Existence, Functional Impairment and Lung Repair Potential of Endothelial Colony Forming Cells in Oxygen-Induced Arrested Alveolar Growth

Running title: Alphonse et al.; Endothelial colony forming cells for lung repair

Rajesh S. Alphonse, MD, PhD\textsuperscript{1}; Arul Vadivel, PhD\textsuperscript{2}; Moses Fung, BSc\textsuperscript{1}; William Chris Shelley, BSc\textsuperscript{3}; Paul John Critser, MD, PhD\textsuperscript{3}; Lavinia Ionescu, PhD\textsuperscript{1}; Megan O’Reilly, PhD\textsuperscript{1}; Robin K. Ohls, MD\textsuperscript{4}; Suzanne McConaghy, MSc\textsuperscript{4}; Farah Eaton, BSc\textsuperscript{1}; Shumei Zhong, MSc\textsuperscript{2}; Merv Yoder, MD\textsuperscript{3}; Bernard Thébaud, MD, PhD\textsuperscript{2}

\textsuperscript{1}Dept of Pediatrics, Women and Children’s Health Research Institute, Cardiovascular Research Center and Pulmonary Research Group, University of Alberta, Edmonton, Canada; \textsuperscript{2}Ottawa Hospital Research Institute, Regenerative Medicine Program, Sprott Center for Stem Cell Research, Dept of Pediatrics, Children’s Hospital of Eastern Ontario, University of Ottawa, Ottawa, Ontario, Canada; \textsuperscript{3}Dept of Pediatrics, Herman B Wells Center for Pediatrics Research, Division of Neonatal-Perinatal Medicine, Indiana University School of Medicine, Indianapolis, IN; \textsuperscript{4}Dept of Pediatrics, University of New Mexico, Albuquerque, NM

Address for Correspondence:
Bernard Thébaud, MD, PhD
Ottawa Hospital Research Institute
501 Smyth Road
Ottawa, ON K1H 8L6, Canada
Tel: 613-737-8899
Fax: 613 739-6294
E-mail: bthebaud@ohri

Journal Subject Codes: Basic science research:[129] Angiogenesis, Vascular biology:[97] Other vascular biology
Abstract

Background—Bronchopulmonary dysplasia (BPD) and emphysema are life-threatening diseases resulting from impaired alveolar development or alveolar destruction. Both conditions lack effective therapies. Angiogenic growth factors promote alveolar growth and contribute to alveolar maintenance. Endothelial colony forming cells (ECFCs) represent a subset of circulating and resident endothelial cells capable of self-renewal and de novo vessel formation. We hypothesized that resident ECFCs exist in the developing lung, are impaired during arrested alveolar growth in experimental BPD and that exogenous ECFCs restore disrupted alveolar growth.

Methods and Results—Human fetal and neonatal rat lungs contain ECFCs with robust proliferative potential, secondary colony formation upon replating, and de novo blood vessel formation in vivo when transplanted into immunodeficient mice. In contrast, human fetal lung ECFCs exposed to hyperoxia in vitro and neonatal rat ECFCs isolated from hyperoxic – alveolar growth arrested rat lungs mimicking BPD – proliferated less, showed decreased clonogenic capacity and formed fewer capillary-like networks. Intra-jugular administration of human cord blood-derived ECFCs after established arrested alveolar growth restored lung function, alveolar and lung vascular growth and attenuated pulmonary hypertension. Lung ECFC colony- and capillary-like network forming capabilities were also restored. Low ECFC engraftment and the protective effect of cell-free ECFC-derived conditioned media suggest a paracrine effect. Long-term (10 months) assessment of ECFC therapy showed no adverse effects with persistent improvement in lung structure, exercise capacity, and pulmonary hypertension.

Conclusions—Impaired ECFC function may contribute to arrested alveolar growth. Cord blood-derived ECFC therapy may offer new therapeutic options for lung diseases characterized by alveolar damage.

Key words: angiogenesis, oxygen, endothelial cell, lung, injury, stem cell, endothelial progenitor cell
Despite improvements in the ability to control fertility, treat infertility, and reduce maternal, fetal, and infant mortality, neither the preterm birth rate nor the rate of long-term disabilities associated with extreme preterm birth has declined. Preterm delivery remains a major health care problem, affecting 10% of all births and accounting for more than 85% of all perinatal complications and death. Each year, 5000-10,000 newborns suffer from bronchopulmonary dysplasia (BPD), a chronic lung disease that follows ventilator and oxygen therapy for acute respiratory failure after preterm birth. BPD is characterized by interrupted development of alveolar structures and can be complicated by pulmonary hypertension. BPD has long-term consequences that extend into adulthood, including early onset emphysema, and currently lacks specific therapeutic options. Understanding how alveoli and the underlying capillary network develop and how these mechanisms are disrupted in disease states are critical for developing effective therapies for lung regeneration.

Emerging evidence indicates that endothelial-derived angiocrine signals induce and sustain regenerative lung alveolarization. Vascular Endothelial Growth Factor (VEGF) is absolutely critical for vascular development and embryonic survival. VEGF signaling contributes to normal lung development and early disruption of VEGF signaling leads to structural abnormalities seen in experimental and human BPD and emphysema. Angiogenic growth factor activation protects against O2-induced arrested alveolarization and stimulates lung vascular growth in newborn rats. Compensatory lung growth following unilateral pneumonectomy stimulates pulmonary capillary endothelial cells to produce angiocrine growth factors that induce proliferation of epithelial progenitor cells to support alveolarization.

Recent observations suggest that the lung microvascular endothelium is enriched with resident progenitor cells that exhibit vasculogenic capacity. These highly proliferative cells
resemble the endothelial colony forming cells (ECFCs) described in human peripheral and umbilical cord blood\textsuperscript{10}. The role of lung ECFCs during normal and impaired lung growth is unknown.

We hypothesized that ECFC-driven angiogenesis contributes to normal lung alveolar development and ECFC supplementation restores disrupted lung alveolar and vascular growth in hyperoxia-induced experimental BPD.

**Materials and Methods**

Expanded methods are available in the Supplement. All procedures were approved by the Animal Health Care Committee of the University of Alberta. Human fetal tissue collection was reviewed by the Institutional Review Board at the University of New Mexico.

**Lung ECFC isolation and culture**

Human fetal lungs (n=3, 17-20 weeks gestational age) were collected within 60 minutes after termination, washed in sterile PBS with antimicrobials, and suspended for further processing in a-MEM with 10\% FCS and antimicrobials. Rat lungs were collected at postnatal day (P)14 (n=5/group). Under aseptic conditions, the peripheral rims of the lungs were cut out, chopped into 1-2 mm\textsuperscript{2} pieces and suspended in digestive solution (0.1 U collagenase and 0.8 U dispase/mL) (Roche Applied Science, Laval, QC) at 37 °C for 1 hr with intermittent shaking. The lung digest was strained through 70mm and 40mm cell strainers in tandem and washed twice with DMEM plus 10\% fetal calf serum (FCS), at 300g and 4 °C for 10 min. After washing, the cells were resuspended in phosphate buffered saline (PBS) containing 0.1\% (w/v) bovine serum albumin (BSA) and incubated with streptavidin tagged dynabeads (Dynal, Invitrogen, Burlington, ON) that were pretreated with biotinylated anti-rat or anti-human CD31 antibody (Abcam, Cambridge, MA). The dynabead tagged CD31 positive cells were selected using a
magnetic separator and plated in a 6-well plate (4000-5000 cells/well) precoated with rat tail collagen type I and placed in a 37 °C, 5% CO2 humidified incubator. After 24 hrs of culture, non-adherent cells and debris were aspirated, adherent cells were washed once and added with complete Endothelial Growth Medium-2 (cEGM-2). Medium was changed daily for 7 days and then every other day up to 14 days. ECFC colonies appeared as a well-circumscribed monolayer of cobblestone-appearing cells, between 5 and 14 days. ECFC colonies were identified daily from day 5 and enumerated on day 7 by visual inspection using an inverted microscope (Olympus, Lake Success, NY), under 20X magnification. Individual ECFC colonies were marked with a fine tipped marker and clonally isolated using cloning cylinders (Fisher Scientific, Ottawa, ON) and plated in T25 flasks pretreated with collagen type I. Upon confluence, ECFCs were plated and expanded in type I collagen coated T75 flasks. ECFCs between passages 4-8 were used for all experiments.

**DiI-acetylated-low density lipoprotein (DiI-Ac-LDL) uptake and Ulex europaeus-lectin binding**

Experiments were performed as previously described\(^\text{10}\).

**Immunophenotyping of ECFCs**

ECFCs were phenotyped by flow cytometry analysis\(^\text{10}\).

**Retroviral mediated eGFP labelling of ECFCs**

Rat lung ECFCs in passage 4-5 were incubated overnight with 2 x 10^6 TU/ml of lentiviral vector and 7 μg/ml protamine sulfate in cEGM-2. ECFCs in wells with uniform green fluorescence were trypsinized, expanded and sorted for green fluorescence protein using FACS.

**In Vitro Cell Viability Assay**

ECFC viability was evaluated at various time points using the MTT assay as previously...
described\textsuperscript{11}.

**Capillary-like network formation in Matrigel**

The formation of cord-like structures by ECFCs was assessed on Matrigel (BD Biosciences, Mississauga, ON) coated 96-well tissue culture plates as previously described\textsuperscript{7}.

**Single cell clonogenicity**

The FACSAria cell sorter (BD Biosciences, Mississauga, ON) was used to place one ECFC per well in a flat-bottomed 96-well tissue culture plate precoated with type I collagen and containing 200 \( \mu \)L complete EGM-2 media. At day 14, Hoechst 33258 (Sigma) was added at 3 \( \mu \)g/mL to each well for 10 min for nuclear detection. The culture plate was examined with a fluorescent microscope at 20X magnification, well by well, for the growth of endothelial cells.

**De novo angiogenesis in vivo**

ECFCs were loaded on collagen-fibronectin matrices and implanted subcutaneously in NOD/SCID mice to assess their capacity to contribute to \textit{de novo} vasculogenesis as described\textsuperscript{12}.

**Oxygen-Induced BPD Model**

We used three rodent models of oxygen-induced BPD: 1) For comparison of lung ECFC function, newborn Sprague Dawley (SD) rat pups were exposed to room air (21%; control group) or hyperoxia (95% oxygen; BPD group) from birth to P14 in sealed Plexiglas chambers (OxyCycler; BioSpherix, Lacona, NY) with continuous oxygen monitoring and ECFCs were isolated at P14 for comparative analysis\textsuperscript{7}; 2) To test the therapeutic potential of human cord blood-derived ECFCs, immune-compromised newborn \( \text{rag}^{-/-} \) mice were exposed to 85% oxygen from P4 to P14. Human cord-derived ECFCs, isolated, expanded, and quality controlled as previously described\textsuperscript{10} were administered at P14 through the jugular vein (10\(^5\) cells/mouse in 100 \( \mu \)L DMEM). Lungs were harvested at P28. For cell engraftment experiments, ECFCs were
labeled prior to injection with a red fluorophore (CellBrite\textsuperscript{TM} Cytoplasmic Membrane Staining Kit, Biotium Inc, Hayward, CA). A subset of rag\textsuperscript{-/-} mice was kept for long-term assessment until 10 months of age; 3) In subsequent experiments, we used RNU nude rats (exposed to 95% oxygen from P4 to P14 and injected with 2.5x10\textsuperscript{5} cells/rat in 100 \(\mu\)L DMEM) to test if human cord-derived ECFC treatment restored resident lung ECFC function.

**Lung Morphometry**

Lungs were fixed and alveolar structures were quantified on a motorized microscope stage (Leica CTRMIC and openlab software, Quorum Technologies; Guelph, ON) by using the mean linear intercept as previously described\textsuperscript{7}.

**Immunohistochemistry**

von Willebrand Factor (vWF) positive lung capillaries (30-100\(\mu\)m) were quantified on a motorized microscope stage.

**Right Ventricular Hypertrophy**

The right ventricle free wall was separated from the left ventricle and the septal wall. The tissue was dried overnight and weighed the following day\textsuperscript{13}.

**Echocardiography**

All evaluations of pulmonary artery flow were performed with a (maximal) sweep speed of 200 mm/s. Pulsed-wave Doppler of pulmonary outflow was recorded in the parasternal view at the pulmonary valve level. The pulmonary acceleration time (PAAT) was measured from the beginning of the pulmonary flow to its onset and normalized with heart rate for comparisons as described\textsuperscript{13}.

**Lung function testing**

Tests were performed on anesthetized and paralyzed animals using Flexivent (Scireq, Montreal,
QC, Canada) as described.  

**Real-time PCR**

Real-time PCR was performed on frozen lungs from 3 animals/group harvested at various time points after injection as described.

**ECFC-derived Conditioned Media (CdM) experiments**

CdM was obtained as described. Upon preparation, CdM was pooled, frozen at -80°C and thawed right before use. Control cell CdM was obtained from HUVECs.

*In vitro*, AT2 were isolated from time-dated fetal day 19.5 rat lungs as described using serial differential adhesions to plastic and low-speed centrifugations. For wound healing assays, a cell monolayer was scraped with a p200 pipette tip and media replaced with CdM or DMEM as described.

*In vivo*, CdM was administered daily to newborn rats exposed to hyperoxia through intraperitoneal injections at the dose of 7 μl/g from P4 to P21 and lungs were harvested at P22.

**Exercise capacity**

Mice were run according to a predetermined protocol.

**Statistical Analysis**

Values are expressed as means ± standard error of the mean (SEM). Statistical comparisons were made with one-way ANOVA. Time course studies were analyzed using repeated measures ANOVA. *Post hoc* analysis used Fisher’s probable least significant difference test (Statview 5.1; Abacus Concepts, Berkeley, CA). A value of P less than 0.05 was considered statistically significant. All P values were two-sided and no adjustment for multiple comparisons was made. All endpoints were assessed by investigators blinded to the experimental groups.
Results

Human fetal lung harbors ECFCs with self-renewal, high proliferative potential and de novo blood vessel formation capacity

CD31 positive selected cells isolated from human fetal lung tissue yielded cobblestone-like colonies between 4 to 14 days in culture (Figure 1A). These ‘late-outgrowth’ colonies demonstrated basic endothelial cell characteristics such as ingestion of Dil-acLDL, binding Ulex europaeus-lectin (Figure 1B) and tube formation in Matrigel (Figure 1C). Further phenotypic characterization revealed expression of endothelial-specific cell surface markers including CD31, CD105 (endoglin), CD144 (VE-cadherin) and CD146 (M-CAM), (Figure 1D). Human ECFCs were negative for monocyte/macrophage specific CD14 and hematopoietic cell specific CD45 (Figure 1D) and thus, display an ECFC-like phenotype, consistent with the phenotype of circulating human cord blood ECFC\(^1\).

In single cell clonogenic assays, lung endothelial cells generated colonies from single plated cells: 6.0 ± 1.0 % formed colonies with 50-500 cells (low proliferative potential, LPP) and 7.6 ± 2.8 % formed more than 500 cells (high proliferative potential, HPP) in 3 independent experiments (Figure 1E). Upon replating, ECFCs generated secondary colonies with similar frequency (6.0 ± 1.0 % colonies with 50-500 cells and 8.0 ± 2.0 % formed more than 500 cells) in 3 independent experiments (Figure 1F). Thus, the human lung endothelial cells displayed the clonal self-replenishing capacity.

In collagen-fibronectin loaded matrices implanted subcutaneously in NOD/SCID mice, human lung ECFCs formed de novo capillaries identified by antibody specific for human-CD31 that connected with the host vasculature as demonstrated by the presence of circulating red blood cells in the capillary lumen (Figure 1G). Together these observations confirm the existence of
resident ECFCs with properties similar to cord blood ECFC in the developing human lung.

**Hyperoxia impairs human lung ECFC function**

Hyperoxia is one deleterious factor contributing to BPD. In order to mimic the disease condition *in vitro*, we exposed human lung ECFCs to 40% hyperoxia for 24 hours. ECFCs exposed to hyperoxia formed fewer capillary-like structures in matrigel compared to ECFCs exposed to room air (Figure 1H) and generated fewer HPP ECFC colonies compared to ECFCs cultured in room air (Figure 1I). These results are consistent with prior studies reporting disrupted VEGF-nitric oxide signaling in cord blood ECFC exposed to hyperoxia *in vitro* and suggests impaired ECFC function may be associated with decreased lung vascular growth and arrested alveolarization in BPD.

**Lung ECFC function is impaired in experimental hyperoxia-induced BPD in newborn rats**

To further investigate this hypothesis, we explored lung ECFC function in a well-established hyperoxia-induced neonatal rat model of arrested alveolar growth mimicking some of the histological features seen in human BPD.

First, we show that similar to the human fetal lung, the neonatal rat lung harbors ECFCs that form cobble stone-like colonies within 7-14 days, with basic endothelial cell characteristics (Dil-acLDL ingestion and *Ulex europaeus*-lectin binding) and tube-like formation in Matrigel (Figure 2A-C). The lung ECFC also displayed endothelial-specific cell surface markers (CD31, VEGFR2 and vWF) but did not express hematopoietic cell specific CD45, CD14 or CD133 (Figure 2D). In single cell clonogenic assays, rat ECFCs generated LPP and HPP colonies from single plated cells and secondary HPP upon replating (Figure 2E).

Rat lung ECFCs also formed *de novo* capillaries when implanted subcutaneously in NOD/SCID mice in collagen-fibronectin loaded matrices (Figure 2F).
Next we compared the function of ECFCs extracted from a neonatal rat model mimicking BPD. Following 2 weeks of exposure of neonatal rats to hyperoxia (95% O2), lungs of newborn rats develop a consistent and irreversible histological pattern of alveolar simplification and vascular paucity, characteristic of human BPD (Figure 3A).

When plated at equal densities and cultured in identical conditions, rat lung ECFCs from the hyperoxia-exposed rats showed reduced cell growth (Figure 3B).

ECFCs from hyperoxia-exposed rat lungs formed fewer cord-like endothelial networks compared to controls as assessed by total cord length by 32.7 ± 11.8% (P < 0.05) and number of intersects by 48.2 ± 5.3% (P<0.05) (Figure 3C).

In single cell clonogenic assays, 23.6 ± 2.5% of single plated ECFCs from the control group vs only 9.3 ± 3.2% of ECFCs form the hyperoxia group were capable of forming HPP colonies containing more than 500 cells each (P < 0.05, Figure 3D). No significant difference between the groups was observed in the percentage of cells that formed LPP colonies containing fewer than 50 cells or 50 to 500 cells.

Together, the above findings suggest a functional deficiency in the ECFCs isolated from lungs of hyperoxia-exposed newborn rats. Since angiogenesis contributes to normal alveolarization and because oxygen-induced arrested alveolarization is associated with decreased ECFC function, we investigated the therapeutic potential of human cord blood-derived ECFCs to restore normal alveolarization in this model.

**Human cord blood-derived ECFCs reverse alveolar growth arrest, preserve lung vascularity, and attenuate pulmonary hypertension in hyperoxia-induced BPD in newborn rodents**

Exposure of newborn rag^{-/-} mice to hyperoxia (85% O2) from P4 to P14 (Figure 4A) increased
lungs (Figure 4B) and induced a histological pattern reminiscent of human BPD, characterized by fewer, enlarged alveolar structures (MLI 41±2.3 vs 25.6±1.9 in room air-controls, P<0.05, Figure 4C-D). Intra-jugular administration of cord blood-derived ECFCs at P14 after established alveolar growth arrest, significantly improved lung compliance (Figure 4B) and alveolar architecture (MLI 30.3±1.6, P<0.05) (Figure 4C-D). ECFCs had no adverse effect on lung function and structure in room air-housed control animals.

Hyperoxia-exposed mice had decreased lung vascular growth (mean number of blood vessels/high power field 3.5±0.3 vs 2.2±0.4, Figure 5A-B) and exhibited pulmonary hypertension as assessed by echo-doppler (PAAT 11.4±0.4 vs 14.9±1.6 in room air-controls, P<0.05) and RVH (RV/LV+S ratio 0.35±0.05 vs 0.22±0.06 in room air-controls, P<0.01, Figure 5C). ECFC therapy restored PAAT almost to control levels (14.1±2) and significantly reduced RVH (RV/LV+S ratio 0.26±0.03, Figure 5D).

Similarly, ECFC therapy restored normal alveolar architecture (Figure S-1A), lung vascular growth (Figure S-1B) in newborn RNU rats that were exposed to 95% hyperoxia from P4-P14 (immunocompromised rat BPD model). In addition, ECFC therapy significantly attenuated RVH in hyperoxia-exposed RNU rats (Figure S-1D).

**ECFC engraftment and contribution to lung neovascularization**

Quantification of human ECFCs using qRT-PCR showed a low rate of engraftment in recipient lungs with a drastic decrease in detected human Alu sequences from the first day after injection to almost undetectable levels within 3 days (Figure 6A). Fourteen days after fluorescent-labeled ECFC administration, very few cells were found in the lung (Figure 6B). However, when lung ECFCs were isolated from RNU rats and tested for clonogenic capacity, resident lung ECFCs from the treated hyperoxia group were capable of forming significantly more HPP colonies.
compared to the untreated hyperoxia-exposed group (P<0.05, Figure S-2A). In addition, lung ECFCs from the treated animals formed more-extensive endothelial networks than the untreated animals in the hyperoxia group as assessed by cord length and number of intersects (P<0.05, Figure S-2B).

**Paracrine effect of human cord blood-derived ECFCs**

Given the low engraftment of ECFCs at the doses administered in these studies and evidence that some EPCs secrete factors that promote tissue repair\(^{18}\), we hypothesized that ECFCs may also exert a paracrine activity that mediates the reparative response in the lung. To verify this, we assessed the effect of cell free human cord blood derived-ECFC CdM *in vitro* and *in vivo*.

*In vitro*, we assessed the protective effects of ECFC-CdM using two experimental models representing the epithelial and endothelial compartments of lung alveoli. First, confluent monolayers of AT2 cells were subject to a pipette tip inflicted scratch injury in the presence of DMEM, HUVEC-CdM or ECFC-CdM. At 12 hours, AT2 cell wound closure was significantly higher with ECFC-CdM compared to DMEM and HUVEC-CdM (Figure 7A, B). Second, human fetal lung ECFCs were suspended in serum-free matrigel overlaid with DMEM, HUVEC-CdM or ECFC-CdM and incubated in room air or 95% oxygen. At 6 hrs, hyperoxia significantly decreased endothelial cord-like structure formation in DMEM and HUVEC-CdM, whereas ECFC-CdM significantly preserved ECFC network formation (Figure 7C, D).

*In vivo*, ECFC-CdM was administered as daily intra-peritoneal injections from P4 to P21 to neonatal rats exposed to hyperoxia. CdM therapy preserved alveolar (Figure. 7E, F), lung vascular growth (Figure 7G) and attenuated pulmonary hypertension as assessed by the PAAT and right ventricular hypertrophy (Fig. 7H, I). ECFC-CdM had no adverse effects on lung structure and lung vascularity in room air control animals.
Long-term effects of ECFC therapy

Intra-jugular delivery of ECFCs at P14 appeared safe and efficient up to 10 months of life. Airspaces remained enlarged (Figure 8A, B) and exercise capacity (Figure 8C) and PAAT (Figure 8D) remained perturbed in hyperoxic-exposed animals compared to room air controls. In contrast, hyperoxic-exposed neonatal mice that had received ECFCs in the neonatal period exhibited preserved alveolar architecture (Figure 8A, B), significantly improved exercise capacity (Figure 8C) and PAAT (Figure 8D) at 10 months of age. Control room air housed animals treated with ECFCs showed no alterations in any of these parameters. Other organs including the brain, heart, liver, kidney and spleen had no signs of impaired architecture (data not shown).

Discussion

Here we show that the developing human and rat lung harbors resident ECFCs capable of self-renewal and de novo vessel formation in vivo. We also provide evidence that hyperoxia perturbs human and rat lung ECFC function in vitro and in vivo in experimental oxygen-induced BPD respectively. Furthermore, exogenous, human cord blood-derived ECFCs restore normal lung alveolar and vascular growth, function, diminishes pulmonary hypertension and restores resident lung ECFC function in this model compared to control animals. This effect is mediated through a paracrine effect. The therapeutic benefit persisted at 10 months with no adverse effects on lung structure and exercise capacity in hyperxia-exposed animals.

Increasing evidence suggest that blood vessels contribute to normal lung growth as opposed to passively following the development of the airways5. This has led to the current working hypothesis that preservation of vascular growth and endothelial survival promotes
alveolar growth and sustains the architecture of the alveoli. The consistent association between arrested alveolar development and impaired capillary formation in human and experimental BPD and the demonstration that angiogenic growth factors promote normal alveolar development and repair further supports this. Thus, if angiogenic growth factors and adequate lung vascularization contribute to lung development and integrity, then circulating or resident ECFCs are appealing candidate cells likely to be involved in the same mechanisms. The isolation of a circulating cell that gives rise to cells appearing endothelial-like in vitro and with the potential to incorporate at sites of neoangiogenesis in vivo by Asahara et al challenged the paradigm that vasculogenesis is a process restricted to embryonic development and opened exciting new therapeutic avenues. A current limitation is the ability to define an EPC based on a set of markers that can unambiguously identify this cell type. To date, at least three different methodologies exist for the isolation of EPCs. Schematically, EPCs appear to represent two distinct cell populations- the minimally proliferative but proangiogenic hematopoietic or macrophage-like phenotype (that include circulating angiogenic cells and colony forming unit-Hill colonies) and the highly proliferative non-hematopoietic phenotype (ECFCs). Based on their timing of emergence in culture, these populations have also been described as early and late outgrowth EPCs, respectively. ECFCs, which are phenotypically indistinguishable from cultured endothelial cells, demonstrate high proliferative potential, capacity for self-renewal and de novo vessel formation. ECFCs reside throughout the vascular endothelium contributing to vascular integrity and are mobilized into a circulating pool of endothelial progenitors, putatively involved in neovasculogenesis via tissue recruitment and homing. Recently, Alvarez et al described a population of resident pulmonary microvascular EPCs in adult rat lungs that display a high proliferative potential and capable of de novo angiogenesis in vivo. These cells resemble the
ECFCs previously isolated from the peripheral circulation and in human umbilical cord blood. Similar cells have now been described in the mouse\textsuperscript{23} pulmonary circulation. This suggests that ECFCs could be the primary progenitor population participating in neovasculogenesis in the lung. The existence of these resident lung ECFCs in the developing lung and their role in arrested alveolar growth in BPD is unknown. We reasoned that ECFCs dysfunction could underlie the pathogenesis of diseases such as BPD, characterized by disrupted vascular growth.

We adopted a procedure based on the already existing protocol for recovering circulating ECFCs from bone marrow or peripheral blood\textsuperscript{10} to demonstrate the presence of resident ECFCs in fetal human and neonatal rat lung. These cells displayed the stringent characteristics of ECFCs including (i) the ability to generate colonies with 500 or more daughter cells derived from cultured single cells and (ii) some of the clonogenic cells are capable of self-renewal, and (iii) the capacity to form vessels \textit{de novo}, when impregnated in collagen-fibronectin gels and implanted subcutaneously in recipient immunodeficient mice.

Given the importance of angiogenesis in normal alveolar growth, we hypothesized that ECFC function would be impaired in disease conditions associated with arrested alveolar growth such as BPD. Earlier observations reported on the susceptibility of cord blood derived-ECFCs to oxidant-damage\textsuperscript{24}. Furthermore, cord blood-derived ECFCs are lower in number in preterm infants who subsequently develop BPD versus those who do not develop BPD\textsuperscript{25,26}. Here we show that resident human lung ECFCs exposed to hyperoxia \textit{in vitro} and rat ECFCs isolated from the lung of a hyperoxia-induced BPD model that display arrested alveolar growth were significantly hampered in their ability to proliferate, form vascular networks \textit{in vitro} and generate colonies with HPP compared to ECFCs isolated from control lungs. While there are no specific cell surface markers to identify the resident ECFC within the vascular endothelial intima \textit{in vivo},
it is interesting that endothelial replacement or expansion within the aorta has long been known to emerge within clusters of endothelial cells rather than a dispersed pattern during normal embryonic growth or in animals with hypertension\textsuperscript{27,28}. Confirmation of the ECFC within the vascular endothelial intima will require further work to identify a unique and specific marker.

These data formed the rationale for testing the therapeutic potential of umbilical cord blood-derived ECFC supplementation in experimental BPD. Here we provide first evidence for the therapeutic benefit of ECFCs in restoring lung vascular and alveolar growth as well as lung function in an established model of BPD. ECFCs also attenuated pulmonary hypertension, a complication commonly associated with severe BPD. In addition, treatment with cord blood-derived ECFCs restored colony forming and capillary-like network forming capabilities of resident lung ECFCs of newborn rats with experimental BPD. These data are consistent with the beneficial effect of angiogenic factors in experimental BPD\textsuperscript{29-31} and underscore the therapeutic benefit of promoting lung angiogenesis to repair the lung. Altogether, these observations suggest an increased susceptibility of ECFCs in the developing lung to hyperoxia that may contribute to impaired lung vascular and alveolar growth in BPD and that ECFC supplementation represents a promising strategy for lung repair.

In this study, we have employed ECFCs derived from human cord-blood to document the therapeutic effect of these cells in a model of impaired lung development. This clinically relevant source of ECFCs appears particularly appealing for treating neonatal diseases. In order to test ECFCs of human origin we used immunodeficient rag-/- mice, in which hyperoxia-mediated lung injury appeared less pronounced. Practical constraints in obtaining ECFCs from the rat cord blood limited us from using the more robust model of in newborn Sprague-Dawley rats. Despite this limitation, the demonstrated findings are consistent and convincing with regard to the
therapeutic effect of ECFCs in BPD.

In this study, we administered ECFCs after established arrested alveolar growth and after hyperoxic exposure. The rationale was based on previous in vitro studies suggesting that hyperoxia impairs cord blood-derived ECFC function\textsuperscript{24,32}.

Lung injury in neonatal mice exposed to hyperoxia is also associated with decreased early outgrowth EPCs\textsuperscript{33} and can be overcome by bone marrow-derived angiogenic cell infusion\textsuperscript{34}. Further evidence for the role of EPCs in lung repair is provided by experimental and clinical data showing that (i) bone-marrow derived EPC attenuate lipopolysaccharide (LPS)-induced acute lung injury\textsuperscript{35} and elastase-induced emphysema\textsuperscript{36,37}, (ii) EPCs are higher in patients with acute lung injury compared to healthy control subjects\textsuperscript{38}, and (iii) the increase in circulating EPCs correlates with improved outcome and illness severity and improved survival in acute\textsuperscript{39,38} and chronic\textsuperscript{40} lung disease, suggesting a prognostic role similar to ischemic heart disease.

Very few engrafted cells were detected by immunofluorescence and analysis of human-specific Alu sequences. We did not pursue a dose dependent study to find a concentration of ECFC that engrafted into regenerating vasculature in this model. Others have noted that human ECFC can form up to 27\% of the vascular endothelium in recovering ischemic muscle tissue of nude mice subjected to a hindlimb ischemic injury\textsuperscript{41}. Additional work may determine that higher doses of administered human cells may lead to sustained engraftment. Alternatively, this observation may suggest that, similar to other cell-based strategies including early outgrowth EPCs and mesenchymal stromal cell therapy, ECFCs exert their therapeutic benefit mainly through a paracrine activity. This paracrine activity is supported by the protective effect of cell-free ECFC-derived CdM on alveolar epithelial cell wound healing and human lung ECFC vascular network formation in vitro. As well, in vivo, ECFC-derived CdM restored alveolar and
lung vascular growth comparable to whole cell therapy.

In our in vivo studies, CdM was administered as daily intraperitoneal injections from P4-21 with the reasoning that repeated dispensation would be necessary to ensure continuous availability of protective factors. With cell therapy, on the other hand, we expected a more sustained release of protective factors from the transfused cells even after a single intravenous dose. However, recent observations suggest that a single injection of CdM may be sufficient to prevent oxygen-induced lung injury in neonatal mice\textsuperscript{1,2}. The biologically active component of mesenchymal stromal cell-derived CdM seems to be contained in exosomes, membrane derived nanoparticles, as recently demonstrated in a hypoxia-induced model of pulmonary hypertension\textsuperscript{42}. It is likely that ECFCs, as many other cells, produce exosomes. Whether these exosomes can be used as biomarker or therapeutic tool remains to be studied.

A concern with cell-based therapy, especially in the neonate, is the risk of long-term adverse effects including tumor formation. Here we show that the therapeutic benefit in the ECFC-treated mice was still present at 10 months of age without adverse effects on lung structure or exercise capacity. No tumors were detectable by examining serial histological sections of major organs including the lung, brain, heart, liver, kidney and spleen at 10 months post-ECFC injection.

In conclusion, we show for the first time that ECFCs exist in the distal vasculature of the developing mammalian lung and their functional capacity is impaired in oxygen-induced lung damage. We also show that therapeutic supplementation with human umbilical cord blood derived-ECFCs is feasible, efficacious and apparently safe in this experimental O\textsubscript{2}-induced model of BPD in neonatal mice.
**Funding Sources:** This work was supported by a grant from the Canadian Institutes for Health Research (CIHR, BT) and the Riley Children's Foundation (MY). RSA was the recipient of an Alberta Innovate Health Solutions (AIHS) stipend. BT was supported by a Canada Research Chair and AIHS clinical investigator award and by the Canadian Foundation for Innovation (CFI).

**Conflict of Interest Disclosures:** None.

**References:**


endothelium is enriched with progenitor cells that exhibit vasculogenic capacity. *Am J Physiol

10. Mead LE, Prater D, Yoder MC, Ingram DA. Isolation and characterization of endothelial

the paracrine effect of mesenchymal stem cells in preventing oxygen-induced neonatal lung injury

12. Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT,
Ingram DA. Redefining endothelial progenitor cells via clonal analysis and hematopoietic

alveolar growth and pulmonary hypertension in hyperoxia-induced lung injury. *Am J Respir Crit

Aprahamian TR, Walsh K, Thebaud B. Airway delivery of soluble factors from plastic-adherent

Eaton F, Peault B, Mosca F, Lazzari L, Thebaud B. Short-term, long-term and paracrine effect of
human umbilical cord-derived stem cells in lung injury prevention and repair in experimental

16. van Haaften T, Byrne R, Bonnet S, Rochefort GY, Akabutu J, Bouchentouf M, Rey-Parra GJ,
B. Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung

17. Mund JA, Estes ML, Yoder MC, Ingram DA, Jr., Case J. Flow cytometric identification and
functional characterization of immature and mature circulating endothelial cells. *Arterioscler

Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and

Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis.
*Science.* 1997;275:964-967.

Yang HK, Oh BH, Park YB, Kim HS. Synergistic neovascularization by mixed transplantation of


Figure Legends:

Figure 1. ECFCs exist in the developing human fetal lung and are perturbed by hyperoxia. A. Phase contrast microscopy showing characteristic cobblestone-like colonies of CD31 positive cells obtained by beads isolation. B. These cells demonstrate Dil-acLDL uptake (red) within 4 hrs of incubation and Ulex europaeus-lectin binding (green) within 1 hr of incubation after fixation. Counterstaining with Hoechst 33258 (blue) illustrates that all adherent cells are positive for LDL-uptake and Ulex-lectin binding. C. These cells form tube-like structures when suspended in matrigel. D. Fluorescent activated cell sorting. Isolated cells are positive for endothelial-specific cell surface antigens CD31, CD105 (endoglin), CD144 (VE-cadherin), CD146 (M-CAM), and negative for monocyte/macrophage specific CD14 and hematopoietic cell specific CD45. Filled gray histograms represent antigen staining with negative isotype controls overlaid in white. All experiments were performed in triplicate. E. Single cell clonogenic assay. Single cells are capable of giving rise to clusters (up to 50 cells) or colonies 50-500 cells (low proliferative potential, LPP) or more than 500 cells (high proliferative potential, HPP) in 96-well plates when plated at a seeding density of 1 cell per well. Results represent the mean ± standard error of mean (SEM) of 3 independent experiments. F. Upon replating, HPP ECFCs were able to form clusters or secondary colonies with LPP and HPP. G. Subcutaneous Matrigel Plug Assay. Human fetal lung ECFCs form blood vessels de novo when seeded in fibronectin-collagen plugs (10⁶ ECFCs per implant) and implanted subcutaneously into the flanks of NOD/SCID mice. Fourteen days post implantation, the cellularized implants were excised, paraffin embedded and stained with hematoxylin and eosin (H&E) and anti-human CD31 (brown). Black arrows indicate red blood cell-perfused anti-human CD31+ vessels within the gel implant. H. Hyperoxia
impairs network formation in vitro. Human fetal lung ECFC exposed to 40% hyperoxia in vitro show a significant decrease in number of intersects compared to RA exposed ECFCs (n=5 for each group, *P<0.05). I. Comparative single cell clonogenic assay. Human fetal lung ECFC exposed to 40% hyperoxia in vitro and plated in 96-well plates at a seeding density of 1 cell per well formed clusters and gave rise to colonies with LPP, but formed significantly fewer HPP compared to RA-exposed ECFCs (*P<0.05, 3 independent experiments).

Figure 2. ECFCs exist in the developing rat lung. A. Phase contrast microscopy showing characteristic cobblestone-like colonies of CD31 positive cells obtained by beads isolation. B. These cells demonstrate Dil-acLDL uptake (red) within 4 hrs of incubation and Ulex europaeus-lectin binding (green) within 1 hr of incubation after fixation. Counterstaining with Hoechst 33258 (blue) illustrates that all adherent cells are positive for LDL-uptake and Ulex-lectin binding. C. These cells form tube-like structures when suspended in matrigel. D. Fluorescent activated cell sorting. Isolated endothelial cells are positive for endothelial-specific cell surface antigens- CD31, vWF and VEGFR2 and negative for monocyte/macrophage specific CD14 and hematopoietic cell specific CD45 and CD133. E. Single cell clonogenic assay. Rat lung endothelial cells are capable of giving rise to clusters (up to 50 cells) or colonies 50-500 cells (low proliferative potential, LPP) or more than 500 cells (high proliferative potential, HPP) in 96-well plates when plated at a seeding density of 1 cell per well. Results represent the mean ± standard error of mean (SEM) of 3 independent experiments. Upon replating, HPP ECFCs were able to form clusters or secondary colonies with LPP and HPP. F. Subcutaneous Matrigel Plug Assay. Rat lung ECFCs form blood vessels de novo when seeded on fibronectin-collagen plugs (10^6 ECFCs per implant) and implanted subcutaneously into the flanks of NOD/SCID mice.
Fourteen days post implantation, the cellularized implants were excised, paraffin embedded and stained with hematoxylin and eosin (H&E) and anti-human CD31 (brown). The arrow points to a rat endothelial cell; the rat endothelial cell lines a vessel filled with mouse red blood cells indicating that the rat endothelial cells have inoculated with the mouse circulation. The arrowhead points to a murine endothelial cell lining a mouse blood vessel.

**Figure 3.** Rat lung ECFC function is perturbed in hyperoxia-induced experimental BPD in newborn rats. A. Schematic describing the rat model of hyperoxia-induced BPD. Newborn rats are housed in 95% \( \text{O}_2 \) from portnatal day (P)-4 to P-14 and studied in comparison with room air (RA) raised control rats. Exposure of rat pups to hyperoxia during the alveolar stage of lung development results in arrested alveolar growth characterized by larger and fewer alveolar structures as shown in H&E stained representative lung slides. ECFCs were isolated from RA and hyperoxia-exposed rat lungs on P-14 and their proliferative, clonogenic and vessel forming potentials were assessed. B. MTT Assay. Lung ECFCs form RA and hyperoxic animals were plated at equal cell densities and cultured under identical culture conditions. ECFCs from the hyperoxia-exposed group showed decreased cell growth as assessed by MTT assay (\( n=6 \), \( P<0.05 \) on days 12 and 16, \( P<0.01 \) on day 8 and \( P<0.001 \) on day 20). C. Matrigel assay. Quantitative assessment of the ability to form tube-like structures on Matrigel reveals a significant decrease in total cord length and number of intersects in the hyperoxia-exposed ECFCs compared to RA ECFCs (\( n=6 \) for each group, \( *P<0.05 \)). D. Comparative single cell assay. Colony forming potential of single cell plated rat lung ECFCs was assessed by measuring the percentage of single ECFCs capable of generating colonies after 14 days in culture. Significantly fewer ECFCs from the hyperoxia group are capable of generating colonies with 500 or more cells in comparison.
with RA controls (n=6 lungs/group, *P<0.05).

**Figure 4.** Human umbilical cord ECFC therapy improves lung function and reverses alveolar growth arrest in hyperoxia-exposed newborn mice. A. Experimental design. Newborn rag<sup>/-</sup> mice were housed in room air (RA) or 85% O<sub>2</sub> from postnatal day P-4 to P-14. At P14, mice were treated with intra-jugular injections of human umbilical cord blood-derived ECFCs and housed in room air until assessment of endpoints on P-28. B. Lung function testing. Untreated mice exposed to hyperoxia had significantly increased lung compliance compared to RA and ECFC-treated RA controls (n=4-7, P<0.05). Lung compliance was significantly improved in hyperoxia-exposed mice treated with ECFCs in comparison to untreated hyperoxia-exposed controls (P<0.05). C. Representative H&E–stained lung sections showing larger and fewer alveoli in untreated hyperoxia-exposed (O<sub>2</sub>) lungs as compared to control mice housed in RA at P28. Intra-jugular administration of ECFCs in O<sub>2</sub>-exposed animals preserved alveolar growth. ECFCs did not affect lung structure in control RA mice. D. Quantitative assessment of alveolar architecture by mean linear intercept confirms the protective effect of ECFCs on alveolar growth (n=5/group, *P<0.05).

**Figure 5.** Human umbilical cord ECFCs improve lung angiogenesis and prevents pulmonary hypertension in hyperoxia-exposed newborn mice. A, B. Effects of ECFC treatment on pulmonary vessel density assessed on lung slides stained with von Willebrand Factor (vWF) at P28. Pulmonary vessel density of 30-100μm sized blood vessels per 10 high power fields (40X) was significantly decreased in the lungs of O<sub>2</sub>-exposed animals compared to RA. Intra-jugular injection of ECFCs significantly improved pulmonary vessel density (n=5/group, *P<0.01). C.
Pulmonary arterial acceleration time (PAAT) was significantly decreased in O2-exposed animals compared to controls. Intra-jugular ECFCs restored the PAAT almost to control levels (n=4-6/group, *P<0.01). D. Hyperoxia-exposed mice had significant RVH as indicated by the increase in right ventricle/left ventricle plus septum (RV/LV+S) ratio compared with controls. ECFC therapy significantly reduced RVH (n=4-9/group, *P<0.01).

**Figure 6.** Low Engraftment rate after intra-jugular injection of Human umbilical cord ECFCs. A. Quantitative RT-PCR for human Alu sequences in the lung revealed a rapid decline during the first day after injection. Human DNA became almost undetectable 3 days after injection. B. Representative frozen lung section at P21 depicting rare fluorescent-labeled ECFCs.

**Figure 7.** Paracrine effect of human umbilical cord ECFCs. A. Representative confluent monolayers of freshly isolated rat alveolar type 2 epithelial cells (AT2) damaged using a pipette tip, washed to remove damaged cells, and treated with DMEM, ECFC-derived conditioned medium (CdM) or HUVEC CdM. B. The percent of wound closure showed that ECFC CdM accelerated AT2 wound closure as compared with DMEM and HUVEC CdM (n=6/group, *P<0.05). C. Representative endothelial network formation assay on matrigel of human fetal lung ECFCs treated with DMEM, ECFC-derived conditioned medium (CdM) or HUVEC CdM in room air (RA) and hyperoxia (O2). D. Quantitative assessment of cord-like structure formation shows a significant decrease in the number of intersects in hyperoxia in DMEM and HUVEC CdM treated ECFCs compared to RA exposed ECFC. ECFC CdM preserved the number of intersects in hyperoxia. (N=3-6/group, *P<0.05). E. Representative H&E section showing arrested alveolar growth in O2 exposed newborn rats compared to RA controls. ECFC CdM
significantly preserved alveolar growth. F. Quantitative assessment of the mean linear intercept confirms the protective effect of ECFC CdM on alveolar growth (n=6/group, *P<0.001). G. Mean data showing decreased pulmonary vessel density in O$_2$ exposed newborn rats compared to RA controls as assessed by the number of barium filled pulmonary vessels. ECFC CdM significantly attenuated the loss of pulmonary vessels in hyperoxia (n=5/group, *P<0.01). H. Pulmonary arterial acceleration time (PAAT) was significantly decreased in O$_2$-exposed animals compared to controls. ECFC CdM preserved the PAAT compared to untreated O$_2$-exposed animals (n=5-6/group, *P<0.05).

I. Hyperoxia-exposed rats had significant RVH as indicated by the increase in right ventricle/left ventricle plus septum (RV/LV+S) ratio compared with controls. ECFC CdM significantly reduced RVH compared to untreated O$_2$-exposed animals (n=5-6/group, *P<0.05).

**Figure 8.** Long term safety and efficacy of ECFC cell therapy. A. Representative H&E–stained lung sections at 10 months of age shows persistent alveolar simplification in hyperoxia-exposed animals as compared to lungs from rats housed in room air (RA). Oxygen exposed animals treated with ECFCs have improved lung histology. B. The mean linear intercept confirms arrested alveolar growth in untreated O$_2$-exposed animals compared to RA and RA+ECFC treated animals and preserved alveolar structure with ECFC therapy compared to untreated O$_2$-exposed animals (n=3-6/group, *P<0.05). C. Oxygen exposed animals experienced reduced exercise capacity compared to RA housed animals. Oxygen exposed animals treated with ECFC had improved exercise capacity (n=3-6 animals/group, * P<0.05). D. Oxygen exposed animals had decreased pulmonary arterial acceleration time (PAAT) compared to RA and RA+ECFC animals. Oxygen exposed animals treated with ECFC had improved PAAT compared to
untreated O₂-exposed animals (n=3-6 animals/group, * P<0.05). E. Representative lung section of an ECFC treated mouse lung at 10 months of age showing no presence of CD31 positive structures. In contrast, CD31 staining is abundant in a human adult lung.
Figure 1

**Figure 1A**

Di-Ac-LDL Ulex Hoechst

**Figure 1B**

**Figure 1C**

**Figure 1D**

CD31 CD105 CD144 CD146 CD14 CD45

**Figure 1E**

Generation 1

- 2-50 cells/well
- 50-500 cells/well
- >500 cells/well

(Division on d14)

Mean No. of Colonies

(3 independent trials)

**Figure 1F**

Generation 2

- 2-50 cells/well
- 50-500 cells/well
- >500 cells/well

(Division on d14)

Mean No. of Colonies

(3 independent trials)

**Figure 1G**

- Human Fetal Lung ECFCs
- Subcutaneous implant

**Figure 1H**

Number of Intersections

n=5 for both groups

*P < 0.05 vs. RA

**Figure 1I**

- Implant vascularization

- No. of colonies per 96-well plate

* P < 0.05 vs. RA

3 Independent trials in each group
Figure 2

A. Di-Ac-LDL, Ulex Hoechst

B. vWF

C. VEGFR2

D. CD31, CD45, CD133

E. Generation 1 and Generation 2

F. Rat Lung ECFCs

Implant vascularization

Subcutaneous implant

d0, d14

Mean No. of Colonies

(No of cells per colony/cluster)
Figure 3

- **A**
  - P-0, P-4, P-14: Alveolar stage
  - Hyperoxia (BPD model) or Room air

- **B**
  - MTT Assay
  - Normoxia
  - Hyperoxia
  - Cell Viability (a.u.)
  - n=6 trials for both groups
  - *P<0.05, †P<0.01, §P<0.001 vs. Normoxia

- **C**
  - Cord Length (μm)
  - No of Intersections
  - n=6 for each group
  - *P<0.05 vs. Control

- **D**
  - Colonies per 96-well plate
  - * P < 0.05 vs. Control
  - 3 Independent trials

Figure 4

A. Rag-/- mice hUCB-ECFC (intra-jugular) and endpoints assessed:

- P-0
- P-4
- P-14
- P-28

Hyperoxia (BPD model) or Room air

B. Dynamic Compliance (mL/cm H2O/Kg)

- RA
- RA+ECFC
- O2
- O2+ECFC

*P<0.05 vs all other groups
n=4-7/group

C. Mean Linear Intercept

- RA
- RA+ECFC
- O2
- O2+ECFC

*P<0.05 vs all other groups
n=5/group

D. μm
Figure 5

A. Microscopic images showing mean vessel count/HPF.

B. Bar graph comparing mean vessel count/HPF with RA, RA+ECFC, O₂, O₂+ECFC groups. *P<0.01 vs other groups, n=5/group.

C. Pulmonary Arterial Acceleration Time.

D. Right Ventricular Hypertrophy.

*P<0.01 vs other groups, n=4-9/group.
Figure 6

(A) Alu/18s (control as 1)

(B) 14 days
Figure 7

A

0 hr 9 hr 18 hr

DMEM ECFC-CdM HUVEC-CdM

RA O2

B

% Wound Closure

* P<0.05 vs other groups
n=6/group

DMEM ECFC-CdM HUVEC-CdM

C

DMEM ECFC-CdM HUVEC-CdM

D

* P<0.05; n=3-6/group

Branching-Points

DMEM ECFC-CdM HUVEC-CdM

RA O2
Figure 7, cont’d

Mean Linear intercept

RA

O₂

*P<0.001 vs other groups
n=6/group

RA

O₂

*P<0.01 vs O₂-DMEM
n=5/group

RA

O₂

*p<0.05 vs other groups;
n=5-6/group

RA

O₂

*p<0.05 vs other groups
n=5-6/group

RA

O₂

*p<0.05 vs other groups
n=5-6/group

RA

O₂

*p<0.05 vs other groups
n=5-6/group
Figure 8

Mean Linear intercept

*P < 0.05 vs other groups
n=3-6/group

Exercise Capacity

*P < 0.05 vs O₂
**P < 0.01 vs all RA groups
n=3-6/group

PAAT

*P < 0.05 vs all groups
n=3-6/group

Human Lung  Mouse Lung
Existence, Functional Impairment and Lung Repair Potential of Endothelial Colony Forming Cells in Oxygen-Induced Arrested Alveolar Growth
Rajesh S. Alphonse, Arul Vadivel, Moses Fung, William Chris Shelley, Paul John Critser, Lavinia Ionescu, Megan O'Reilly, Robin K. Ohls, Suzanne McConaghy, Farah Eaton, Shumei Zhong, Merv Yoder and Bernard Thébaud

Circulation. published online April 7, 2014;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2014/04/02/CIRCULATIONAHA.114.009124

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2014/04/02/CIRCULATIONAHA.114.009124.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

Existence, Functional Impairment And Lung Repair Potential of Endothelial Colony Forming Cells In Oxygen-Induced Arrested Alveolar Growth

First Author: Alphonse

Short title: Endothelial colony forming cells for lung repair

Rajesh S Alphonse, MD, PhD, Arul Vadivel, PhD, Moses Fung, BSc, William Chris Shelley, BSc, Paul John Critser, MD, PhD, Lavinia Ionescu, PhD, Megan O’Reilly, PhD, Robin K Ohls, MD, Suzanne McConaghy, MSc, Farah Eaton, BSc, Shumei Zhong, MSc, Merv Yoder, MD, Bernard Thébaud, MD, PhD

1 Department of Pediatrics, Women and Children’s Health Research Institute, Cardiovascular Research Center and Pulmonary Research Group, University of Alberta, Edmonton, Canada

2 Ottawa Hospital Research Institute, Regenerative Medicine Program, Sprott Center for Stem Cell Research, Department of Pediatrics, Children’s Hospital of Eastern Ontario, University of Ottawa, Ottawa, Ontario, Canada

3 Department of Pediatrics, Herman B Wells Center for Pediatrics Research, Division of Neonatal-Perinatal Medicine, Indiana University School of Medicine, Indianapolis, Indiana, USA

4 Department of Pediatrics, University of New Mexico, Albuquerque, New Mexico, USA

Correspondence:
Dr. Bernard Thébaud
Ottawa Hospital Research Institute
501 Smyth Road
Ottawa, ON K1H 8L6, Canada
Phone: 613-737-8899, Fax: 613 739-6294
E-mail: bthebaud@ohri
Materials and Methods

All procedures were approved by the Animal Health Care Committee of the University of Alberta. Human fetal tissue collection was reviewed by the Institutional Review Board at the University of New Mexico and was determined to not constitute human subject research, as no identifying information was collected with tissues that would otherwise be discarded. The investigators collecting the tissues were not involved in the consent process and had no contact with women consenting to the termination procedure.

Lung ECFC isolation and culture

Human fetal lungs (n=3, 17-20 weeks gestational age) were collected within 60 minutes after termination, washed in sterile PBS with antimicrobials, and suspended for further processing in a-MEM with 10% FCS and antimicrobials. Rat lungs were collected at postnatal day (P)14 (n=5/group). Under aseptic conditions, the peripheral rims of the lungs were cut out, chopped into 1-2 mm$^2$ pieces and suspended in digestive solution (0.1 U collagenase and 0.8 U dispase/mL) (Roche Applied Science, Laval, QC) at 37 °C for 1 hr with intermittent shaking. The lung digest was strained through 70mm and 40mm cell strainers in tandem and washed twice with DMEM plus 10% fetal calf serum (FCS), at 300g and 4 °C for 10 min. After washing, the cells were resuspended in phosphate buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin (BSA) and incubated with streptavidin tagged dynabeads (Dynal, Invitrogen, Burlington, ON) that were pretreated with biotinylated anti-rat or anti-human CD31 antibody (Abcam, Cambridge, MA). The dynabead tagged CD31 positive cells were selected using a magnetic separator and plated in a 6-well plate (4000-5000 cells/well) precoated with rat tail collagen type I and placed in a 37 °C, 5% CO2 humidified incubator. After 24 hrs of culture, non-adherent cells and debris were aspirated, adherent cells were washed once and added with complete Endothelial Growth Medium-2 (cEGM-2). Medium was changed daily for 7 days and then every other day up to 14
days. ECFC colonies appeared as a well-circumscribed monolayer of cobblestone-appearing cells, between 5 and 14 days. ECFC colonies were identified daily from day 5 and enumerated on day 7 by visual inspection using an inverted microscope (Olympus, Lake Success, NY), under 20X magnification. Individual ECFC colonies were marked with a fine tipped marker and clonally isolated using cloning cylinders (Fisher Scientific, Ottawa, ON) and plated in T25 flasks pretreated with collagen type I. Upon confluence, ECFCs were plated and expanded in type I collagen coated T75 flasks. ECFCs between passages 4-8 were used for all experiments.

**Dil-acetylated-low density lipoprotein (Dil-Ac-LDL) uptake and Ulex europaeus-lectin binding**

Attached cells at passage 4-5 were incubated with 20 µg/mL Dil-Ac-LDL (Biomedical technologies, Stoughton, MA) in cEGM-2 media for 4 hrs at 37 °C, 5% CO₂ in a humidified incubator. Cells were washed several times and fixed with 2% paraformaldehyde for 10 min. After washing with PBS, the cells were reacted with 10µg/ml fluorescein tagged *Ulex europaeus*-lectin (Vector Laboratories, Burlingame, USA) for 1h. Following nuclear counterstaining with Hoechst 33258, double-stained DilAc-LDL+/FITC-Lectin+ cells were imaged with an inverted fluorescence microscope (Leica Microsystems, Richmond Hill, ON, Canada).

**Immunophenotyping of ECFCs**

Early passage (4-5) ECFCs (0.25-0.5 million per sample) were washed in flow buffer (PBS containing 0.05% sodium azide and 0.1% BSA) and blocked by incubation for 1 hr in 100µL of flow buffer containing 5% milk. ECFCs were then incubated at 4 °C for 30-60 minutes in dark with appropriate concentrations of primary or isotype control antibody, as outlined below, in 50µL of 5% milk-flow buffer. After washing, if necessary, the cells were incubated in dark, with comparable concentrations of secondary antibody in 50µL of 5% milk-flow buffer. After washing, antibody-labeled ECFCs were
analyzed by fluorescence-activated cell sorting (FACS) (FACSCalibur, BD Biosciences, San Diego, CA)\textsuperscript{1}.

**Retroviral mediated eGFP labelling of ECFCs**

By screening multiple Human Immunodeficiency Virus (HIV)-1 based lentiviral vectors for optimal transduction efficiency to ECFCs, we selected a vesicular stomatitis virus (VSV)-pseudotyped lentiviral vector harboring enhanced Green fluorescence protein (eGFP) under the control of ubiquitous Elongation factor (EF)-1 promoter (LV-EF-eGFP). Rat lung ECFCs in passage 4-5 were incubated overnight with $2 \times 10^6$ TU/ml of lentiviral vector and 7 µg/ml protamine sulfate in cEGM-2. ECFCs in wells with uniform green fluorescence were trypsinized, expanded and sorted for GFP fluorescence using FACS.

**In Vitro Cell Viability Assay**

Lung ECFCs obtained from hyperoxia-exposed and room-air (control) rat pups were plated in equal numbers and grown in identical culture conditions. Cell viability was evaluated at various time points using the MTT assay as previously described.

**Capillary-like network formation in Matrigel**

The formation of cord-like structures by ECFCs was assessed on Matrigel (BD Biosciences, Mississauga, ON) coated 96-well tissue culture plates as previously described\textsuperscript{2}. The capillary-like networks were quantified by measuring the number of intersects and the total length of cord-like structures in random fields from each well using OpenLab (Quorum Technologies Inc, ON, Canada) software.

**Single cell clonogenicity**
The FACSAria cell sorter (BD Biosciences, Mississauga, ON) was used to place one ECFC per well in a flat-bottomed 96-well tissue culture plate precoated with type I collagen and containing 200 µL complete EGM-2 media. Cells were cultured at 5% CO2 and 37 °C in a humidified incubator and culture media was replaced twice/week. At day 14, Hoechst 33258 (Sigma) was added at 3 µg/mL to each well for 10 min for nuclear detection. The culture plate was examined with a fluorescent microscope at 20X magnification, well by well, for the growth of endothelial cells. Wells with 2 or more endothelial cells were scored as positive. The number of cells per well was enumerated by visual inspection at 40X magnification. Colonies with more than 500 cells were trypsinized and resuspended in cEGM-2. Based on the number of cells in that colony, an appropriate amount of cEGM-2 was added and mixed well such that each 200 µL would approximately contain one ECFC. From this suspension, cells were again seeded at single cell density in 96-well plates precoated with collagen type-I, cultured for 2 weeks and evaluated for secondary colonies (or second generation colonies), as described earlier. ECFCs from colonies with more than 500 cells were serially passaged in 24-well and 6-well tissue culture plates followed by T25 and T75 tissue culture flasks.

De novo angiogenesis in vivo

ECFCs were loaded on collagen-fibronectin matrices and implanted subcutaneously in NOD/SCID mice to assess their capacity to contribute to de novo vasculogenesis as described\(^3\). ECFCs were identified by antibody specific for human CD31 (Dako, Carpenteria, CA) that cross-reacts with rat but no mouse endothelial cells. Cellularized collagen-fibronectin implants were excised after 14 days post-implantation and examined for vascularization by immunohistochemical staining. Red blood cell perfused anti-human CD31+ vessels were identified in implants loaded with human or rat ECFCs respectively.

Oxygen-Induced BPD Model
We used two rodent models of oxygen-induced BPD: 1) For comparison of lung ECFC function, newborn rat pups were exposed to room air (21%; control group) or hyperoxia (95% oxygen; BPD group) from birth to P14 in sealed Plexiglas chambers (OxyCycler; BioSpherix, Lacona, NY) with continuous oxygen monitoring and ECFCs were isolated at P14 for comparative analysis; 2) To test the therapeutic potential of human cord blood-derived ECFCs, immune-compromised newborn rag/mice were exposed to 85% oxygen from P4 to P14. Human cord-derived ECFCs, isolated, expanded, and quality controlled as previously described were administered at P14 through the jugular vein (10⁵ cells/mouse in 100 μL DMEM). Lungs were harvested at P28. For cell engraftment experiments, ECFCs were labeled prior to injection with a red fluorophore (CellBrite™ Cytoplasmic Membrane Staining Kit, Biotium Inc, Hayward, CA). A subset of mice was kept for long-term assessment until 10 months of age.

**Lung Morphometry**

Lungs were fixed with a 4% glutaraldehyde solution through the trachea under a constant pressure of 20 cm H₂O. The trachea was ligated, and the lungs were immersed in fixative overnight at 4°C. Lung volume was measured by water displacement. Lungs were embedded in paraffin and serial step sections, 4 mm in thickness, were taken along the longitudinal axis of the lobe. The fixed distance between sections was calculated so as to allow a systematic sampling of 10 sections across the whole lobe. Lungs were stained with hematoxylin and eosin (H&E). Alveolar structures were quantified on a motorized microscope stage (Leica CTRMIC and openlab software, Quorum Technologies; Guelph, ON) by using the mean linear intercept (MLI) as previously described.

**Immunohistochemistry.** Sections were incubated with a polyclonal rabbit anti-von Willebrand Factor (vWF) antibody (Catalog #A0082; Dako) and a biotinylated secondary antibody (Catalog #B2770; Life Technologies Inc., Burlington, ON). A streptavidin-HRP conjugate (Catalog #S-911; Life Technologies
Inc., Burlington, ON) was used to link the DAB Chromogen (DAB Catalog # D4293; Sigma Aldrich, Oakville, ON) for visualization. Lung capillaries (30-100µm) were quantified on a motorized microscope stage.

**Right Ventricular Hypertrophy**

The right ventricle free wall was separated from the left ventricle and the septal wall. The tissue was dried overnight and weighed the following day\(^5\).

**Echocardiography**

All evaluations of pulmonary artery flow were performed with a (maximal) sweep speed of 200 mm/s. Pulsed-wave Doppler of pulmonary outflow was recorded in the parasternal view at the pulmonary valve level. The pulmonary acceleration time (PAAT) was measured from the beginning of the pulmonary flow to its onset and normalized with heart rate for comparisons as described\(^5\).

**Lung function testing**

Tests were performed on anesthetized and paralyzed animals using Flexivent (Scireq, Montreal, QC, Canada) as described\(^6\).

**Real-time PCR**

Total RNA was extracted from pulverized frozen lungs using Qiagen RNeasy kit (Qiagen, Mississauga, ON). RNA was quantified using a Nanodrop system (ND-1000 ThermoFisher Scientific, Wilmington, DE) and cDNA was prepared from lung RNA using random hexamers. PCR was performed on an ABI 7900 and using Taqman Universal PCR master mix (Applied Biosystems), Human Alu sequence primers and values were determined from a standard curve prepared from pure ECFCs to detect human cells. All results are expressed as a ratio of Alu sequences normalized to
human 18S. Three animals/group were harvested at 2 hours, 3, 7, 14 and 21 days after injection.

**ECFC-derived Conditioned Media (CdM) experiments**

Human umbilical cord-blood ECFCs in passages 4-6 were grown in T75 flasks up to 90% confluence in cEGM-2. Following removal of the respective culture medium, cells were rinsed 3 times with PBS and serum free DMEM was added. After 24 hours, supernatants were collected, concentrated (25x) and desalted by centrifugal filtration (Amicon - Millipore, Billerica, MA) as described\(^6\). Upon preparation, CdM was pooled, frozen at -80°C and thawed right before use. Control cell CdM was obtained from HUVECs.

*In vitro*, AT2 were isolated from time-dated fetal day 19.5 rat lungs as described using serial differential adhesions to plastic and low-speed centrifugations\(^7\). For wound healing assays, 10^6 cells/mL AEC2 were seeded into a plastic 24-well cell culture plate. At ~80 hours, the cell monolayer was scraped with a p200 pipette tip and media replaced with CdM or DMEM. The surface area of the wound was recorded over time using OpenLab (Quorum Technologies Inc, ON, Canada)\(^7\).

*In vivo*, CdM was administered daily to newborn rats exposed to hyperoxia through intraperitoneal injections at the dose of 7 µl/g\(^6\) from P4 to P21 and lungs were harvested at P22.

**Exercise capacity**

Mice were run according to a predetermined protocol. Exhaustion was defined as the animal running exclusively on the lower third of the treadmill coupled with hitting of the shock panel twice within 30 seconds\(^6\).

**Statistical Analysis**

Values are expressed as means ± standard error of the mean (SEM). Statistical comparisons were made with ANOVA. *Post hoc* analysis used Fisher’s probable least significant difference test (Statview 5.1;
Abacus Concepts, Berkeley, CA). A value of P less than 0.05 was considered statistically significant. All endpoints were assessed by investigators blinded to the experimental groups.
**Figure S-1**

**A**  
Mean Linear Intercept  
*P < 0.01 vs all other groups; n = 4-6 animals/group

**B**  
Vessel Count  
*P < 0.05 vs all other groups; n = 4-6 animals/group

**C**  
Right Ventricular Hypertrophy  
* P < 0.05 vs all other groups; n = 4-6 animals/group
Figure S-2

A

*P < 0.05; n = 3 trials per group

B

*P < 0.05 vs all other groups; n = 6 per group
Figure S-1. Human umbilical cord ECFC therapy reverses alveolar and lung vascular growth arrest and attenuates RVH in hyperoxia-exposed newborn RNU rats. A. Representative H&E-stained lung sections at P28 show the characteristic arrested alveolar growth with larger and fewer alveolar structures in untreated hyperoxia-exposed (O$_2$) lungs as compared to control rats housed in RA. Intra-jugular administration of ECFCs in O$_2$-exposed animals restored alveolar growth as compared to untreated O$_2$-exposed animals. Quantitative assessment of alveolar architecture by the mean linear intercept confirms the protective effect of ECFCs on alveolar growth compared to untreated O$_2$-exposed animals (n=4-6/group, *P<0.01). ECFCs did not alter lung structure in control RA rats. B. Effects of ECFC treatment on pulmonary vessel density assessed on lung sections stained with von Willebrand Factor (vWF) at P28. Pulmonary vessel density of 30-100µm sized blood vessels per 10 high power fields (40X) was significantly decreased in the lungs of O$_2$-exposed rats compared to RA. Intra-jugular injection of ECFCs significantly improved pulmonary vessel density compared to untreated O$_2$-exposed animals (n=4-6/group, *P<0.05). C. Hyperoxia-exposed rats displayed significant RVH compared to RA housed animals as indicated by the increase in right ventricle/left ventricle plus septum (RV/LV+S) ratio compared with controls. ECFC therapy significantly reduced RVH in hyperoxic exposed rats compared to untreated O$_2$-exposed rats (n=4-6/group, *P<0.05).

Figure S-2. Human umbilical cord ECFC therapy restores resident lung ECFC function in hyperoxia-exposed newborn RNU rats. A. Comparative single cell clonogenic assay. Colony forming potential of single plated ECFCs from RNU rat lungs was assessed by measuring the percentage of single ECFCs capable of generating colonies after 14 days in culture. Significantly fewer ECFCs from the hyperoxia group were capable of generating colonies with 500 or more cells in comparison with RA controls (*P<0.05, n=3 trials/group). Treatment with cord blood-derived ECFCs
significantly restored the colony forming potential of resident lung ECFCs isolated from treated RNU lungs exposed to hyperoxia (*P<0.05, n=3 trials/group). Cell therapy did not alter the colony forming properties of ECFCs from control RA rat lungs. **B. Quantitative assessment of the *in vitro* capillary-like network forming ability of lung ECFCs.** Lung resident ECFCs from untreated hyperoxic rats formed fewer capillary networks compared to lung ECFCs from untreated hyperoxia-exposed rats as assessed by the cord length and number of branch points (n=6/group, *P<0.05). Cord blood ECFC therapy restored the ability of hyperoxic resident lung ECFCs to form capillary-like networks as assessed by improved total cord length and number of intersects compared to lung ECFCs from untreated hyperoxia-exposed RNU rats (n=6/group, *P<0.05).
References


