containing gene 2 (Sox2), Kruppel-like factor 4 (Klf4), and c-Myc. iPS cell pluripotency is highly similar to ESCs [30,31]. The ability of mouse iPS cells to generate an entire mouse, and of human iPS cells to form teratomas, which is the ultimate standard for pluripotency for human cells, *in vivo* [32] indicates that they are pluripotent cells and suggests that the defined factor reprogramming approach produces cells with a developmental potential similar to that of ESCs. Teratoma formation while indicating pluripotency, is a major limitation of iPS technology. In addition, the complexity of the technique, cost-effectiveness and the length of time required may pose as limitations [28, 29].

One of the numerous advantages of iPS cells over ESCs is the ability to engineer patient-specific iPS cells which could be useful to provide (1) unprecedented insights into disease mechanisms and (2) a useful platform for drug discovery [32].

1.4.2. Endothelial Progenitor Cells (EPCs). Circulating EPCs in adult human peripheral blood were originally identified in 1997 by Asahara et al [33], which challenged the paradigm that vasculogenesis is a process restricted to embryonic development. The ability to isolate a circulating cell that displays potential to give rise to cells appearing endothelial-like *in vitro* and with the potential to incorporate at sites of neoangiogenesis *in vivo*, spawned a new field of investigation. The isolation and further characterization defined the basic and translational properties of these presumed bone marrow-derived circulating EPCs. Numerous preclinical studies in animal models suggest a high probability for successful clinical translation of EPCs as biomarkers, or cell therapies to treat ischemic disorders via new vessel formation. Hence, there is considerable interest in the potential of these cells to promote vasculogenesis and overcome endothelial dysfunction in PH.

Role of EPCs in respiratory health and disease. In lipopolysaccharide-induced murine lung injury, EPCs (Sca-1+, Flk-1+) are rapidly released into the circulation and contribute together with other bone marrow-derived progenitor cells to lung repair [34]. In elastase-induced emphysema, bone marrow-derived cells develop characteristics of endothelial cells and contribute to the repair of alveolar capillary wall [35,36]. Patients with acute lung injury have 2-fold higher

number of circulating EPCs than healthy control subjects [37], suggesting the mobilization of EPCs may play some biological role in lung disease. Similar to the prognostic role of EPCs described in ischemic diseases, illness severity and improved survival [38] appear to correlate with increased circulating EPCs in acute lung injury as well. Circulating EPCs are significantly increased in patients with pneumonia while patients with low EPC counts have persistent fibrotic changes even after recovery from pneumonia. In patients with chronic lung disease, the EPC (CD34+, CD133+, KDR+, kinase domain region, also known as VEGF receptor 2 or fetal liver kinase-1/Flk-1) count is decreased and correlates with disease-severity [39]. In the developing lung, arrested alveolar growth in hyperoxia-induced BPD in neonatal mice is associated with decreased circulating, lung and bone marrow EPC (CD45-, Sca-1+, CD133+, VEGFR-2+) [40].

Controversy around the definition of EPCs. A major limitation has been the lack of a clear definition of how to unambiguously identify circulating EPCs, and thus, deciding which cell population to infuse into patients. Various methods exist for the isolation and identification of EPCs [41,42]. Most of the above mentioned studies relied on the expression of cell surface markers such as CD34, CD133, and VEGFR2; however, there is yet no clear agreement on what markers define a "true" EPC. Alternatively, EPCs can be derived by culturing mononuclear cells (MNCs) in conditions that promote EC specification. Schematically, there are three major methods to culture of EPCs from circulating MNCs. In one assay, nonadherent MNCs cultured on fibronectin form so-called colony forming unit-Hill (CFU-Hill) within 5-9 days [43]. CFU-Hills display some phenotypic and

functional characteristics of endothelial cells, including expression of cell surface antigens (CD31, CD105, CD144, CD146, vWF, and VEGFR2) and uptake of AcLDL. However, they also express hematopoietic-specific antigens CD45 and CD14, display nonspecific esterase and phagocytic capabilities consistent with monocytes/macrophages, and cannot be propagated long term in culture [42]. A second assay identifies adherent, so called "circulating angiogenic cells" (CACs) following 4 days of culture in endothelial specific conditions [44,45]. Likewise, CACs resemble endothelial cells phenotypically but are also enriched for hematopoietic-derived monocytes/macrophages. Less studied cells are the so-called endothelial colony forming cells (ECFCs) [46,47,48,49,50]. Cells plated on collagen I in endothelial growth media form cobblestone-like adherent colonies within 6 days from umbilical cord blood MNCs, or 14-21 days from adult peripheral MNCs [49]. A very similar population can also arise from the prolonged (~2 week) culture of MNCs on fibronectin and have been termed late outgrowth EPCs. By definition, a true EPC is a cell that can be clonally and serially re-plated in culture and will give rise to endothelium by differentiation *in vitro* or *in vivo*. In contrast to CFU-Hills and CACs, which display various monocyte/macrophage phenotypes and function [51], ECFCs are characterized by (i) robust proliferative potential [52], (ii) secondary and tertiary colony formation upon re-plating, and (iii) de novo blood vessel formation *in vivo* when transplanted into immunodeficient mice [42]. Current efforts focus on improved characterization of circulating EPCs. Using a combined protocol including polychromatic flow cytometry, colony assays, immunomagnetic selection, and

electron microscopy, Mund et al were able to reliably identify ECFCs and mature circulating endothelial cells (CD34+, CD31+, CD146+, CD105+, CD45-) in circulating peripheral blood and cord blood, with ECFCs being increased in cord blood and extremely rare in the peripheral blood of healthy adults [53]. In summary, evidence suggests that CFU-Hill and CACs are not true EPCs, but modified myeloid lineage cells that participate in neoangiogenesis. Nonetheless, these cells still display therapeutic benefit, but they do not require direct transdifferentiation into the endothelial monolayer of new vessels. In contrast, ECFCs display all the features of a true EPC: clonal proliferative capacity, hierarchy of proliferative potential, and *de novo* vessel forming ability *in vivo*. Thus, while the highly proliferative forms of "true" EPCs may be potentially exciting, both their efficacy and safety need to be defined in rigorous preclinical testing before any consideration to use these cell populations in translational clinical studies.

All together, these findings suggest that EPCs leave the bone marrow, enter the circulation and then migrate to the pulmonary vasculature and perivascular tissue where they contribute to repair the injured endothelium and help by restoring lung integrity.

Chapter 2 of this thesis outlines stem cells and their role in BPD.

1.5. Long-term complications of BPD

BPD is the most common and major long-term pulmonary complication of prematurity [54]. Due to advances in neonatal care, post-partum survival of preterm babies and infants that develop BPD has increased. This presents with the long-term consequences that come with this increased survival. It has been shown the ex-preterm babies with BPD have an increased rate of re-hospitalization and increased respiratory symptoms later in life [55]. In addition, adult survivors of BPD have early onset emphysema and may have residual functional and structural abnormalities [56]. Chapter 3 of this thesis discusses the long-term implications of BPD and the role of stem cells in it.

1.6. Paracrine mediators and exosomes – an alternative to cell based therapy

Recent advances in stem cell research suggest that stem cells may act through paracrine mediators and most of their beneficial effect may be because of a paracrine mechanism [57,58]. Therefore, a lot of research has been focused around finding those paracrine mediators and exploring their therapeutic potential. In the quest for such mediators of stem cell action, exosomes have gained a lot of attention recently. They are small vesicles that contain both proteins and RNAs as their cargo and have shown to be secreted by most cells of the body. They have been studied as a diagnostic and therapeutic modality [59]. Chapter 4 of this thesis discusses exosomes and their isolation from stem cells.

1.7. Overall hypothesis

The main objective of our study is to investigate the presence of Endothelial Colony Forming Cells (ECFCs), which are late outgrowth Endothelial Progenitor Cells (EPCs), in the developing human lung and explore the effect of hyperoxia on these cells. We also aim to look into emphysema as a long-term complication of BPD and to study the presence of exosomes in ECFCs which will provide a platform for future investigations into the mechanisms through which stem cells act.

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Chapter 2

Existence of Endothelial Progenitor Cells in Human Fetal Lungs With Clonogenic Potential and the Effect of Hyperoxia On These Cells

2.1. Introduction

BPD is a chronic lung disease of prematurity that results in alveolar simplification and respiratory distress in the newborn. Despite of being identified more than 45 years ago as severe complication of prematurity, BPD still does not have an effective therapy[1][2]. Extreme prematurity and perinatal injury in BPD results in decreased alveolar formation and decreased vascularity with formation of abnormal structure[3]. Vascular component has been increasingly implicated in BPD recently [4,5] and since it is the main focus of our research, it is discussed in greater detail in the following section.

2.1.1. Pulmonary vascular development

Pulmonary vascularization is dependent on vasculogenesis, the de novo formation of blood vessels and angiogenesis, the formation of new blood vessels from pre-existing vessels[6]. The mesenchyme that surrounds the embryonic lung has blood lakes present from which vasculogenesis occurs initially. Endothelial precursors have been identified in the splanchnic mesenchyme as early as 4 weeks gestation[7]. Capillary network is observed at 7 weeks around each potential main bronchus. The capillaries join together to form small blood vessels along the developing airways. This addition of new tubules to existing vessels occurs until 17^{th} week of gestation that marks the end of pseudoglandular stage of lung development. The next stage of lung development, the canalicular stage, is accompanied by angiogenesis which results in the widespread growth of the capillaries[8]. In addition, the capillaries at this stage are lined by alveolar type 1

and type 2 pneumocytes and form alveolar-capillary gas exchange surface at about 24 weeks gestation. In the final stage of lung development, the alveolar stage, maturation of distal lung microvasculature takes place and a single capillary network is formed[9].

Initially the pulmonary artery acquires smooth muscle from adjacent bronchi for their vessel wall formation. With the increasing size of the arteries, the muscles are derived from the surrounding mesenchyme. Endothelial cells have been implicated as another source of smooth muscle cells. Veins derive their muscle lining from mesenchymal fibroblasts and endothelial cells. At birth, postnatal modification of blood vessels occur due to the decrease in pulmonary vascular resistance and pulmonary arterial pressure and an increase in pulmonary blood flow[7,8].

The exact mechanism by which the regularization of the lung vasculature takes place is not known. There are several factors implicated in this process. Vascular endothelial growth factor (VEGF) is one of the main regulators of blood vessel growth[10]. The assembly and stabilization of the formed vessels is brought on by Tie2 which is a tyrosine kinase receptor, and by angiopoietin which is its ligand[11]. Insulin like growth factor also plays a role in airway and blood vessel development[12]. Nitric oxide (NO) which is a gaseous transmitter has been shown to play an important role in fetal lung development[13]. Recently, EPCs have been shown to play a role in lung development[14]. A lot more needs to be known about the molecular events and the factors that control and regulate normal lung vascular development.

2.1.2. Vascular abnormalities in BPD

In BPD, the interaction between pro and antiangiogenic factors is thought to be disrupted which may result in vascular abnormalities[15]. It has been found that alveolar formation and angiogenesis are interdependent[16] and primary abnormality in one process may result in secondary abnormality in the other[17].

Alterations in angiogenic growth factors may be responsible for microvascular disruptions that occur in the developing lung because of hyperoxia and prolonged mechanical ventilation. VEGF has been shown to play an important role in the development of the lung microvasculature and in postnatal development and maintenance of alveolar structure[16]. Administration of VEGF inhibitors during lung development have shown to result in disrupted alveolar and capillary development in distal lung. Interventions that alleviate alterations in VEGF levels brought on by hyperoxia, improve alveolar injury[18]. Angiopoietin promotes vascular maturation and stabilization by enhancing the response of endothelial cells to VEGF. Transcription factors hypoxia inducible factor 1/2 (HIF 1/2) and activation protein 1 regulate angiogenic growth factor expression. Animals deficient in HIF 2α exhibit defective lung morphogenesis and neonatal respiratory distress[19].

Endothelial nitric oxide synthase (eNOS) is important for alveolar regeneration and maintenance. Genetically modified mice deficient in eNOS exhibit severely impaired lung growth[20]. NO mediates several angiogenic growth factors and may also be responsible for the transcriptional control of their expression[21].

Nitric oxide synthase (NOS) is responsible for the release of NO. Preterm baboons with ventilator-induced BPD have markedly decreased levels of pulmonary NOS and therefore decreased NO production. Exogenous NO supplementation preserves alveolar architecture and lung vascular remodeling in hyperoxia and mechanical ventilation-induced BPD[22,23]. NO therapy has shown to reverse pulmonary hypertension and alveolar arrest in neonatal rats that occurs because of VEGF receptor blockage since VEGF-induced angiogenesis is partly mediated by NO[24].

Conversely, anti-angiogenic factors contribute to BPD[25]. Endothelial monocyte activating polypeptide-II has found to be elevated in infants with BPD and its expression is negatively correlated with vascular development[26]. Another anti-angiogenic protein, endostatin, antagonizes VEGF receptor-2 activation and is positively correlated with the development of BPD in preterm neonates[27]. In BPD, disrupted lung vascular growth appears to be a result of impaired angiogenic factors, altered expression of anti-angiogenic factors and NOS. Since EPCs play a role in vessel formation, they are likely to play a role in the disease process.

Recently, the use of stem cells has been implicated in the pathogenesis of BPD and has gained attention as a prevention and therapeutic strategy. The next section discusses the role of stem cells, with particular focus of the role of EPCs in BPD.

2.1.3. Stem cells in BPD

The rationale for use of exogenous stem cells in BPD is that the lung damage may involve a deficiency in number and function of progenitor cells. Since these progenitors contribute to the growth and maintenance of the lung architecture, including the epithelial surfaces and pulmonary microvasculature, replenishing the injured cells with multipotent stem cells may be an important therapeutic strategy. Recently, a number of studies have shown the benefits of different types of stem cells in rodent models of oxygen-induced lung injury.

Mesenchymal stem cells

Studies have shown that intravenous and airway delivery of bone-marrow derived MSC alleviates inflammation and prevents lung vascular and alveolar damage in oxygen-induced BPD in newborn rats [28,29]. In addition, exercise tolerance and survival is also improved. These studies suggest that since the engraftment rate of MSCs was observed to be low and not in proportion to the benefit observed, these cells may act through a paracrine mechanism. Supporting this further is the finding that the conditioned media from MSCs is as protective as the cells. MSCs from umbilical cord blood have also been studied [30]. MSCs from this source is easily accessible and clinically relevant. MSCs from human cord blood mitigate fibrotic changes and prevent alveolar growth arrest in neonatal rats challenged by oxygen.

Human amniotic fluid stem cells (hAFSC)

Human amniotic fluid derived, multipotent stem cells that were selected via c-kit home and integrate into the injured lungs of nude mice with hyperoxic injury. They also show markers of epithelial and alveolar type 2 (AT2) differentiation [31]. It is reported that the damaged AT2 cells create a microenvironment for the chemotactic recruitment of uncommitted hAFSC that then differentiate into AT2 cells [32].

Endothelial Progenitor Cells (EPC)

Recently studies have explored the role of EPCs in BPD. These studies have mostly looked at circulating, cord blood derived ECFCs.

Safranow et al. found higher circulating early and late outgrowth EPC counts in preterm infants as compared to term infants. They also found a positive association between early outgrowth EPC count and BPD and an inverse association between the EPC count and Apgar score in preterm babies [33]. Baker et al. also found increased ECFC colonies in preterm umbilical cord blood derived ECFCs than term. In addition, preterm ECFCs exhibited increased proliferative potential and were more susceptible to hyperoxia as compared with term ECFCs. Antioxidants played a protective role in preterm ECFCs [34]. Another study [35] found that hyperoxia not only decreases ECFC growth but is responsible for disrupting VEGF-NO signaling in human preterm ECFCs and VEGF and NO treatment improves growth in hyperoxia. In contrast, Borghesi et al. found that circulating ECFCs are low at low gestational ages and increase during gestation.

Furthermore, extremely premature infants with lower numbers of ECFCs at birth have increased risk of developing BPD [36]. However, Paviotti et al. did not find any difference that the levels of EPC may contribute to the development of BPD in extremely premature infants [37].

The effect of hyperoxia on EPCs has also been studied in neonatal mice [46]. It was found that moderate hyperoxia reduced EPCs in the blood, bone marrow and lungs of neonatal mice. Whereas, in the adult mice, hyperoxia increased the number of EPCs. Also, lung VEGF, VEGFR-2, endothelial NO synthase and Epo receptor expression was reduced in neonatal mice but not in the adult. This observation points towards a developmental difference that may be responsible for making the developing lung more susceptible to hyperoxia.

None of these studies have explored the presence of ECFCs in the developing lung and their role during BPD. We hypothesized that resident lung ECFCs exist in fetal lungs and their clonogenic potential is impaired in hyperoxia.

2.2. Materials and Methods

All procedures were approved by the Human Research Ethics Board of the University of Alberta.

2.2.1. Collection of lung tissue samples

We acquired human fetal lung tissues from our collaborators at the University of New Mexico (Albuquerque, NM, USA). The fetuses were from elective abortions and had no abnormalities, nor were they were aborted for any medical conditions. The fetal lungs were shipped to us in Dulbecco's Modified Eagle Media (DMEM) that contained 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin and amphotericin B (PSF) on ice and arrived 24-36 hours after abortion. Immediately after receiving the tissue, the isolation process was started. All experiments were done with an *n* of 3 in triplicates unless otherwise stated.

2.2.2. ECFC isolation and culture

Under aseptic conditions, the lung tissue was washed with serum-free DMEM with 1% v/v PSF. Thereafter, it was chopped finely using McIlwain Tissue Chopper. The chopped tissue was then suspended in collagenase/dispase digestive solution (0.1 U collagenase & 0.8 U dispase/mL) (Roche Applied Science, Laval, QC) at 37°C with intermittent shaking for 1 hour. The enzyme-tissue mixture was quenched with an equal volume of DMEM with 10% FBS. The digested sample was strained through 70 μm and 40 μm cell strainers followed by centrifugation for 10 minutes at 300 g and 4°C. The cell pellet obtained upon centrifugation was

then resuspended in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Anti-human CD31 antibody (Abcam, Cambridge, MA) coated streptavidin tagged dynabeads (Dynal, Invitrogen, Burlington, ON) were added and the mixture was incubated for 30 minutes at 4°C. The CD31 positive cells were selected using a magnetic separator and plated in 6-well plates that had been previously coated with rat tail collagen type I. The cells were plated at densities of 50,000 and 100,000 cells/well. The plates were placed in a 37°C, 5% CO_2 humidified incubator. For the first 7 days, the media was changed every day and then every other day thereafter. The media used for the entire culture process was Endothelial Basal Medium-2 (EBM-2) that was supplemented with Endothelial Growth Medium-2 (EGM-2) singlequots (Lonza, Basel, Switzerland), 10% FBS and 1% PSF. ECFC colonies were identified from day 10 onwards as well circumscribed "cobblestone" colonies morphologically using an inverted microscope (Olympus, Lake Success, NY). The colonies were isolated using cloning cylinders (Fisher Scientific, Ottawa, ON) and plated in 25-cm^2 flasks, and upon confluence expanded in 75-cm^2 flasks that had been pretreated with type I collagen. A second bead selection with anti-human CD31 antibodies was performed at this point for further purification of ECFCs.

2.2.3. ECFC Characterization

2.2.3.1. *Immunophenotyping of ECFCs by FACS*

ECFCs were characterized by endothelial specific surface markers that have been described by Mead et al, 2008.

At passage 4-5, 0.15 – 0.20 million cells/sample were washed twice with flow buffer (PBS containing 0.05% sodium azide and 0.1% BSA). After washing, ECFCs were incubated in darkness for 60 minutes at 4°C with primary or isotype control antibody. Cells were then washed twice and resuspended in 300 μl flow buffer and analyzed by Fluorescence-activated cell sorting (FACS) (FACSCanto, BD Biosciences, San Diego, CA).

2.2.3.2. *Dil-acLDL uptake and Ulex europaeus-lectin binding*

At passage 4-5, attached cells were incubated with 20 μg/mL Dil-Ac-LDL (Biomedical technologies, Stoughton, MA) in EGM-2 media at 37°C in 5% CO_2 humidified incubator for 4 hours. The cells were then washed a few times and fixed with 4% paraformaldehyde for 10 min. Cells were washed again with PBS and 10 μg/ml of fluorescein tagged Ulex europaeus-lectin (Vector Laboratories, Burlingame, USA) was added for 1 hour. Nuclear staining with Hoechst 33258 dye was done and then the double-positive stained cells were observed under an inverted fluorescence microscope (leica Microsystems, Richmond Hill, ON, Canada) (Mead et al, 2008).

2.2.3.3. *Single cell clonogenic assay*

This assay was performed to assess the ability of ECFCs to form colonies when plated at single cell density. Pure ECFCs from passages 5-7 were sorted and one ECFC per well was plated using FACS Aria cell sorter (BD Biosciences, Mississauga, ON) into 96-well plates in triplicates. The plates had been pre-

coated with type I collagen and the wells contained 200 μL of media. Cells were incubated at 37°C in 5% CO_2 humidified incubator and media was changed 3 times per week. At day 14, the wells were inspected and classified according to the cells/well that a single cell was able to generate. Colonies with 2-50 and 50-500 cells were classified as low proliferative and colonies with more than 500 cells were classified as high proliferative. A high proliferative colony was then trypsinized and cells were again seeded at single cell density in 96-well plates to assess for second generation colony formation. Media was changed three times per week and wells were analyzed at day 14 as described earlier.

2.2.3.4. *In vitro cord formation on matrigel*

To assess the cord forming ability of ECFCs, 40 μL/well of thawed matrigel was added to three wells in a 96 well plate for each group and the matrigel was allowed to set for 10 minutes at 37°C in the incubator. 10,000 cells per well were seeded into the matrigel coated wells and incubated in 5% CO_2 humidified incubator at 37°C for 6-8 hours. Cells were analyzed every 2 hours for cord formation with an inverted phase contrast microscope. The capillary-like networks usually formed between 4-6 hours in our experiments. Using 10X magnification, three fields were randomly chosen and used for quantification of cord length and number of intersections using OpenLab software (Quorum Technologies Inc, ON, Canada).

2.2.4. *In vitro* cell viability by MTT assay

This assay was used to measure the mitochondrial reduction of the yellow tetrazole 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT; Invitrogen, Eugene, OR) to purple colored formazan. The absorbance of this colored product after dissolution is proportional to the number of metabolically viable cells in the sample.

The cells were plated at equal densities in 96 well plates and maintained in uniform culture conditions while being exposed to experimental conditions. The cells were washed with PBS and topped with serum-free DMEM. MTT was dissolved in PBS to give an effective MTT concentration of 0.5mg/mL in the medium. The cells were incubated with MTT for 2 hours in 5% CO_2 humidified incubator at 37°C. The media was then removed and cells were washed with PBS. Dimethyl sulfoxide was then added to dissolve the formazan crystals and the absorbance was measured spectrophotometrically at 550nm with Spectra Max 190 microplate reader (Molecular Devices, Downington, PA).

2.2.5. *In vitro* exposure of ECFCs to hyperoxia

Human fetal lung ECFCs were plated in 6-well plates previously coated with type 1 collagen. When they achieved 80% confluence, the media was changed and the plates were placed in sterile, humidified *ex vivo* perfusion chamber with oxygen levels set to 60% to mimic hyperoxic conditions *in vivo* with 5% CO_2 at 37°C. The cells were exposed to hyperoxic conditions for 48 hours after which they

were trypsinized and used for various experiments. The control cells were simultaneously incubated in room air at 37°C in 5% CO_2 humidified incubator.

2.2.6. Administration of MSC conditioned media (CdM)

Human fetal lung ECFCs were plated in 24-well plates previously coated with type 1 collagen. The O_2 + MSC CdM group was administered MSC CdM at the start of the 48 hour exposure to hyperoxia (60% O_2). 200μL of concentrated MSC CdM was diluted up to three times with EGM-2. The controls used were ECFCs in room air and ECFCs exposed to hyperoxia without CdM. At the end of 48 hours, the cells were trypsinized and used for single cell clonogenic assay as described above.

Statistical analysis. Statistical comparisons were made with the use of ANOVA. A value of $p<0.05$ was considered statistically significant.

2.3. Results

2.3.1. ECFCs exist in human fetal lungs and exhibit "cobblestone-like" colonies morphologically

ECFCs isolated from human fetal lungs by enzymatic digestion and selection of $CD31^+$ cells, yielded "cobblestone-like" colonies between days 7-10 in culture. They were identified morphologically and separated out using cloning rings and expanded further. ECFCs from human fetal lungs younger than 17 weeks of gestational age required $CD31^+$ and $CD45^-$ FACS selection to obtain pure cultures. (Figure 2-1)

2.3.2. Endothelial surface antigens were positive in human fetal ECFCs

ECFCs were characterized phenotypically and strongly expressed the endothelial specific cell surface markers CD31, CD105, CD144 and CD146. They did not express the haematopoeitic cell surface marker CD45, nor CD14 which is a macrophage/monocyte marker. There were no contaminating cells on FACS analysis. (Figure 2-2)

2.3.3. ECFCs demonstrated uptake of Dil-acLDL and UEA-1

Human fetal ECFCs exhibited basic endothelial cell characteristics such as Dil-acLDL uptake and binding to *Ulex europaeus*-lectin. (Figure 2-3)

2.3.4. Human fetal lung ECFCs demonstrated clonogenic and self renewing properties

When plated at a single cell density, human fetal lung ECFCs demonstrated a clonogenic property, which is one of the most characteristic and unique properties of ECFCs. Of single plated ECFCs, 56.9% formed colonies in 3 independent experiments each time with an *n* of 3. When the high proliferative colonies (colonies with 500 or more cells) were trypsinized, human fetal lung ECFCs generated secondary colonies. (Figure 2-6-1 & 2-6-2)

2.3.5. Human fetal lung ECFCs form complex networks *in vitro*

To assess their functional characteristics, ECFCs from human fetal lungs were plated on matrigel to observe their cord forming ability. The ECFCs formed complex networks on matrigel. (Figure 2-4A)

2.3.6. Human fetal lung ECFCs exposed to hyperoxia *in vitro* are functionally impaired

In vitro hyperoxia exposed ECFCs, when plated on matrigel, formed less complex networks as compared to normoxic ECFCs both on gross examination and when quantitatively. The hyperoxic ECFCs formed significantly less intersections compared to normoxic ECFCs. Hyperoxic ECFCs were observed to form longer cords; however, the difference was not statistically significant. (Figure 2-4B & Figure 2-5)

On single cell plating, hyperoxic ECFCs formed fewer first generation colonies from a single cell as compared to normoxic ECFCs – 56.9% vs. 44.4% respectively. 2.34% of the colonies formed by hyperoxic ECFCs were high proliferative colonies as compared to 16.4% of normoxic ECFCs that formed high proliferative colonies. (Figure 2-6-1)

The second generation colonies showed similar results with hyperoxic ECFCs forming 56.2% of the colonies as compared to 72.2% by normoxic ECFCs. 11.48% of the colonies formed by hyperoxic ECFCs were high proliferative colonies as compared to 15.6% of normoxic ECFCs that formed high proliferative colonies. (Figure 2-6-2)

2.3.7. Hyperoxia-exposed ECFCs show reduced survival and cell growth

Normoxic and hyperoxic ECFCs were plated at equal cell densities and cultured under identical conditions. Cell viability was measured using MTT assay. Hyperoxia-exposed ECFCs showed lesser survival. (Figure 2-7)

2.3.8. MSC CdM improves clonogenic potential of hyperoxic ECFCs

ECFCs, when exposed to hyperoxia while being cultured in MSC CdM as a preventative strategy, formed more first generation colonies (50.34%) on single cell plating as compared to hyperoxic ECFCs without MSC CdM (44.4%). The second generation colonies formed by re-plating of high proliferative first generation colonies showed a more marked difference with hyperoxic ECFCs,

exposed to MSC CdM, forming 70.8% colonies from a single cell and non-MSC CdM exposed hyperoxic ECFCs forming 56.2% colonies from a single cell.

In the first generation colonies, there was no significant difference observed in the number of high proliferative colonies between MSC CdM and non-MSC CdM hyperoxic groups. (Figure 2-8-1) However, in second generation, MSC CdM exposed hyperoxic ECFCs formed more high proliferative colonies as compared to non-MSC CdM hyperoxic group. (Figure 2-8-2)

2.4. Discussion

In this study, we show that ECFCs exist in human fetal lung and express characteristic surface antigens and incorporate endothelial specific staining. They possess clonogenic potential and form complex networks on matrigel.

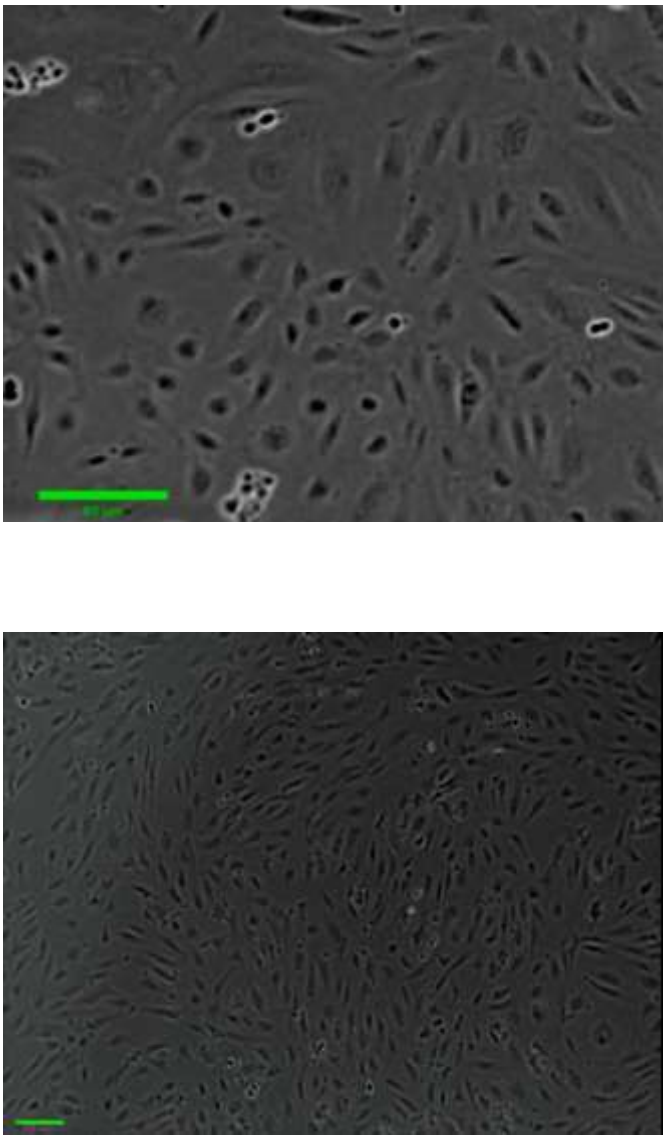
Since hyperoxia is implicated in the pathogenesis of BPD, we hypothesized that hyperoxia perturbs human fetal lung ECFCs *in vitro*. We exposed human fetal lung ECFCs to 60% oxygen and compared them with ECFCs kept in room air (normoxia) and investigated their phenotype and function. Both groups strongly expressed endothelial surface markers and incorporated endothelial specific staining. Functionally, on matrigel and clonogenic assay, hyperoxic ECFCs were found to be impaired. Hyperoxic human fetal lung ECFCs formed less complex cords on matrigel. Hyperoxic human fetal lung ECFCs also showed decreased proliferative potential on first and second generation forming less high proliferative colonies as compared to normoxic ECFCs. In addition, hyperoxic ECFCs showed decreased viability on MTT assay. These findings suggest that hyperoxia affects resident lung ECFCs and this may play an important role in the pathogenesis of BPD. In fact, it has been found that hyperoxia exposure induces an inflammatory response in the lungs that disrupts alveolarization leading to inflammation, alveolar simplification and vascular remodeling. The effect of hyperoxia on the lungs has been studied in neonatal rodents and found to be similar to immature human lungs. Furthermore, circulating ECFCs have been shown to be susceptible to oxidant-damage [34].

The discovery of EPCs by Asahara et al. [45] opened a new avenue for research and now it is generally accepted that vasculogenesis also occurs in postnatal life contrary to the earlier belief that only angiogenesis occurred postnatally [38,39]. Because of the recent advances, we have come to understand that ECFCs are the "true" EPCs [40] that participate in neovasculogenesis [44]. Because of the role ECFCs play in neovasculogenesis, it is likely that ECFC dysfunction plays an important role in the development of BPD. As shown by our study, it is probable that hyperoxia affects the function and survival of resident lung ECFCs that in turn are unable to participate in the normal alveolarization of the lung, leading to the characteristic features of BPD such as alveolar simplification.

In recent years, MSCs have been shown to prevent the lung from hyperoxic injury. Furthermore, it has been shown that that the MSC work through a paracrine effect [41,42,43]. We speculated that some of these beneficial effects are mediated via protection of endogenous lung ECFC and therefore used MSC CdM to assess this. We administered MSC CdM to ECFCs at the start of exposure to hyperoxia and tested them for their clonogenic potential. We found that in the first generation colonies, there was no significant difference in the number of high proliferative colonies formed between MSC CdM and non-MSC CdM hyperoxic groups. However, in second generation, MSC CdM exposed hyperoxic ECFCs formed more high proliferative colonies as compared to non-MSC CdM hyperoxic group. This suggests that despite initial hyperoxic injury and damage, over time MSC CdM provides a protective effect on the ECFCs.

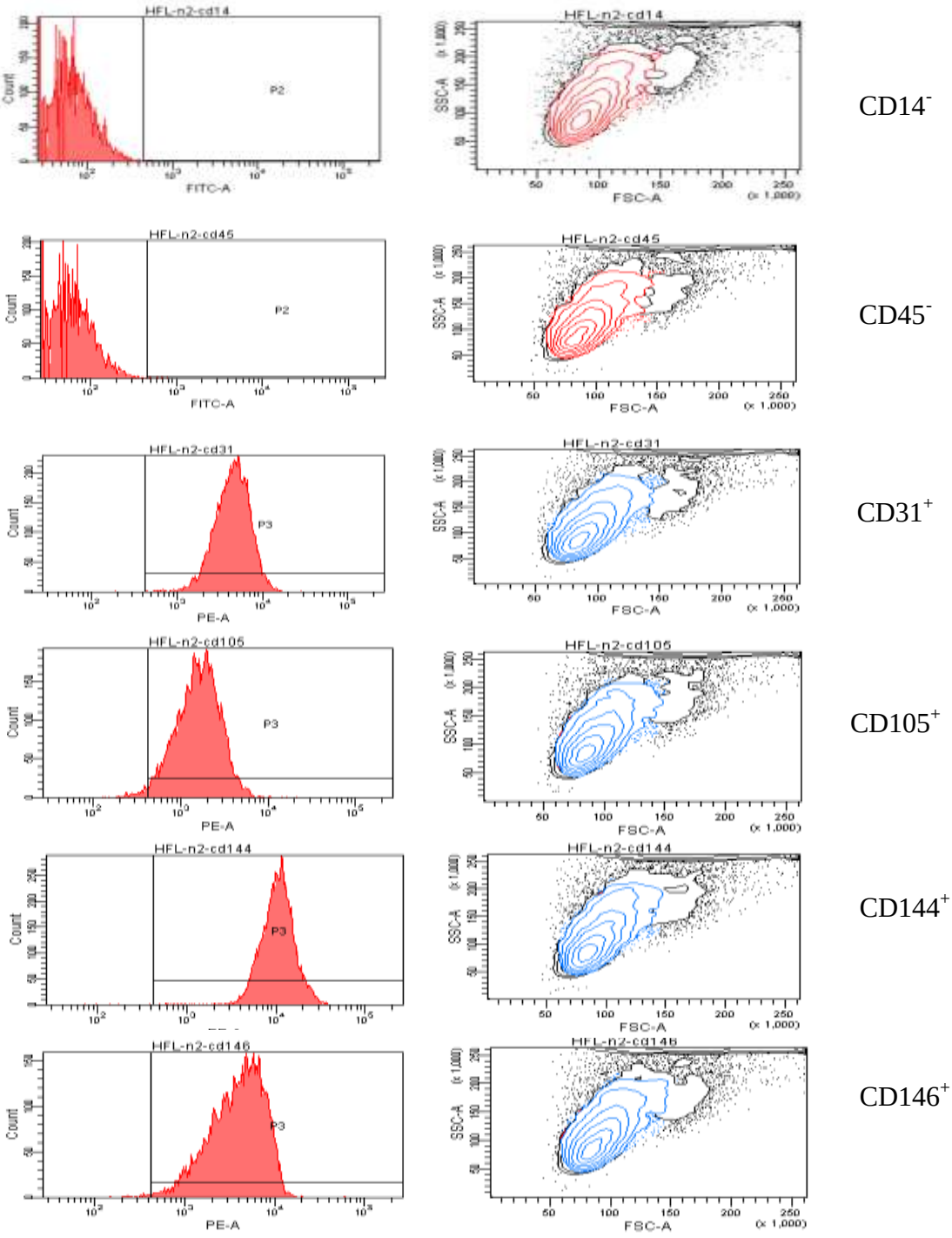
In conclusion, we show that ECFCs exist in the fetal human lung and hyperoxia impairs their survival and functional capacity. We also show that MSC CdM over time improves the functional capacity of hyperoxic ECFCs. These observations suggest that therapies should be targeted to prevent and/or rescue ECFC damage in diseases such as BPD. More research is required in this area to study in depth the mechanism by which ECFCs are implicated in the pathogenesis of BPD.

Figure 2-1. ECFC “cobblestone” colonies



ECFC colonies with cobblestone appearance at day 10 in culture.

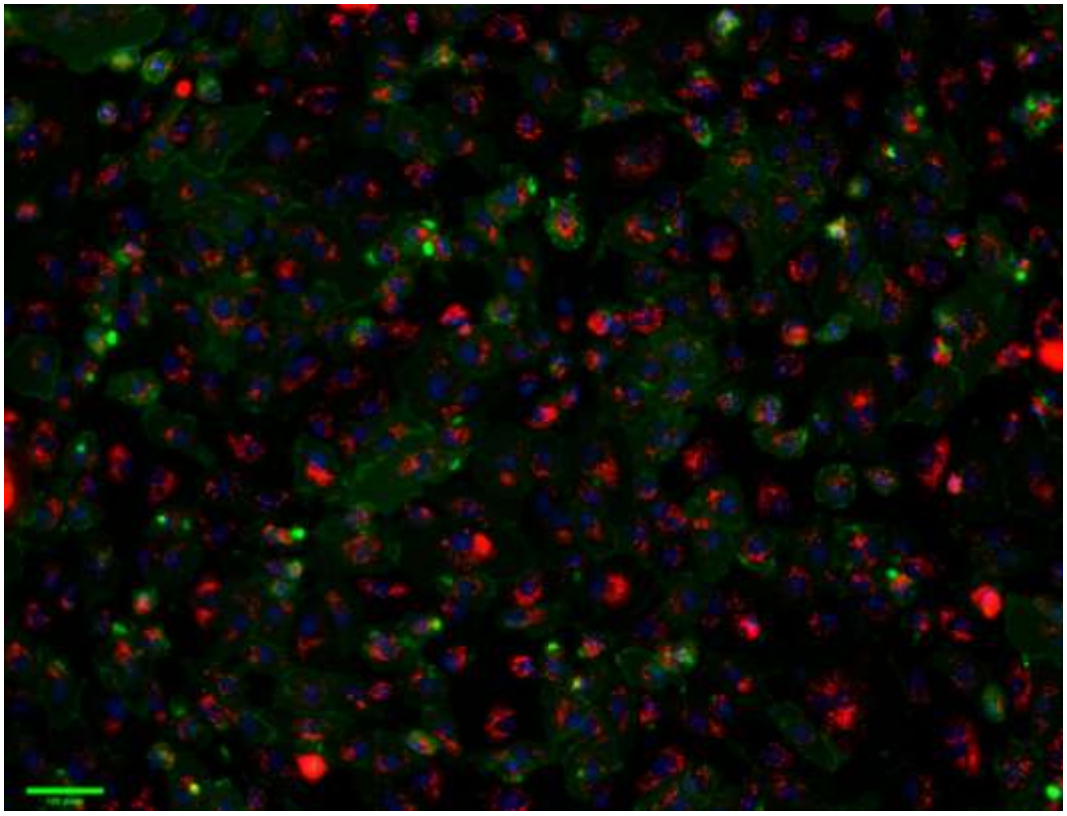
Figure 2-2. Representative FACS analysis of human fetal lung ECFCs



Representative images from 3 different human fetal lung isolations.

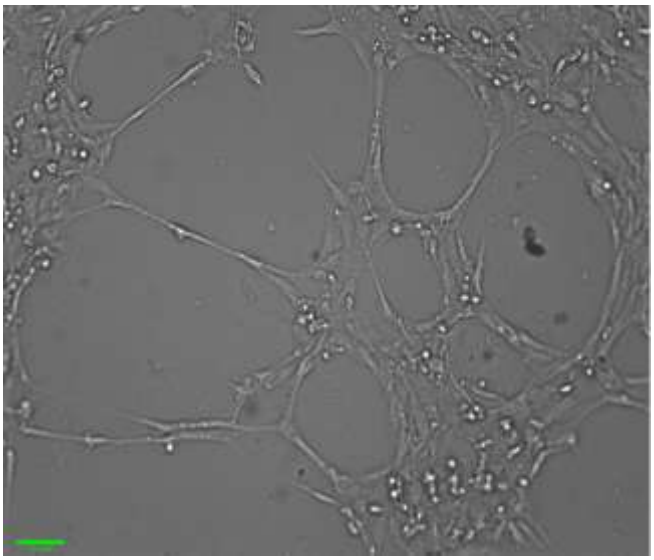
ECFC stained positive for endothelial cell surface markers CD31, CD105, CD144 and CD146. They were negative for hematopoeitc marker CD45 and macrophage/monocyte marker CD14.

Figure 2-3. Human fetal lung ECFCs uptake Dil-acLDL and UEA-1

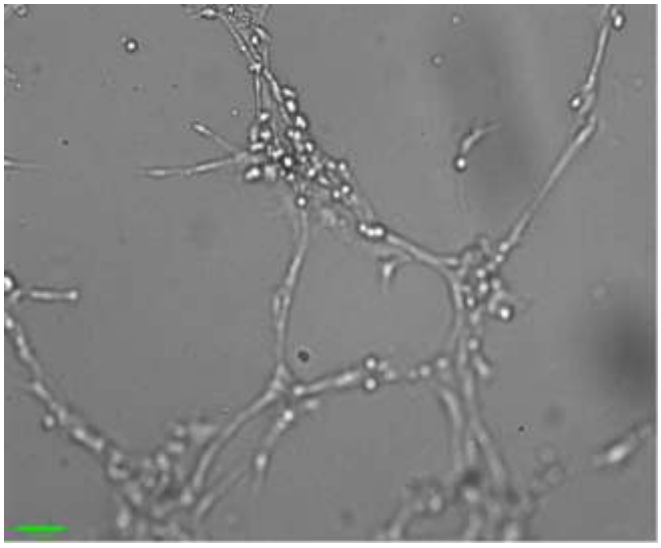


Human fetal lung ECFCs stained positively for endothelial specific stains, Dil-acLDL (red) and UEA-1 (green). They were also positive for Hoechst stain (blue).

Figure 2-4. In vitro capillary-like network formation of human fetal lung ECFCs – Morphology

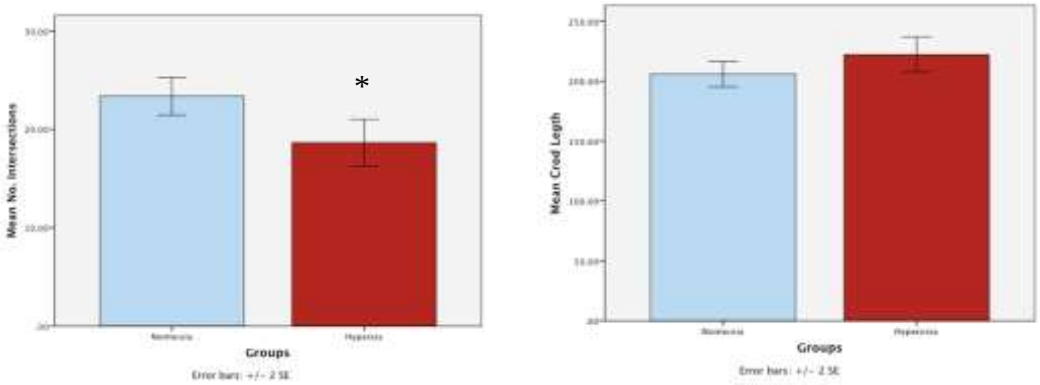


A. Human fetal lung ECFCs form complex networks on matrigel



B. The network forming ability of the human lung ECFCs is impaired in hyperoxia

Figure 2-5. In vitro capillary-like network formation of human fetal lung ECFCs – Number of intersections and mean cord length



In vitro cord forming potential assessed by matrigel assay.

n=3 in duplicates

Mean number of intersections as compared to normoxia $p<0.05$

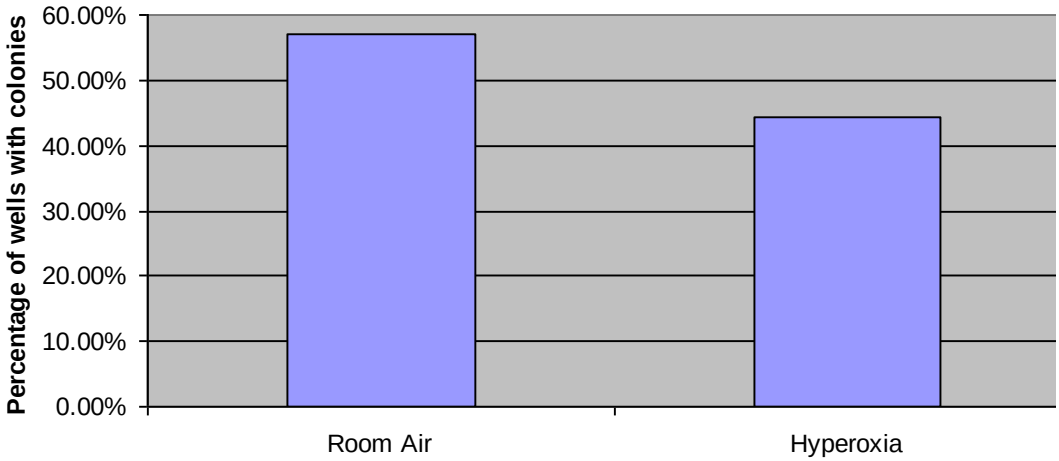
Cord length as compared to normoxia $p<0.08$

Significant difference observed in the number of intersections between normoxic and hyperoxic ECFCs. No significant difference between cord lengths.

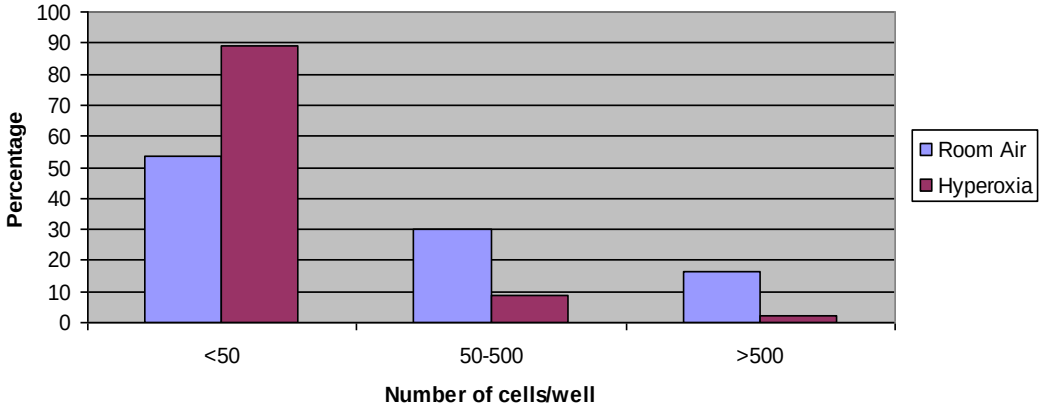
Figure 2-6-1. Self renewal and clonogenic potential – single-cell assay

First generation

(n =3)



A. Room air ECFCs formed a greater number of colonies as compared to hyperoxia-exposed ECFCs.

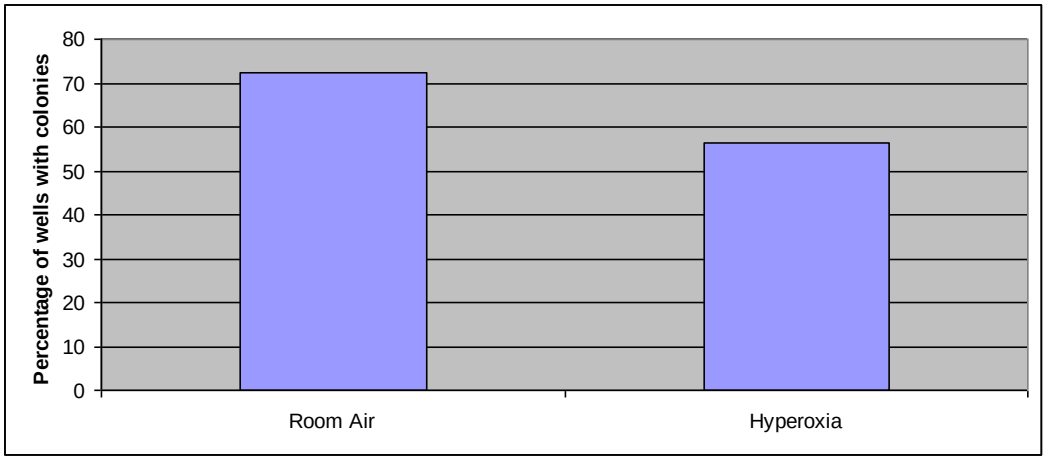


B. Hyperoxia-exposed human fetal lung ECFCs formed fewer high proliferative colonies (> 500 cells/well)

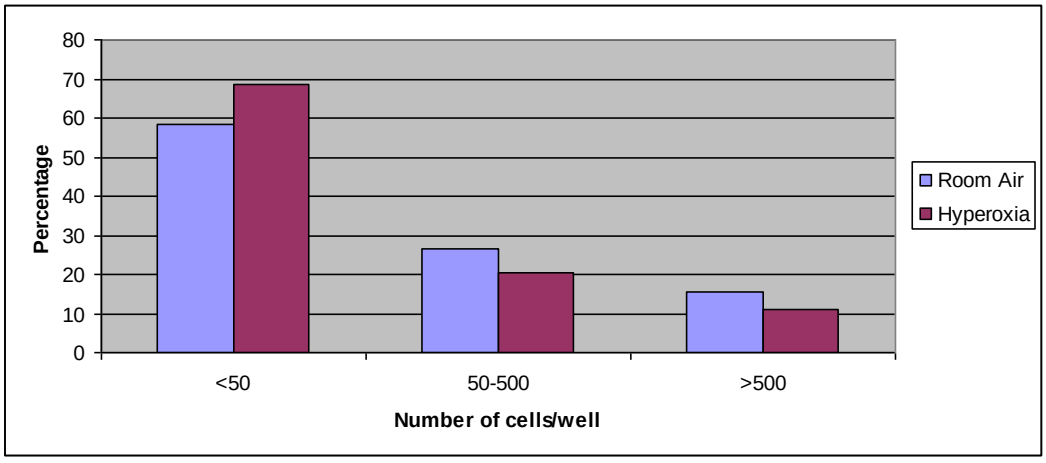
Figure 2-6-2. Self renewal and clonogenic potential – single-cell assay

Second generation

(n=3)

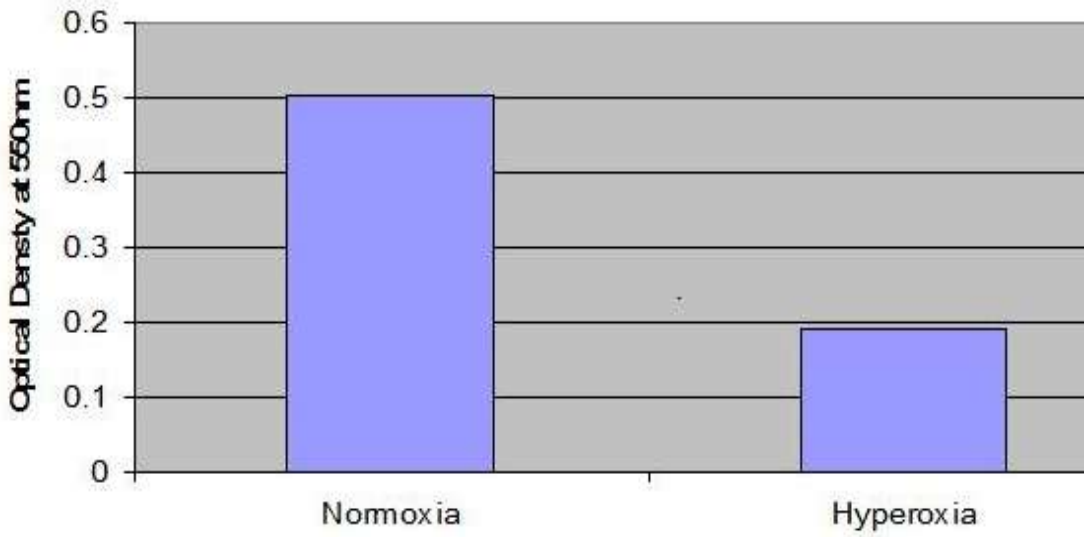


A. Room air ECFCs formed a greater number of colonies as compared to hyperoxia-exposed ECFCs.



B. Hyperoxia-exposed human fetal lung ECFCs formed fewer high proliferative colonies.

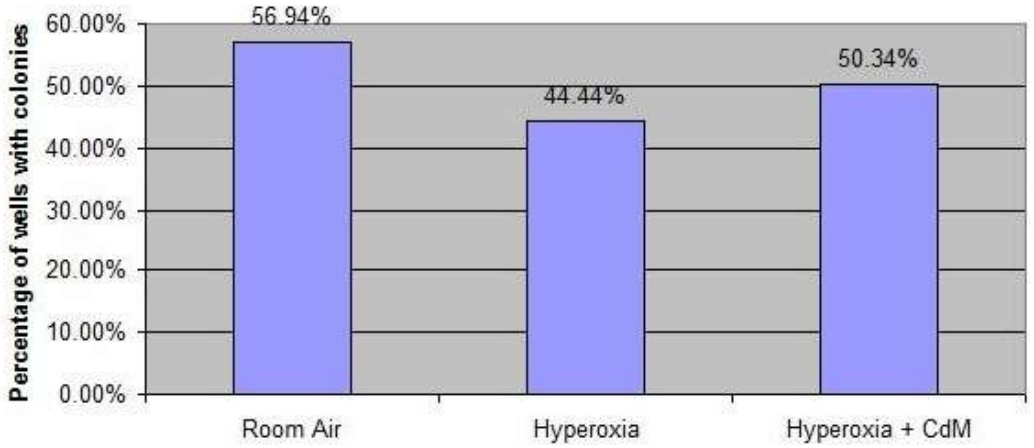
Figure 2-7. MTT assay for assessment of cell viability



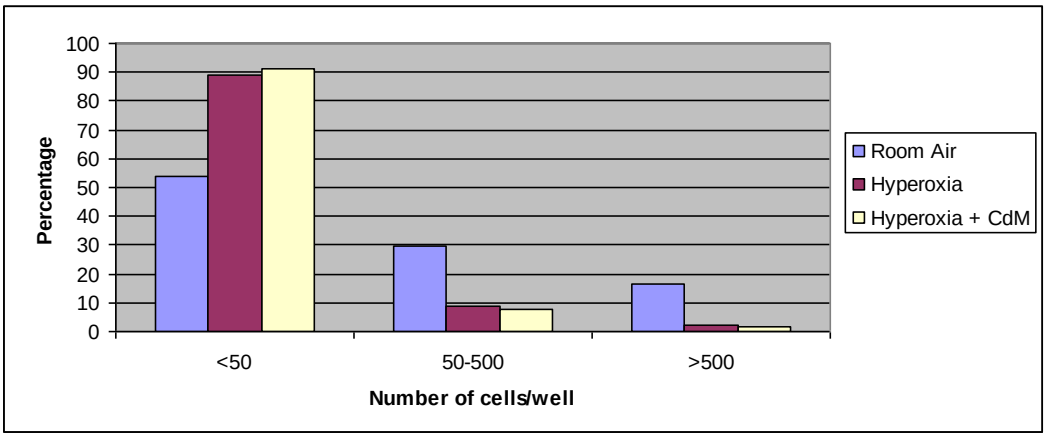
Hyperoxia-exposed ECFCs showed reduced survival as measured by MTT assay. The higher the absorbance as measured by optical density, the higher the viability of the cells.

Figure 2-8-1. MSC CdM improves self renewal and clonogenic potential – single-cell assay (n=3)

First generation



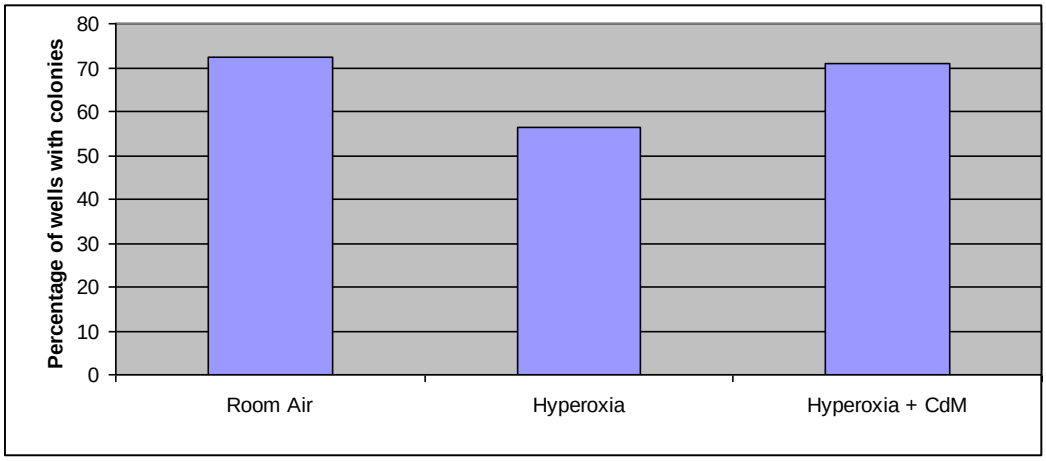
A. Hyperoxia-exposed ECFCs, administered CdM, formed increased number of colonies as compared to hyperoxic ECFCs without CdM



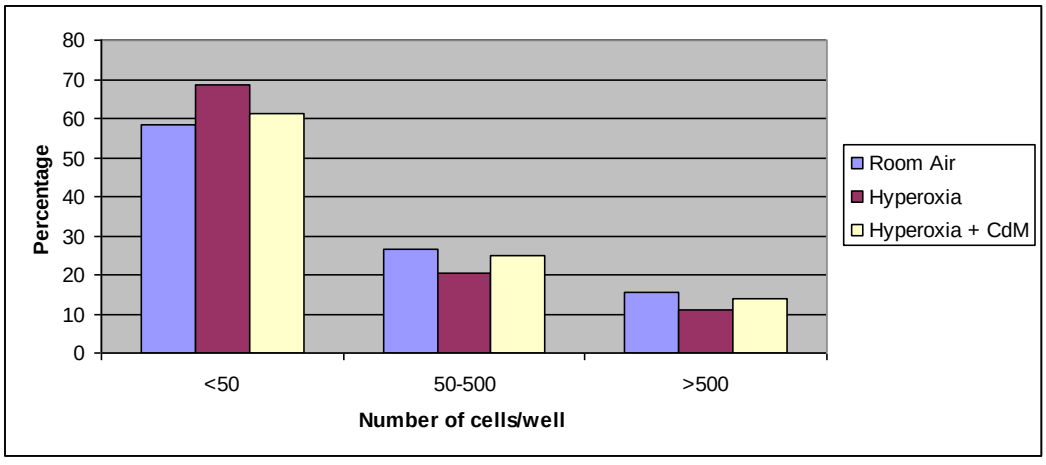
B. Hyperoxia-exposed human fetal lung ECFCs formed fewer high proliferative colonies. A significant difference was not observed in the number of high proliferative colonies between MSC CdM and non-MSC CdM group in first generation.

Figure 2-8-2. MSC CdM improves self renewal and clonogenic potential – single-cell assay

Second generation



A. Hyperoxia-exposed ECFCs, administered CdM, formed increased number of colonies as compared to hyperoxic ECFCs without CdM



B. Hyperoxia-exposed human fetal lung ECFCs formed less high proliferative colonies. The second generation CdM exposed hyperoxic ECFCs formed more high proliferative colonies than non-MSC CdM group.

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Chapter 3

Endothelial Colony Forming Cells in Emphysema

Cancerous vs. Pneumothorax “Normal” Adult Human Lung

In Search of a Better Control for Human Lung ECFC Studies

3.1. Introduction

Due to advances in neonatal care, survival of infants born more and more preterm has increased. BPD remains the main complication of extreme prematurity [1]. These preterm babies are living well in to adulthood. This makes it important to understand the long-term consequences and the disease sequelae of BPD.

Several studies have shown the association between BPD and reduced lung function (reduced forced expiratory flow rates and gas trapping) and increased respiratory symptoms during childhood [2,3,4]. The impairment in pulmonary function is shown to exist with residual abnormalities in pulmonary structure [5]. Airway obstruction, hyper-reactivity and hyperinflation have been described in babies with BPD that survived to adulthood [6]. Doyle et al. found that babies with very low birth weight that developed BPD had poorer lung function in late adolescence than babies without BPD [7,8]. Some other studies found similar abnormalities in lung function in babies born prematurely and with low birth weight [9,10]. In a study by Hennessy et al., pulmonary outcomes of extremely premature babies were measured at 30 months and 6 years of age. It was found that children who were premature at birth had greater prevalence of respiratory symptoms and use of respiratory medications than their peers, and those that had BPD within that group reported much higher use of respiratory medications and respiratory symptoms than those who did not have BPD. However, respiratory symptoms, re-hospitalization and use of respiratory medications declined over the study period showing improvement occurs over a period of time [11]. It has also been found that babies with BPD, are commonly re-hospitalized in the first 2

years of life but the rate of hospitalization decreased over time[12]. Gross et al. reported that preterm babies with BPD were twice as likely as non-BPD babies to require re-hospitalization at 2 years of age and show abnormal pulmonary function at 7 years of age. However, preterm babies without BPD show similar pulmonary function as that of non-preterm babies at 7 years of age[13]. In addition, in ex-preterm babies, both with and without BPD, exercise capacity is compromised[14]. These poor pulmonary outcomes, with respiratory symptoms such as cough, wheeze and asthma have shown to persist well into adolescence and young adulthood of ex-preterm babies [9,15].

Emphysema is characterized by progressive airflow limitation that is not fully reversible and is associated with an abnormal inflammatory response and extra-pulmonary effects [16].

BPD is characterized by the impairment of alveolar formation with fewer and poorly vascularized alveoli and a reduction in gas exchange surface area [17,18].

Both emphysema and BPD share a number of factors in their pathophysiology. Both are marked by decreased alveolar number and increase airspace size leading to respiratory insufficiency [19]. Both diseases have similar precipitating factors including oxidative stress [20,21,22,23], sustained inflammation [24,25] and protease-antiprotease imbalance [26,27]. In addition, these precipitating factors promote apoptosis of epithelial and alveolar cells [28,29,30]. In “new BPD” the vascular component is implicated with poorly vascularized alveoli and impaired microvascular development. Also, there is dysregulated angiogenesis and exogenous VEGF is shown to improve alveolarization in hyperoxic newborn rats

[18,31,32,33,34]. VEGF is also implicated in the pathogenesis of emphysema. Chronic blockade of VEGF receptors causes septal cell apoptosis, resulting in emphysema and lung capillary loss causing some degree of pulmonary hypertension [35]. Liebow et al. have also noted earlier that alveolar septa in centrilobular emphysema are remarkably thin and almost avascular probably because of reduction of blood supply of the pre-capillary blood vessels [36]. Voeskel et al. have also pointed out the pulmonary vascular involvement and the need to address the vascular component of the disease [37].

Interestingly, Wong et al. found that adult survivors of BPD have early onset emphysema and may have residual functional and structural abnormalities [38]. Recent studies suggest that stem/progenitor cells are perturbed in both BPD and emphysema and may be implicated in the pathogenesis of these diseases leading to impaired lung vascular and alveolar growth [39,40,41,42,43]. In addition, stem/progenitor cells have shown beneficial effects in various lung diseases [44]. In recent years, numerous studies have shown beneficial effects of stem/progenitor cells in BPD [45].

Given the common pathophysiology and precipitating factors of both BPD and emphysema it makes sense to look for similar therapeutic targets. Also, since stem/progenitor cells have been implicated in both disease processes [46] and given that babies with BPD develop emphysema later in life, it will be interesting to see if therapy with stem/progenitor cells in BPD helps in preventing emphysema later in life.

The role of ECFCs that are late outgrowth EPC have recently been extensively studied in BPD[47,48,49]. However, the role and function of resident lung ECFCs in the pathogenesis of emphysema has not been investigated. Given that both diseases have involvement of the vascular component, it will be exciting to see if resident lung ECFCs are perturbed in emphysema and compare the function of lung ECFCs in BPD and emphysema.

In order to study ECFC in emphysema, we need to find a suitable adult human lung control. However, it is difficult to acquire normal human lung that would serve as an ideal control. Given this limitation, we decided to compare the ECFCs from two lung pathologies – lung cancer and pneumothorax, since it is relatively easy to acquire tissues from these sources. The tissue acquired from cancerous lung is distant from the site of cancer and is thought to be normal. However, various mediators of cancer may be affecting the grossly and histologically normal tissue long before the development of the actual disease. Furthermore since EPCs have found to be involved in the neovascularization in cancer [50], "normal" tissue from a cancer lung may not serve as a good control.

Thus we hypothesized that lung ECFCs are perturbed in emphysema. The aim of this project was to determine if lungs from patient with pneumothorax represented a better control than healthy parts of the lung from patients with lung cancer.

3.2. Materials and Methods

All procedures were approved by the Human Research Ethics Board of the University of Alberta.

3.2.1. Collection of lung tissue

Lung tissue from both adult human cancer lungs and pneumothorax lungs were collected immediately after resection and transported in sterile condition in normal saline with 1% penicillin/streptomycin and amphotericin B (PSF). The isolation process was started within an hour of tissue acquisition.

3.2.2. ECFC isolation and culture

The tissue was washed several times using sterile Dulbecco's Modified Eagle Medium (DMEM). The tissue was chopped finely using McIlwain tissue chopper after which they were placed in collagenase/dispase solution (0.1 U collagenase and 0.8 U dispase/mL) for 1 hour at 37°C with intermittent shaking. The suspension was mixed with equal volumes of DMEM containing 10% fetal bovine serum (FBS) to quench the enzyme and was then strained through 70μm and 40μm cell strainers. This was followed by centrifugation at 300 g for 10 minutes at 4°C. The pellet obtained was resuspended in PBS with 0.1% bovine serum albumin (BSA) and incubated with CD31 coated dynabeads that were previously coated. After incubation for 30 minutes, magnetic separation was conducted and CD31 positive cells were plated in 6-well plates. We used Endothelial basal medium-2 (EBM-2) supplemented with EGM-2 singlequots with 10% FBS and 1% penicillin/streptomycin and amphotericin B (PSF) as our

culture media. The media was changed every day for a week; thereafter it was changed every other day. ECFC colonies were identified between day 10 and 14 and were isolated through clonal rings and were expanded in T25 and then T75 flasks.

3.2.3. ECFC characterization

Immunophenotyping and uptake of endothelial stains was carried out using FACS as described in sections 2.2.3.1 and 2.2.3.2

Clonogenic and matrigel functional assays were performed as described in section 2.2.3.3 and 2.2.3.4.

3.3. Results

3.3.1. Cancerous lung and pneumothorax lung ECFCs both expresses endothelial specific surface antigens

On immunofluorescence characterization by FACS, endothelial specific surface antigens CD31, CD105, CD144 and CD146 were strongly expressed in both groups. Neither of the groups expressed hematopoietic marker CD45 or macrophage/monocyte marker CD14. This shows that ECFCs isolated were in fact of endothelial origin and did not contain any contaminating cells. (Figure 3-1 and 3-2)

3.3.2. Dil-acLDL and UEA-1 were incorporated by ECFCS

Both cancerous and pneumothorax lung ECFCs stained positively for endothelial specific stains demonstrating the basic endothelial characteristics of these cells and indicating that they are ECFCs. (Figure 3-4)

3.3.3. Formation of complex networks *in vitro* on matrigel

On matrigel, both groups formed complex networks 4 hours after plating which was observed grossly and quantitatively. Cancerous lung ECFCs formed significantly longer cords than pneumothorax lung ECFCs. However, no significant difference was observed in the number of cord intersections. (Figure 3-5 and 3-6)

3.3.4. High proliferative potential of ECFCs on single cell plating

ECFCs from both groups were placed at single cell densities in a 96-well plate. Based on the number of cells generated per colony from a single cell, the colonies were classified as low proliferative (2-50 and 50-500 cells/well) or high proliferative (>500 cells/well). At day 14, first generation colonies formed by cancerous lung ECFCs had a greater number of high proliferative colonies as compared to the pneumothorax lung ECFCs (37.5% vs. 14.8%). Upon re-plating, the second generation ECFCs showed the same pattern with cancerous lung ECFCs forming an increased number of high proliferative colonies as compared to pneumothorax lung ECFCs (40.6% vs. 27.7%). (figure 3-7)

3.4. Discussion

The pathophysiology of emphysema and BPD share a number of common factors. Recent studies suggest that stem/progenitor cells are perturbed in both BPD and emphysema. Given the common pathophysiology and precipitating factors of both BPD and emphysema it makes sense to look for similar therapeutic targets. In the pathogenesis of emphysema, the role and function of resident lung ECFCs has not been investigated. Since the involvement of the vascular component is implicated in both diseases, it will be interesting to see if resident lung ECFCs are perturbed in emphysema and compare the function of lung ECFCs in BPD and emphysema. Thus we hypothesized that lung ECFC are perturbed in emphysema.

In order to study ECFC from emphysema lungs, we would need adult human lung controls that are normal and disease free. This is very challenging since it is hard to obtain normal human lung tissue. We were able to obtain normal lung tissue from subjects that had lung carcinoma and were undergoing removal of the tumor. We obtained tissue distant from the cancer site. Although lung tissue from cancerous lungs is easier to acquire, however, we were not sure if this would be the best control since factors mediating cancer may have affected the normal appearing part of lung as well despite the tissue appearing normal. We were also able to obtain lung tissue from pneumothorax patients which may be a better control but is more difficult to acquire. Since primary spontaneous pnuemothorax is not associated with any underlying lung pathology, we sought to isolate and

compare cancer “normal” lung ECFCs with that of pneoumothorax lung ECFCs in order to find a good control for our future emphysema ECFC studies.

We found that both the groups expressed ECFC surface markers and stained positively for endothelial specific staining. In addition they both formed complex networks on matrigel. Cancerous lung ECFCs formed significantly longer cords than pneumothorax lung ECFCs. On single cell plating, cancer lung ECFCs formed more high proliferative colonies in both first and second generation. This indicates that since there is increased angiogenesis in cancer, cancer lung ECFCs, even from a site distant from the actual cancer foci, may have ECFCs that are highly proliferative and may not be ideal for use as a control. Although statistics were performed on an *n* of 2, it is not ideal and in future we aim to increase the *n* to draw conclusions that are statistically significant.

EPCs, that are a subtype of stem cells with high proliferative potential and are capable of differentiating into mature endothelial cell, have been shown to play a role in angiogenesis and neovascularization in cancer. It has been shown that EPCs home from the bone marrow to upon the angiogenic switch in solid tumors in addition to migrating to the sites of injury for neovascularization[51]. Also, in cancer EPCs contribute to neovascularization in tumors. EPCs mobilize from the bone marrow to the peripheral blood and home to the tumor site where they differentiate in to mature endothelial cells and secrete pro-angiogenic factors to facilitate vascularization of tumors in response to tumor-secreted cytokines [50].

In comparing cancerous lung ECFCs and pneumothorax ECFCs, age may be considered a bias since patients with lung carcinoma are older and patients with

pneumothorax are younger. Therefore the effects we see may be age-related. However, we found ECFCs from cancerous lung to be more robust despite of the older age of the patients from whom the samples were acquired which is in opposition to the current findings in literature that suggests that natural aging affects the efficacy of stem cells [52]. We can confidently conclude that the effects we observed in our study were because of the underlying pathology rather than the age.

Thus, we show that pneumothorax lung ECFCs may serve as a better control in future adult human lung studies. In future, we aim to study the role of ECFCs in emphysema and learn more about the connection and common pathogenesis of BPD and emphysema.

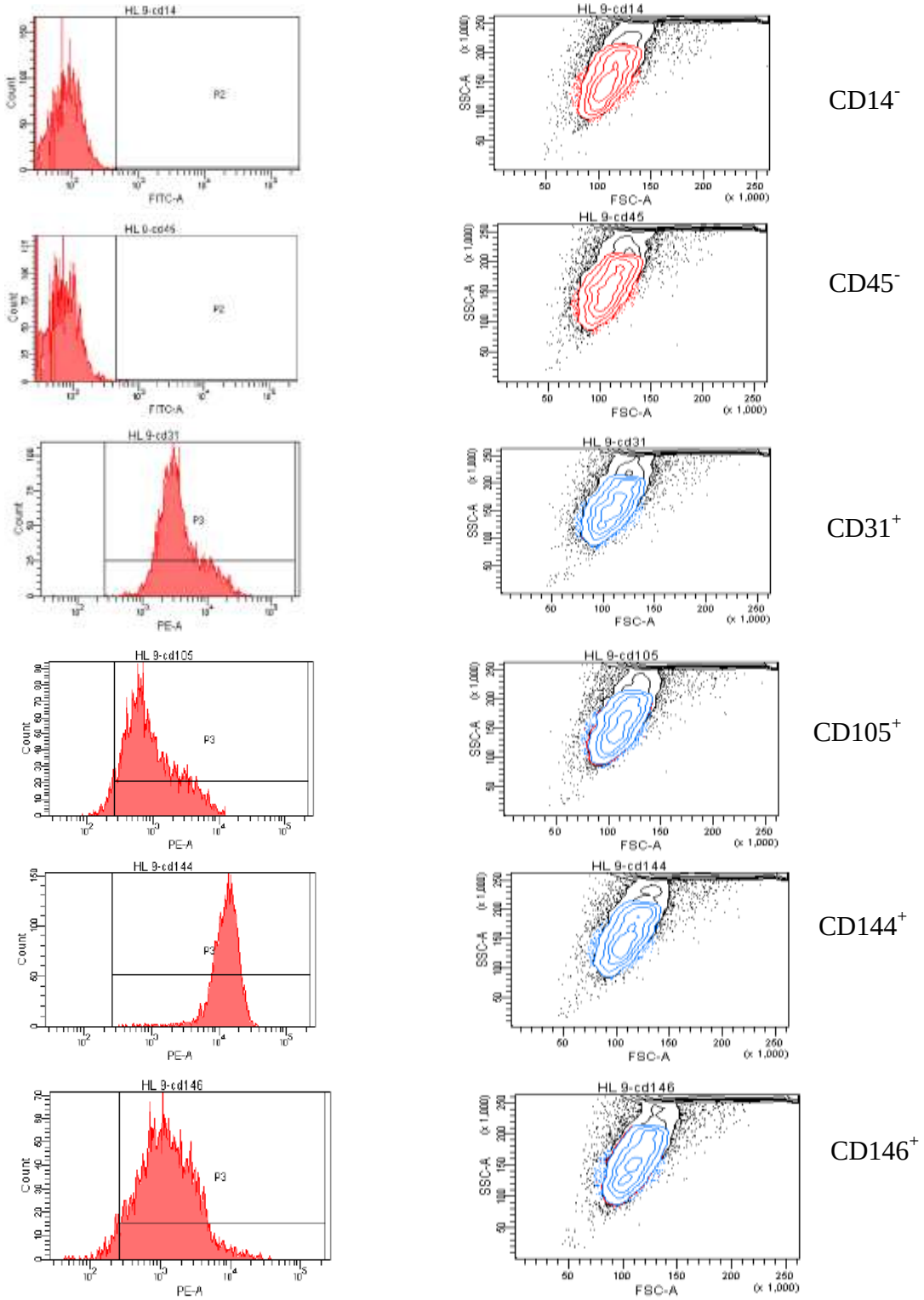
Table 3.1. Clinical characteristics of cancer and pneumothorax groups

Sample No.	Age	Gender	Diagnosis
C-N1	64	F	Cancer and mild COPD
C-N2	84	M	Cancer and mild COPD
P-N1	19	M	Pneumothorax
P-N2	18	M	Pneumothorax

Location of cancer tissue: Remainder of the lung lobe after removal of the tumor

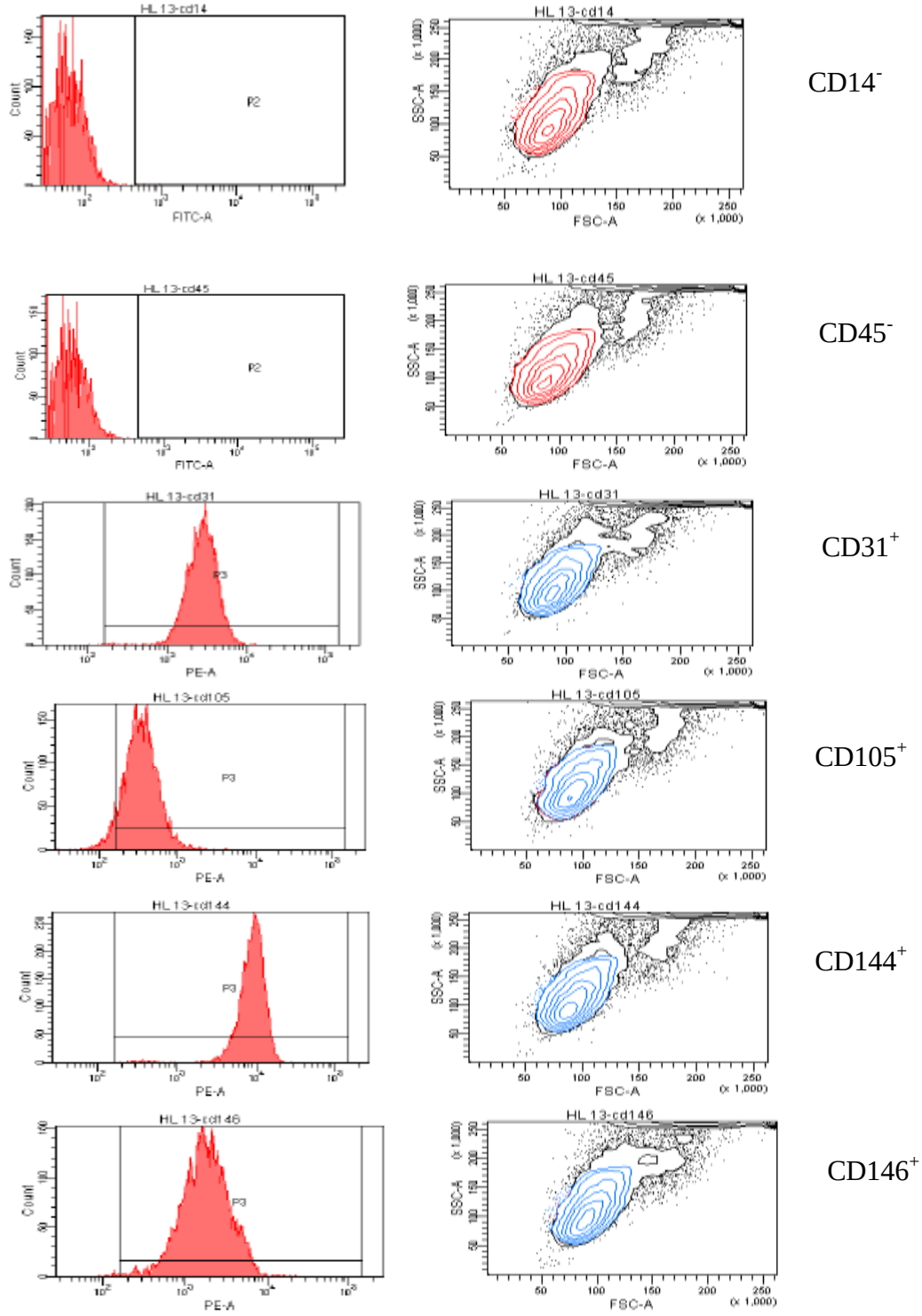
Location of pneumothorax tissue: Left Upper Lobe

Figure 3-1. Representative FACS analysis of Human Cancer Lung ECFCs



Representative images from 2 different human cancer lung isolations ECFC stained positive for endothelial cell surface markers CD31, CD105, CD144 and CD146. They were negative for hematopoeitc marker CD45 and macrophage/monocyte marker CD14

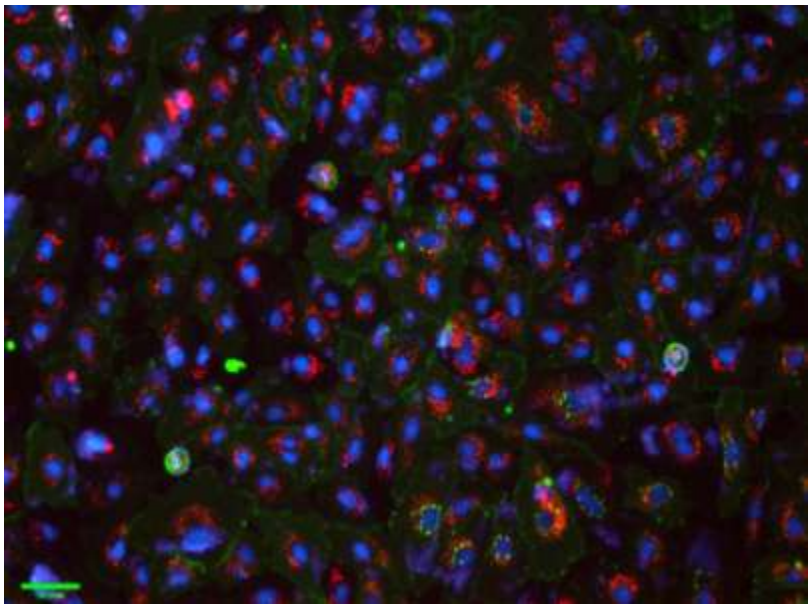
Figure 3-2. Representative FACS analysis of Human Pneumothorax Lung ECFCs



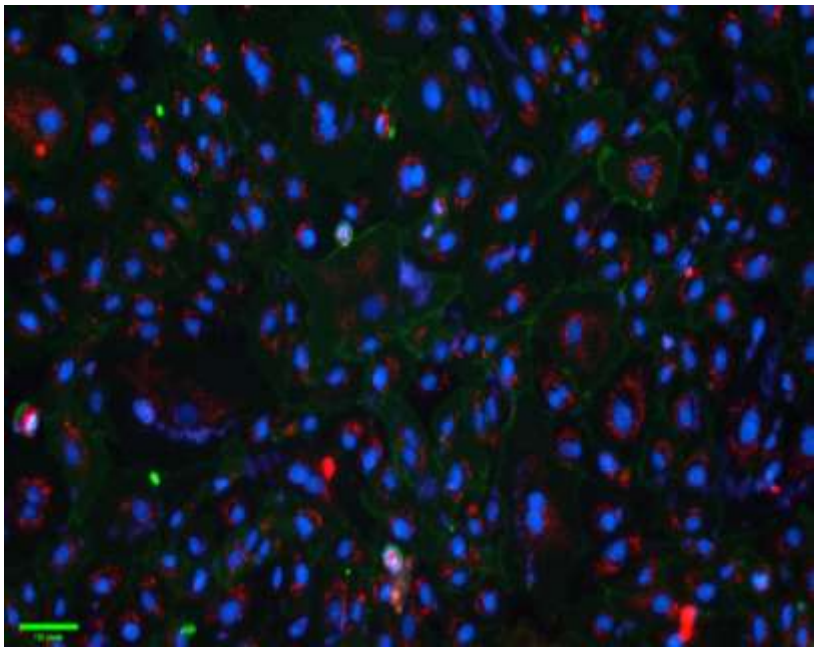
Representative images from 2 different human pneumothorax lung isolations

ECFC stained positive for endothelial cell surface markers CD31, CD105, CD144 and CD146. They were negative for hematopoeitc marker CD45 and macrophage/monocyte marker CD14

Figure 3-3. Human cancer and pneumothorax lung ECFCs uptake Dil-acLDL and UEA-1



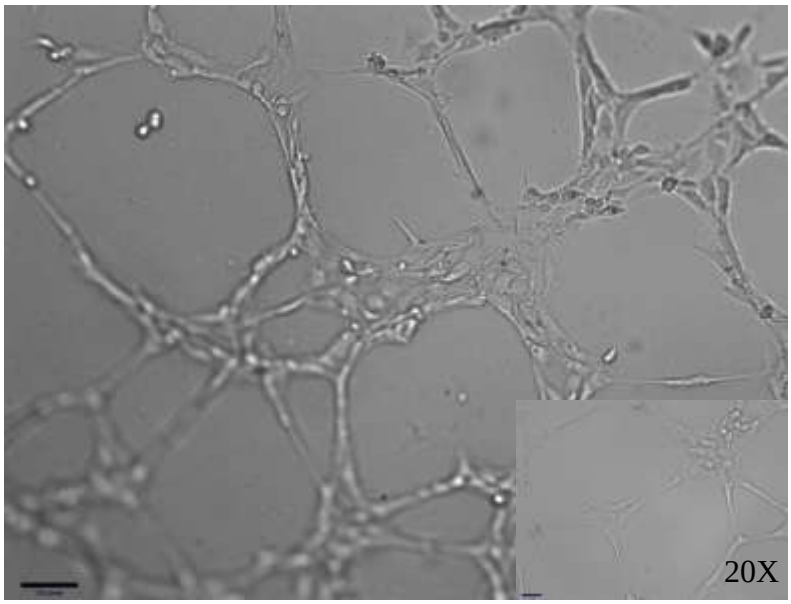
A. Cancer Lung



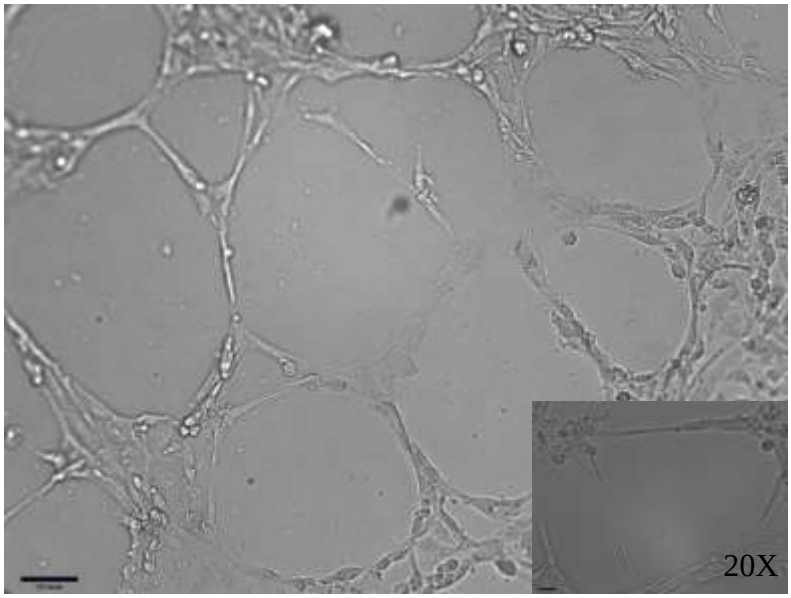
B. Pneumothorax lung

Human cancer and pneumothorax lung ECFCs stain positively for endothelial specific stains, Dil-acLDL (red) and UEA-1 (green). They were also positive for Hoechst stain (blue).

Figure 3-4. In vitro capillary-like network formation of human cancer and pneumothorax lung ECFCs – Morphology



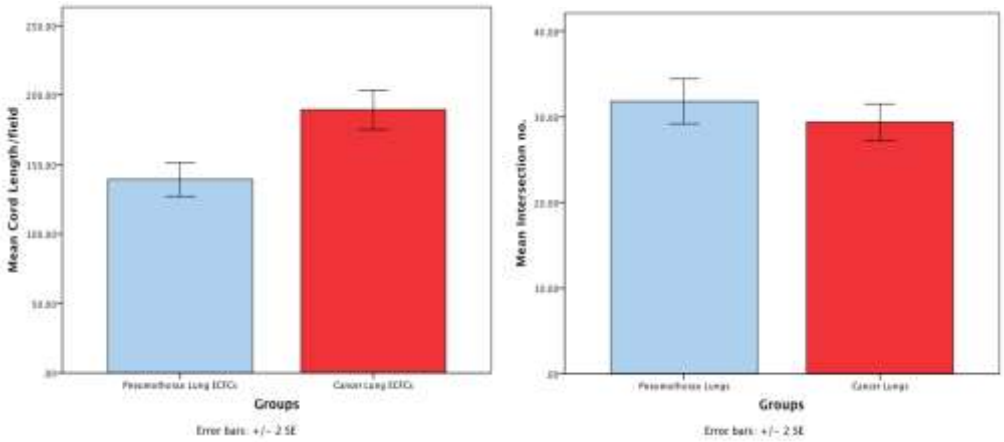
Pneumothorax Lung



Cancer Lung

Human cancer and pneumothorax lung ECFCs form complex networks on matrigel

Figure 3-5. In vitro capillary-like network formation of human fetal lung ECFCs – Number of intersections and mean cord length



In vitro cord forming potential assessed by matrigel assay.

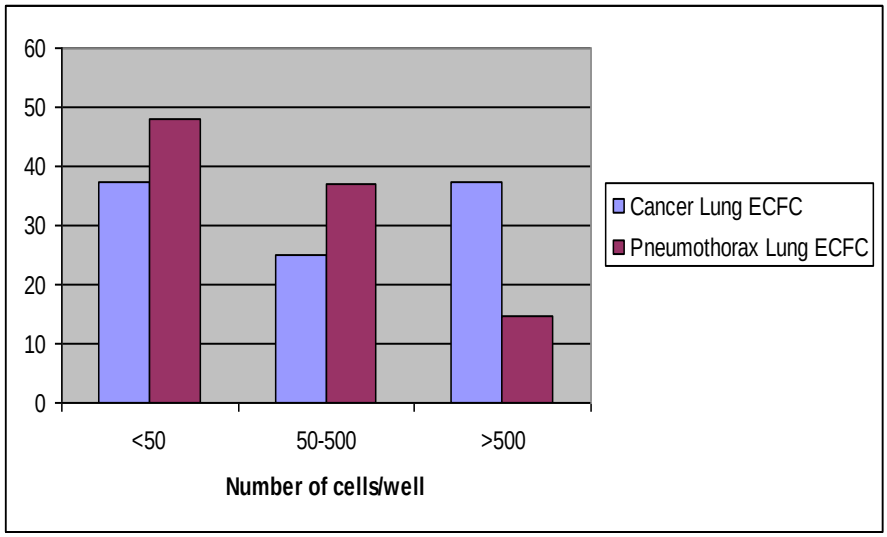
n=2 in duplicates

Human cancer lung ECFCs mean cord length as compared to pneumothorax ECFCs $p<0.006$

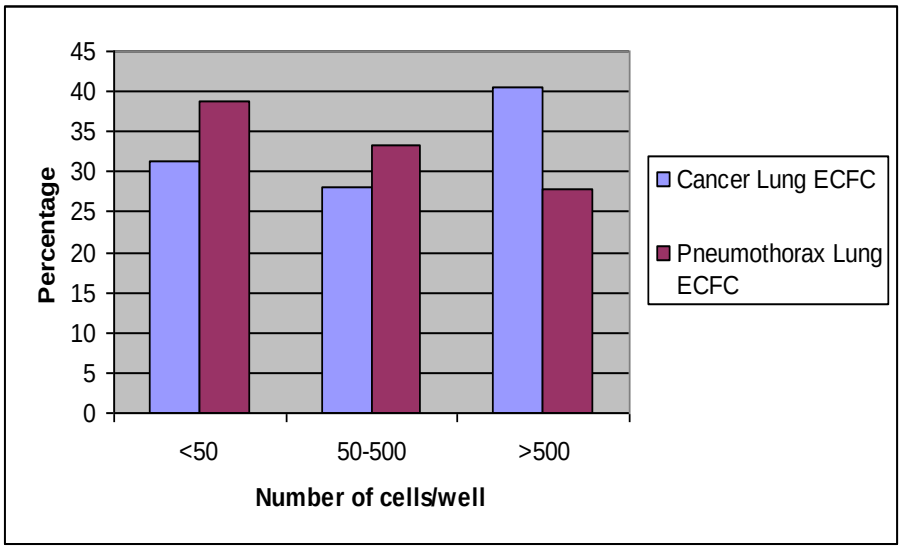
Significant difference observed in the cord length. No significant difference between number of intersections

Figure 3-6. Self renewal and clonogenic potential – single-cell assay

A. First generation



B. Second generation



n=2 in duplicates

Cancer lung ECFCs formed more number of high proliferative colonies in first and second generation as compared to pneumothorax lung ECFCs

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Chapter 4

Existence of Exosomes in Conditioned Media of Endothelial Colony Forming Cells and their therapeutic role in BPD

4.1. Introduction

Exosomes are secreted vesicles belonging to the group that includes microvesicles, ectosomes, membrane particles and apoptotic bodies [1]. The term exosomes was first mentioned in 1981 by Trams et al [2]. Following this, a study by Pan et al. described exocytosis of 50-nm diameter bodies by sheep erythrocytes [3] and were shown to have multiple active enzymes [4].

The current concept regarding exosomes is that they are small vesicles that have a diameter of 40-100nm and a density in sucrose of 1.13-1.19g/ml. On observation with electron microscopy, they have a characteristic cup-shaped or saucer-like morphology [5,6]. Exosomes contain both proteins and RNAs as their cargo [7]. Exosomes are secreted by many types of cells including B cells, dendritic cells, mast cells, T cells, schwann cells, platelets, sperm and tumor cells. They are also secreted in urine, plasma and bronchial lavage fluid [6]. Exosomes have membranes that are enriched in cholesterol, sphingomyelin, ceramide and phospoglycerides with long and saturated fatty-acyl chains [8,9]. They have cell type specific proteins along with tetrspanins, Alix, Tsg101 heat shock proteins (HSsc70, Hsp 90), transport and fusion proteins and phospholipases and lipid-related proteins [10].

Exosomes are thought to be of endosomal origin. They bud off inside endosomes to form multivesicular bodies (MVBs). MVBs contain molecules such as enzymes, nucleic acids, cytokines and bioactive compounds. In response to various stimuli, MVBs fuse with the plasma membrane to release vesicles in the extracellular space. They are thought to act by direct induction of signaling pathways or

through transfer of new receptors and genetic material [11,12]. However, little is known about the molecular mechanisms that may be involved in the fusion of these vesicles with plasma membrane.

4.1.1. Function of exosomes

Exosomes have come to be known as an integral part of intercellular communication, thereby influencing various physiological processes [13,14]. Exosomes are capable of exerting local and remote effects and the message can be targeted to multiple and specific locations. Another important function of exosomes is their role in facilitating immune communication [1,15]. They play a role in antigen presentation [11], apoptosis, angiogenesis, inflammation and coagulation [16]. Exosomes from plasma suppresses Th1-type hypersensitivity and Th2-type allergic response [17]. Exosomes from breast milk contain miRNAs that modulate infant's immune system [18,19,20] and exosomes from placenta modulate T cell activity [21,22]

4.1.2. Exosomes and their role in pathology

Recently, a lot of research has been focused on exosomes and their role in cancer. Exosomes are considered to be the vehicles that carry various factors, such as cytokines, growth factors, adhesion molecules, which mediate the crosstalk within the tumor microenvironment. In addition, they may also activate specific cell signaling pathways that lead to the progression of cancer [23,24]. Exosomes may promote angiogenesis, tumor-induced immune suppression or premetastatic niche

formation by functioning in an autocrine or paracrine manner [25,26,27,28]. Hypoxic tumor cells release more exosomes to promote their survival and angiogenesis [29,30]. Exosomes have been shown to interfere with the action of therapeutics agents, possibly through transfer of mRNAs, miRNAs and proteins that are involved in drug resistance, thereby indirectly contributing to tumor progression and metastasis[31,32]. Interestingly, exosomes may play a therapeutic role in cancer as illustrated by the use of exosomes from tumor peptide-pulsed dendritic cells to suppress tumor growth [33].

Exosomes have been shown to play a role in normal physiological communication between neuronal cells. Exosomes are released by neurons [34], astrocytes [35] and glial cells[36]. They facilitate in the removal of stress proteins and amyloid fibril formation. They have also been implicated in the pathology of neurodegenerative diseases such as Parkinson's [37] and Alzheimer's [38] disease. Exosomes also mediate cellular communication in the pathogenesis of cardiovascular disease. It has been shown that the factors involved in the regulation of cardiac hyperplasia are localized inside exosomes and their levels are increased in patients with acute myocardial infarction and angina pectoris [39,40]. Exosomes have also been implicated in the pathogenesis of atherosclerosis and in vascular dysfunction.[41,42].

In the pathogenesis of autoimmune disease such as Rheumatoid Arthritis (RA), the role of exosomes has been studied. Exosomes have been shown to play a role in the alteration of apoptosis and promotion of T cell activation leading to inflammation, synovial hyperplasia and joint destruction [43,44].

4.1.3. Exosomes in diagnostics and therapeutics

Since exosomes can easily be isolated non-invasively, they appear to be an attractive and promising diagnostic tool. In addition, exosomes contain various proteins, RNA, miRNA and lipids that can serve as markers for disease. Expression patterns of serum miRNA for cancer, colorectal cancer, ovarian cancer, prostate cancer, and diabetes has been studied [45,46,47]. Urinary exosomes could be used as diagnostic markers in acute kidney injury [48] and prostate cancer [49]. The diagnostic value of exosomes from saliva and amniotic fluid has also been studied [50].

Recently, the role of exosomes as a therapeutic modality has also come to light. Exosomes have been studied to deliver small interfering RNA (siRNA) to the mouse brain as a target in Alzheimer's disease [51]. Phase I trials with dendritic cell derived-exosomes(DEX) have been completed in patients with metastatic melanoma[52] and in patients with advanced non-small cell lung cancer[53]. Autologous ascites-derived exosomes (Aex) along with granulocyte-macrophage colony-stimulating factor (GM-CSF) have been tested for colorectal cancer in a Phase I study [54]. DEX vaccination with cyclophosphamide is being tested in a Phase II clinical trial [55].

4.1.4. Exosomes and stem cells

A great deal of interest has been recently generated regarding exosomes derived from stem cells and their use as a replacement to cell based therapies. What makes exosomes attractive for this is that they are metabolically inactive vesicles that are not alive, thus making their handling easier. In addition, they are stable and can be stored at -80°C without losing their biological activity. They can carry large cargo and since they have membrane, they are protected from enzymatic and chemical degradation. They do not undergo host rejection or form tumors and have intrinsic homing ability. However, there are certain challenges that need to be overcome in the field of exosome biology and therapeutics to make them a viable option as a replacement to cell based therapies. The production of exosomes needs to be regularized and needs to pass quality control measures. Their safety and efficacy needs to be extensively tested. Also, technical limitations such as the availability of highly renewable embryonic stem cells to generate MSCs or availability of other sources of stem cells to produce exosomes at a large scale, that may be commercially viable, needs to be overcome [5,56].

Several studies have tested exosomes derived from stem cells. In a study by Xin et al., exosomes from mesenchymal stromal cells (MSC) mediate the transfer of miRNA 133b that promotes neurological remodeling and functional recovery after stroke [57]. Cardiomyocyte progenitors have been reported to secrete exosomes that promote migration of endothelial cells [58]. MSC derived exosomes have been tested in myocardial ischemia/reperfusion injury where they have shown to reduce infarct size, preserve left ventricular geometry and

contractility by activating pro-survival signals and reducing oxidative stress [59,60]. Exosomes from human CD34+ stem cells have been shown to have independent *in vivo* and *in vitro* angiogenic activity and are thought to be mediators of proangiogenic paracrine activity [61]. MSC exosomes have been proposed as a source for drug delivery since MSCs produce exosomes that are immunologically inert and the quality and quantity of exosome production from MSCs is not compromised by the immortalization of these cells [56,62].

The field of exosome research is still new and developing. In light of recent developments, this field holds promise and it seems that in the next few years the basic science of exosome research will be translated into the diagnostics and therapeutics. However, improvements in exosome isolation and purification is required. In addition, further characterization of the protein cargo of the exosomes and the molecular mechanisms may prove useful in understanding the role of exosomes in cell-to-cell communication.

Thus, we hypothesize that exosomes can be isolated from ECFC CdM.

4.2. Methods

4.2.1. ECFC conditioned media (CdM) preparation

Human umbilical cord blood (hUCB)-ECFCs were grown in 175-cm^2 flasks between passages 4-6 using the same seeding density each time. They were grown in cEGM-2 up to 90% confluence. The culture media was then removed and the cells were washed with PBS 3 times and serum free DMEM was added. After 24 hours, supernatants were collected. The supernatant was then subjected to centrifugation at 300 x *g* followed by 2000 x *g*. CdM was then pooled and used directly for exosome isolation. For each exosome isolation, CdM from eight 175-cm^2 flasks was used and from each flask approximately 35mL of CdM was made.

4.2.2. Exosome isolation from hUCB-ECFC CdM

Successive centrifugations were carried out at increasing speeds according to the previously published protocol by Thery et al[63]. All centrifugations were carried out at 4°C and in sterile conditions. The centrifugations were carried out using Beckman 45 Ti rotor using polycarbonate bottles. Up to 10,000 x g centrifugations, the pellet was discarded (which contained dead cells and debris) and the supernatant was used for successive centrifugations. However, after the 100,000 x *g* centrifugations (last two centrifugations), the pellets were kept (exosomes + proteins) and the supernatants discarded. Finally, the pellets were resuspended in 50 - 100µL of fresh PBS. Exosomes were stored in -80°C until further use (for up to 1 year).

4.2.3. Measurement of protein content of exosomes using Bradford assay

All protein quantification for samples and standards were done in duplicates. In a 96-well plate, the wells were loaded with 10μL of PBS (blank) and standard BSA dilutions. Exosome preparations were thawed on ice and the samples were homogenized by pipetting up and down. They were then loaded in wells and 200μL of diluted Bradford solution was added to each blank, standard and sample wells. Optical density was then read at 590 nm. Based on the standard curve, the amount of exosomes is then estimated.

4.2.4. Electron Microscope (EM) analysis of exosomes

Exosome preparations were thawed and resuspended in 50-100μL of 2% paraformaldehyde. 5μL of the resuspended pellets were deposited on formvar-carbon coated EM grids. The membranes were covered and allowed to adsorb for 20 minutes. The grids were then transferred to 100μL drops of PBS on parafilm sheets for washing. The grids were then transferred to 50μL drops of 1% glutaraldehyde for 5 minutes. After which it was transferred to 100μL drop of distilled water and washed a total of eight times in the same manner. For embedding the samples, the grids were transferred to 50μL drops of uranyl oxalate for 5 minutes followed by transferring to 50μL drops of methylcellulose-UA (uranyl acetate) for 10 minutes on ice. The grids were removed with stainless steel loops and excess fluid was blotted. The grid was allowed to air dry for 5 to 10 minutes. They were then observed under the electron microscope at 80 kV.

4.2.5. Immunoblot analysis of exosomes

There are several proteins that are consistently found in exosomes regardless of their source [10]. Therefore, in order to characterize the exosomes, it is important to show that these proteins are present. Proteins from total cell lysates were compared with that of exosomes to show that the protein was specifically enriched in the exosomes. The immunoblot was carried out as described in the protocol[63]. The proteins assessed were CD63 that has high enrichment in exosomes, MFG-E8 that has a very high enrichment in exosomes but not detectable in cell lysates and Grp94 which is absent in exosomes.

4.3. Results

4.3.1. Protein is present in hUCB ECFC exosome preparation

Measuring the protein in the exosome preparation confirmed the presence of protein since at the end of multiple high speed ultracentrifugations, the pellet obtained is very small and not always visible. Secondly, it gives an estimate of the amount to exosomes secreted by the cells. We measured an average of 140μg of exosome per mL. Therefore, eight confluent 175 cm^2 flasks, yielded 140μg of exosomes. (Figure 4-1)

4.3.2. Exosomal proteins are expressed in hUCB ECFC exosome preparation

The exosome preparation expressed CD63 and MFG-E8 proteins that have high expression in exosomes. The exosome preparations were negative for Grp94 which is absent in exosomes but expressed in cell lysates. The immunoblot results showed that the exosomes were present in our preparation. (Figure 4-2-1, 4-2-2 and 4-2-3)

4.3.3. hUCB ECFC exosomes show characteristic cup-shaped morphology on electron microscopy

Under the electron microscope, the exosomes were identified as round vesicles with characteristic cup-shaped morphology. (Figure 4-3)

4.4. Discussion

In this study we found that exosomes can be isolated from ECFCs. The vesicles that we isolated from hUCB ECFCS after a series of ultracentrifugations, were positive for exosomal markers CD63 and MFG-E8 and negative for Grp94 that is absent in exosomes as seen by immunoblotting. In addition, on electron microscopy these vesicles exhibited characteristic cup-shaped morphology of exosomes and were of small size, between 40-100nm.

Exosomes are small vesicles that have a diameter of 40-100nm and a density in sucrose of 1.13-1.19g/ml. Exosomes contain both proteins and RNAs as their cargo and have shown to be secreted by most cells of the body and are also secreted in urine, saliva and bronchial lavage fluid. Since they are membrane-bound their cargo is resistant to damage and acts as a compact vehicle to carry them. Recently, exosomes have gained a lot of attention in facilitating intracellular communication and playing a role in immune response. Their role has been studied in cancer and in the pathogenesis of cardiovascular, neurological and inflammatory disease. Furthermore, they have been studied as a diagnostic and therapeutic modality.

Recent advances in stem cell research suggest that stem cells may act through a paracrine mechanism and most of their beneficial effect may be due to paracrine mediators[64,65,66]. Given that exosomes are metabolically inactive vesicles that are not alive and are stable and can be stored at -80°C without losing their biological activity, it has been proposed that exosomes may be an interesting and viable alternative to cell based therapies. Also, exosomes do not undergo host

rejection or form tumors and have intrinsic homing ability and may also bypass some of the ethical and legal concerns of stem cell based therapies.

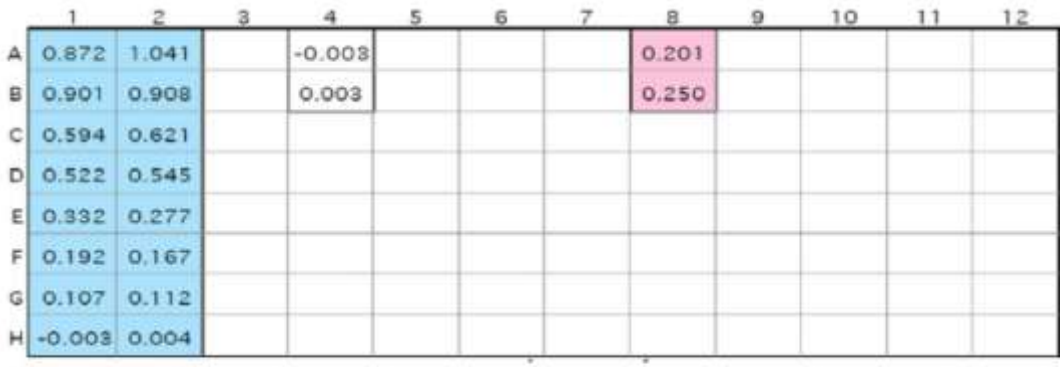
BPD is a chronic lung disease of prematurity that results because of ventilator and oxygen therapy. Currently it lacks effective therapies. The role of stem cells in BPD have been studied extensively in recent years. Since the vascular component is implicated in the disease pathogenesis, attention has been given to the role of EPCs in the pathogenesis of this disease. It has been found that conditioned media from EPCs provide the same therapeutic response as whole cells, indicating a possible paracrine mechanism of EPC action [67]. Interestingly, exosomes from human CD34+ stem cells have been shown to have independent *in vivo* and *in vitro* angiogenic activity and are thought to be mediators of proangiogenic paracrine activity [61]. Given the role of ECFCs in BPD and the fact that they may act through paracrine mechanism that may be brought about by exosomes, we hypothesized that exosomes exist in the conditioned media of ECFC. We also aimed to study their therapeutic role in BPD. In this study, we successfully isolated vesicles from ECFC CdM that exhibit the properties of exosomes. Although our data indicates that the vesicles present in our preparation are most likely exosomes, our Western blot data needs further clarity. The bands observed are not very clear and do not convincingly show the presence of protein markers under study. We would suggest inclusion of more markers and running standards that express these markers in parallel with our exosome preparations.

It will be interesting to explore the therapeutic benefit of exosomes in BPD. If we can overcome the challenges of exosome production, such as consistent extraction

of exosomes from CdM batch after batch and the need for large amounts of CdM to extract exosomes, they may prove to be an exciting therapeutic modality for the treatment of BPD.

In conclusion, exosomes exist in hUCB ECFC CdM and they exhibit characteristic exosome markers and morphology. In future, it will be exciting to study their role as a therapy in BPD.

Figure 4-1. Protein quantification of hUCB ECFC exosome preparation



	1	2	3	4	5	6	7	8	9	10	11	12
A	0.872	1.041		-0.003				0.201				
B	0.901	0.908		0.003				0.250				
C	0.594	0.621										
D	0.522	0.545										
E	0.332	0.277										
F	0.192	0.167										
G	0.107	0.112										
H	-0.003	0.004										

Sample	Wells	Values	Outliers	Result	MeanResult	Std.Dev.	CV%
Un01	A8	0.201		0.124	0.140	0.022	15.7
	B8	0.250		0.155			

The unit is mg/ml

The pink column shows absorbance and the "Result" in the bottom column is the actual protein quantification (average 140 μg/ml)

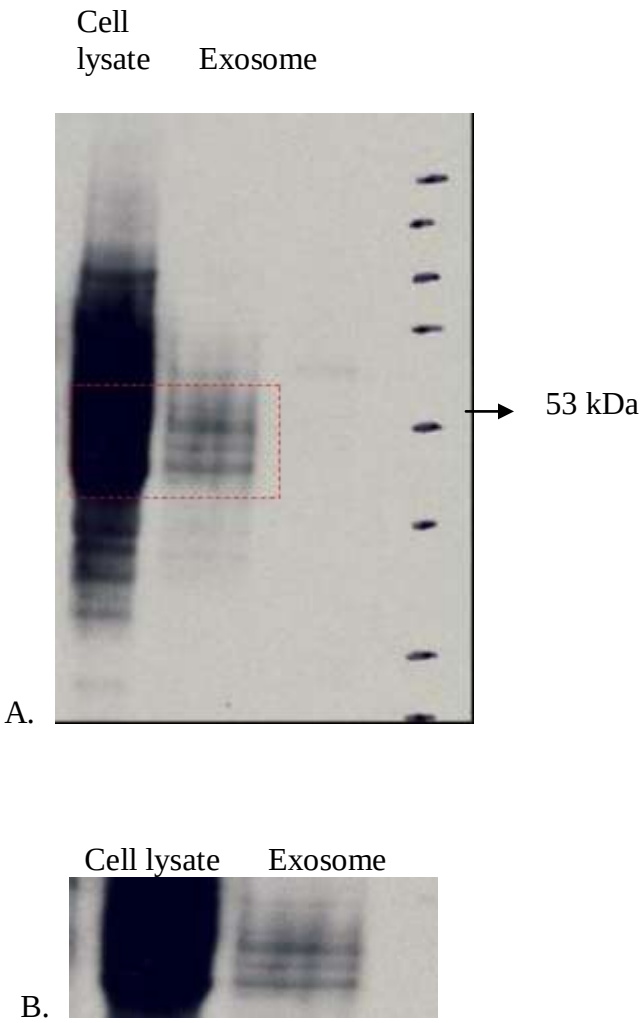
Column 1&2 are standards in duplicate.

Column 4 is blank in duplicate

Column 8 is the sample (exosome preparation) in duplicate

The sample was not diluted

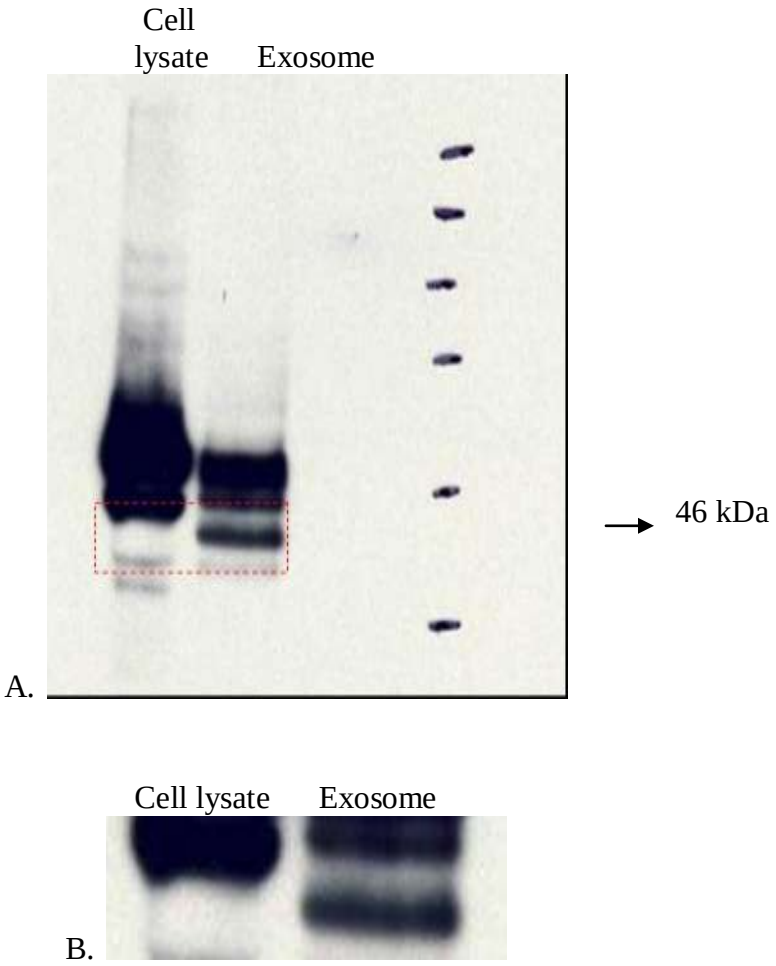
Figure 4-2-1. Immunoblot (Western) analysis of hUCB ECFC derived exosomes – CD63



A. CD63 is present in exosome preparation. The exosome preparation was compared with cell lysate. **B.** Band positive for CD63 is magnified.

Figure 4-2-2. Immunoblot (Western) analysis of hUCB ECFC derived exosomes –

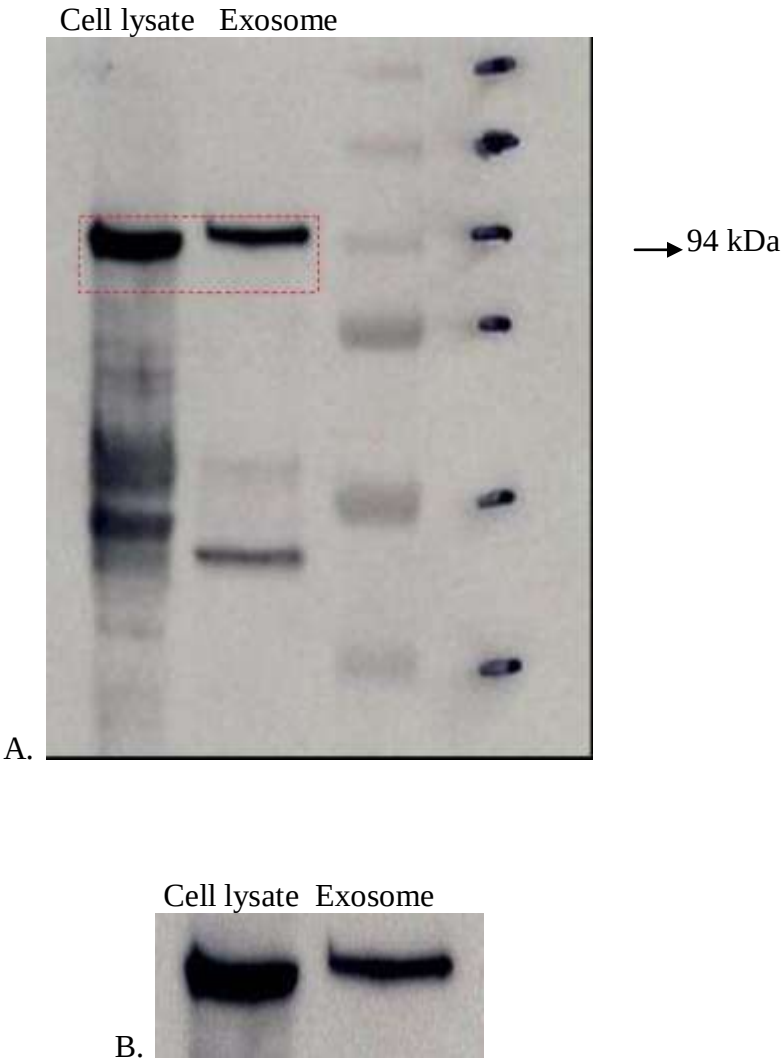
MFG-E8



A. MFG-E8 is present in exosome preparation and absent in cell lysate. **B.** Band positive for MFG-E8 is magnified.

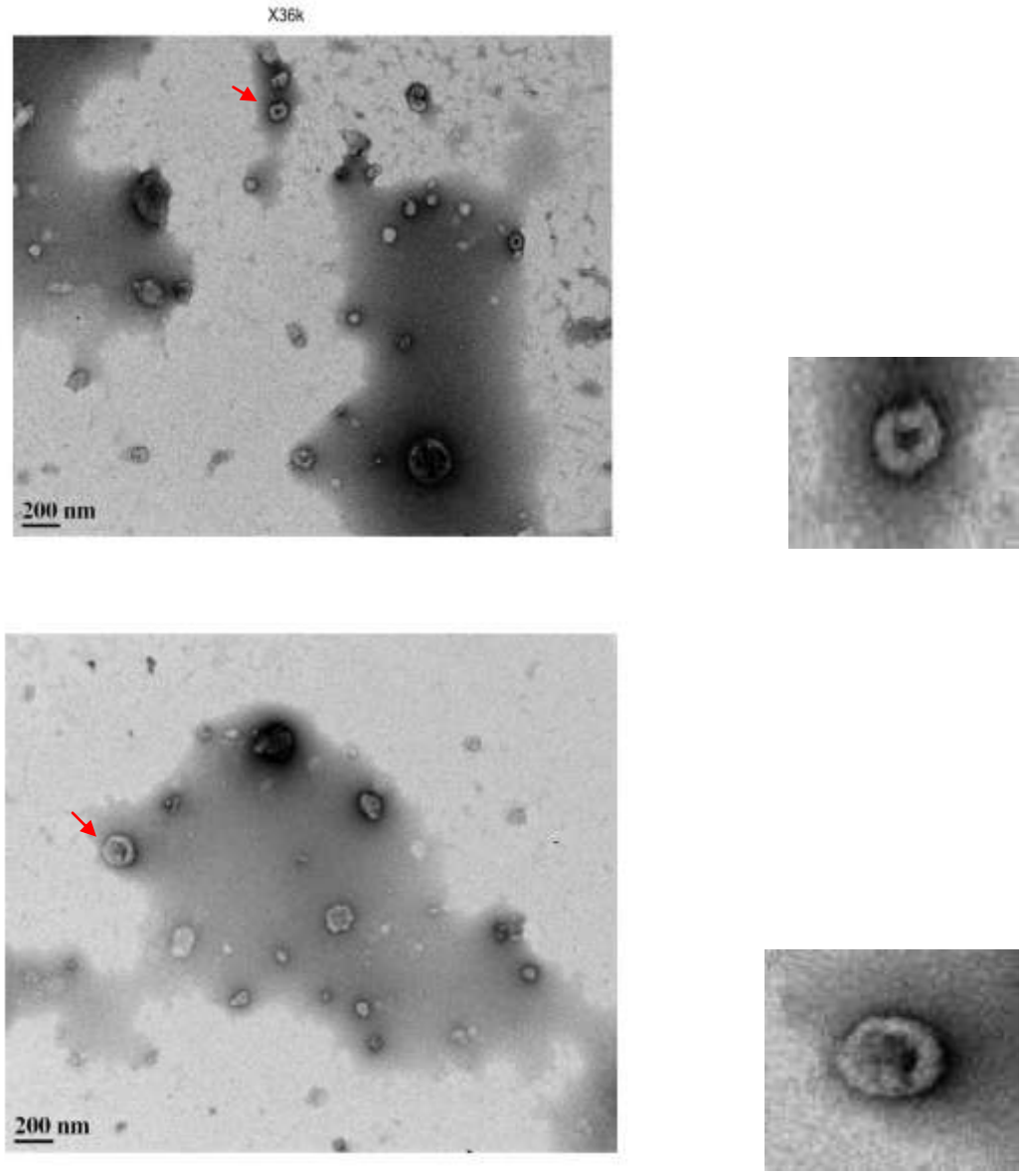
Figure 4-2-3. Immunoblot (Western) analysis of hUCB ECFC derived exosomes –

GRP94



A. GRP94 is absent in exosome preparation and present in cell lysate. **B.** GRP94 band is magnified.

Figure 4-3. Electron microscopic images of hUCB ECFC exosomes



hUCB ECFC exosomes exhibit a characteristic cup-shaped morphology

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Chapter 5

General summary and future directions

5.1. Overview

Bronchopulmonary dysplasia is a chronic lung disease of prematurity that is characterized by alveolar simplification and poorly vascularized alveoli leading to decreased surface area for gas exchange [1]. BPD is a consequence of ventilator and oxygen therapy that does not have any effective treatment as yet. With recent advances in neonatal care, these preterm babies are growing into adulthood and we are now seeing long-term complications of BPD in these ex-preterm babies [2]. The role of stem/progenitor cells have been implicated in the pathogenesis of BPD and seem an exciting therapeutic opportunity for lung diseases, especially BPD [3].

In chapter 2 we showed that resident lung ECFCs exist in the human fetal lung and that their function is impaired in hyperoxia. Administration of MSC CdM improves the clonogenic potential of hyperoxic exposed ECFCs over a period of time. This is a novel finding since ECFCs have not been isolated from human fetal lung before. In addition, these findings suggest that hyperoxic mediated impairment of ECFCs may play an important role in the development of BPD and that these cells may be further investigated as a potential therapy, either whole cells or through their secreted factors, in BPD.

In chapter 3 we aimed to study resident lungs ECFCs in emphysema. In order to find a better control for human lung studies, we compared normal tissue derived from human cancer lung and pneumothorax lung. We speculate that even though

pneumothorax lung is more difficult to acquire than cancer lung, it might serve as a better control given that even the normal tissue from cancer lung, derived from a location distant from the tumor, may be impacted by cancer through chemokines and other factors. The differences we observed between the two groups may be because of the difference in age as patients with pneumothorax were younger. However, as observed earlier in the discussion, aging decreases the efficacy of stem cells but we found the ECFCs derived from normal part of cancer lung from older patients to be more robust.

In chapter 4 we show that exosomes can be isolated from hUCB ECFCs and exhibit the characteristics known to be possessed by these microvesicles. It is an exciting finding that ECFCs produce exosomes and they can be implicated in the mechanism by which ECFCs exert their function. Exosomes derived from ECFCs can be harnessed as a therapy as well as used as biomarkers in ischemic diseases.

5.2. Limitations

There are several limitations to this study that we would like to mention.

Small sample size of three human fetal lungs was used for isolation of ECFCs. However, we performed all the experiments in triplicates unless otherwise stated. Although we consistently and successfully isolated ECFCs from these lungs each time, in order to confirm our findings for hyperoxic exposure studies, we need to increase our sample size.

Another limitation is the acquisition of adult human emphysema tissue. It is challenging to obtain the tissue since we can only get emphysema lung tissues from patients undergoing lung transplant and this surgical process does not happen frequently. In order to study resident lung stem cells and their function in disease, we need to find ways to obtain more and easily accessible human tissue.

In addition, the area of exosome research is relatively new. At present, with the technology available, it is difficult to conclude that each exosome isolation gives the same amount of exosomes each time. Refinement in exosome isolation and characterization techniques needs to be done to produce large amounts of exosomes that can be used for therapy. Instruments like NanoSight (Wiltshire, UK) that allow faster detection, quantitative characterization and visualization of exosomes and other microvesicles, and isolation of exosomes by detection of markers by FACS, are promising and in future may be used for the isolation of exosomes.

5.3. Conclusions

Arrested alveolar development is central to the pathophysiology of BPD. One of the major goals of our research was to gain more insight into one of the key mechanisms responsible for the impairment of alveolar growth - disruption of neovasculogenesis and ECFC dysfunction in the developing lung. We achieved this by isolating resident ECFCs from human fetal lung and assessing their function in *in vivo* hyperoxia exposure.

In addition, we wanted to study the role of ECFC in long-term complications of BPD one of which is early onset emphysema [4]. Furthermore, we attempted to explore better therapies for BPD and alternatives to whole cell therapy. For this, we studied the effect of MSC CdM on hyperoxic fetal lung ECFCs and isolated exosomes from hUCB ECFC CdM. Our research suggests that there are several opportunities for better stem cell therapies in BPD that may not only help to decrease the burden of disease in neonatal period but also help in avoiding the long-term complications of this disease. Further research in this area will hopefully result in effective therapeutic strategies and improve survival and quality of life of babies with BPD.

5.4. Future directions

The findings of our study open up new and exciting avenues for exploration. It will be interesting to assess the function of ECFCs in emphysema and study the effect of administration of ECFCs, ECFC CdM and exosomes in emphysema.

In addition, as we have limitation to studying the effect of hyperoxia on ECFCs *in vivo* in adult human and fetuses, it will be worthwhile to study the effects of hyperoxia on ECFCs in animal models. It will also be beneficial to characterize ECFC CdM and study the contents of it that exert beneficial effect. Also, it will be useful to assess the therapeutic role of exosomes in animal model of BPD. Furthermore, it will be valuable to explore the mechanisms by which ECFCs, their CdM and exosomes act.

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