SUPPLEMENTAL MATERIAL

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

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Short title: Endothelial colony forming cells for lung repair

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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 ^oC 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 ^oC 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 ^oC, 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 T_{25} flasks pretreated with collagen type I. Upon confluence, ECFCs were plated and expanded in type I collagen coated T_{75} 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

Attached cells at passage 4-5 were incubated with 20 μg/mL DiI-Ac-LDL (Biomedical technologies, Stoughton, MA) in cEGM-2 media for 4 hrs at 37 °C, 5% CO_2 in a humidified incubator. Cells were washed several times and fixed with 2% paraformaldehyde for 10 min. After washing with PBS, the cells were reacted with 10μg/ml fluorescein tagged *Ulex europaeus*-lectin (Vector Laboratories, Burlingame, USA) for 1h. Following nuclear counterstaining with Hoechst 33258, double-stained DiIAc-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 ^oC 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)[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 Elogation factor (EF)-1 promoter (LV-EF-eGFP). 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 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[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 T_{25} and T_{75} 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]; 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[1] were administered at P14 through the jugular vein (10^5 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 H_2O. The trachea was ligated, and the lungs were immersed in fixative overnight at 4°C. Lung volume was measured by water displacement[4]. 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[2].

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 1ung 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 T_{75} 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^{o}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

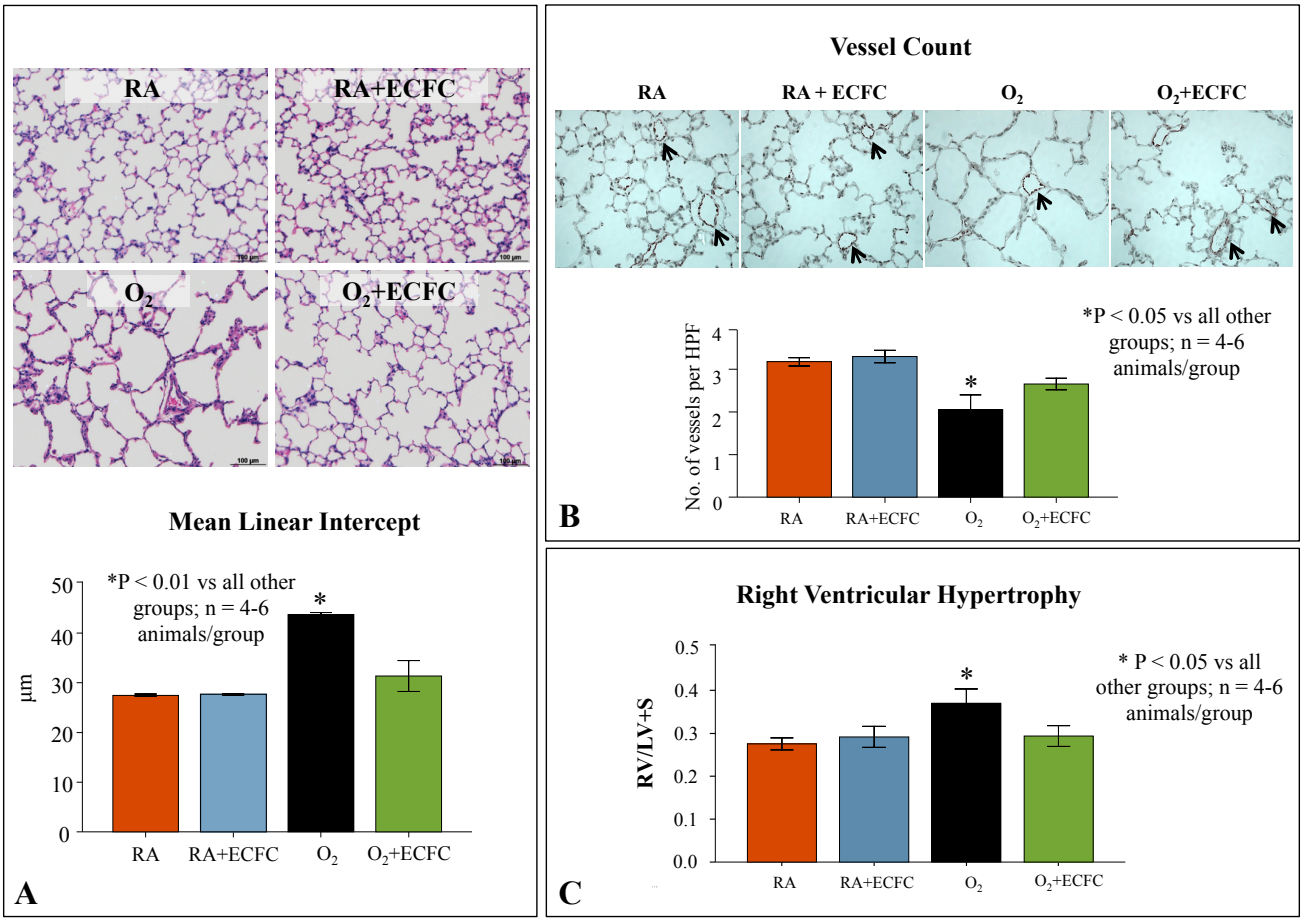
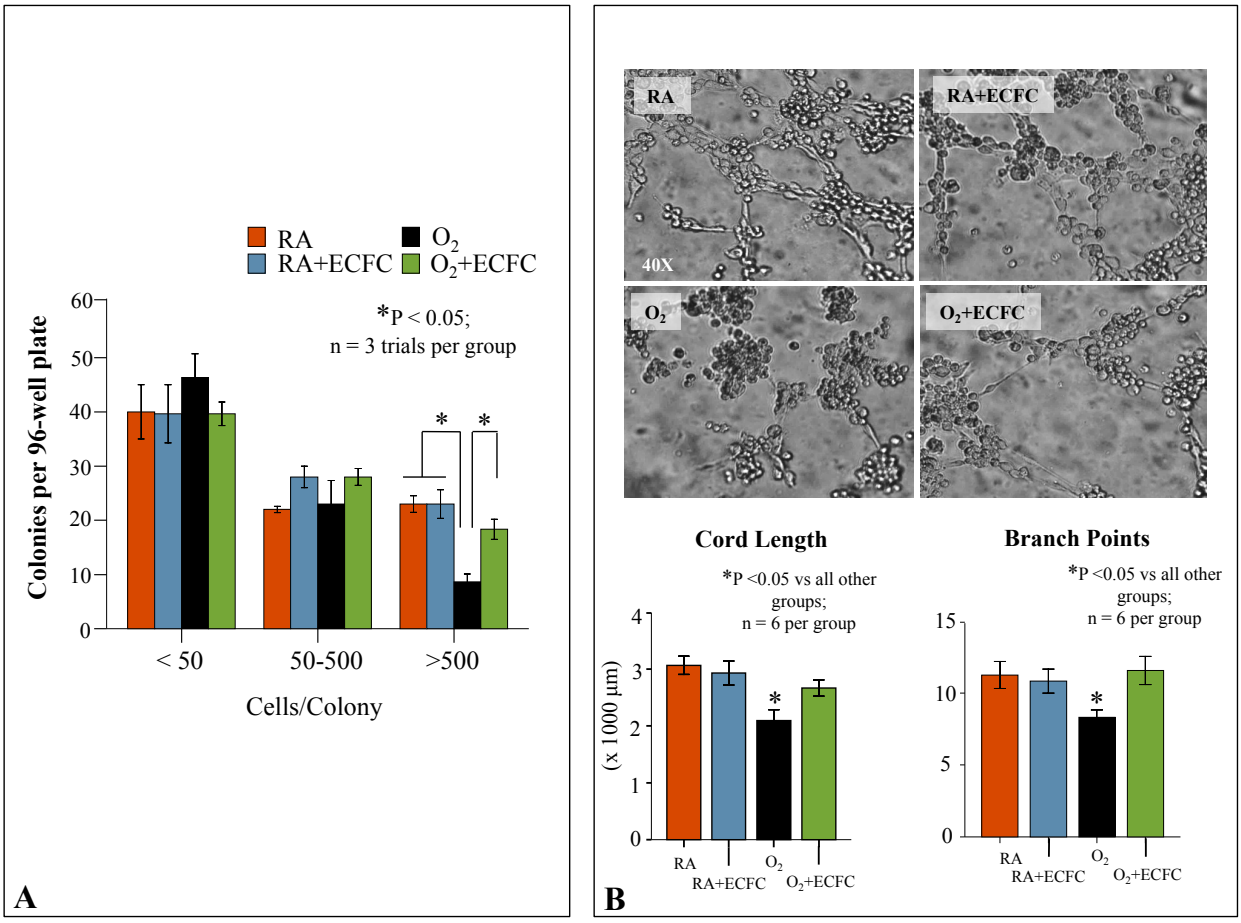

RA
RA+ECFC
O2
O2+ECFC
Mean Linear Intercept
*P < 0.01 vs all other groups; n = 4-6 animals/group
µm
50
40
30
20
10
0
RA
RA+ECFC
O2
O2+ECFC
A
Vessel Count
RA
RA + ECFC
O2
O2+ECFC
*P < 0.05 vs all other groups; n = 4-6 animals/group
No. of vessels per HPF
4
3
2
1
0
RA
RA+ECFC
O2
O2+ECFC
B
Right Ventricular Hypertrophy
* P < 0.05 vs all other groups; n = 4-6 animals/group
RV/LV+S
0.5
0.4
0.3
0.2
0.1
0.0
RA
RA+ECFC
O2
O2+ECFC
C

Figure S-2


A
RA
RA+ECFC
O_2
O_2+ECFC
*P < 0.05;
n = 3 trials per group
Colonies per 96-well plate
60
50
40
30
20
10
0
*
*
< 50
50-500
>500
Cells/Colony
B
RA
RA+ECFC
40X
O_2
O_2+ECFC
Cord Length
*P <0.05 vs all other groups;
n = 6 per group
(x 1000 µm)
4
3
2
1
0
*
RA
RA+ECFC
O_2
O_2+ECFC
Branch Points
*P <0.05 vs all other groups;
n = 6 per group
15
10
5
0
*
RA
RA+ECFC
O_2
O_2+ECFC

Supplemental Figure Legends

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).

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