Endothelial colony-forming cell conditioned media promote angiogenesis in vitro and prevent pulmonary hypertension in experimental bronchopulmonary dysplasia

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Baker CD, Seedorf GJ, Wisniewski BL, Black CP, Ryan SL, Balasubramaniam V, Abman SH. Endothelial colony-forming cell conditioned media promote angiogenesis in vitro and prevent pulmonary hypertension in experimental bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol 305: L73–L81, 2013. First published May 10, 2013; doi:10.1152/ajplung.00400.2012.—Late-outgrowth endothelial colony-forming cells (ECFCs), a type of circulating endothelial progenitor cell (EPC), may contribute to pulmonary angiogenesis during development. Cord blood ECFCs from preterm newborns proliferate more rapidly than term ECFCs but are more susceptible to the adverse effects of hyperoxia. Recent studies suggest that bone marrow-derived EPCs protect against experimental lung injury via paracrine mechanisms independent of vascular engraftment. To determine whether human umbilical cord blood ECFCs from preterm and term newborns have therapeutic benefit in experimental neonatal lung injury, we isolated cord blood ECFCs from full-term and preterm newborns and prepared ECFC-conditioned medium (CM) to test its therapeutic benefit on fetal pulmonary artery endothelial cell (PAEC) proliferation and function as well as alveolar type 2 (AT2) cell growth. PAECs and AT2 cells were isolated from late-gestation fetal sheep. Additionally, we administered both ECFCs and ECFC-CM to bleomycin-exposed newborn rats, an experimental model of bronchopulmonary dysplasia (BPD). Both term ECFC-CM and preterm ECFC-CM promoted cell growth and angiogenesis in vitro. However, when ECFC-CM was collected during exposure to mild hyperoxia, the benefit of preterm ECFC-CM was no longer observed. In the bleomycin model of BPD, treatment with ECFC-CM (or CM from mature EC) effectively decreased right ventricular hypertrophy but had no effect on alveolar septation. We conclude that term ECFC-CM is beneficial both in vitro and in experimental BPD. During oxidative stress, preterm ECFC-CM, but not term ECFC-CM, loses its benefit. The inability of term ECFC-CM to promote alveolarization may limit its therapeutic potential.

endothelial progenitor cells; conditioned medium; angiogenesis; bronchopulmonary dysplasia; pulmonary arterial hypertension

BRONCHOPULMONARY DYSPLASIA (BPD) is a chronic lung disease associated with neonatal respiratory distress syndrome and premature birth (31). Because therapeutic advances such as antenatal steroids, postnatal surfactant, and improved ventilator strategies have led to improved survival at lower gestational ages, the prevalence of BPD has not declined (8, 25, 27). In the postsurfactant era, the “new BPD” is characterized by disrupted pulmonary vascular and alveolar growth resulting in a decreased surface area for gas exchange in the newborn lung (1, 13, 24). Disrupted growth of the pulmonary microcapillary network limits pulmonary blood flow and predisposes preterm infants to pulmonary arterial hypertension (PAH) (30). Increased vascular tone, hypertensive remodeling, and further impaired vessel growth contribute to PAH in preterm infants (20, 30).

For infants with severe BPD, late PAH is associated with poor prognosis and significant mortality (9). In addition to PAH, early lung injury decreases vascular growth, which results in impaired gas exchange and leads to significant postnatal sequelae including respiratory insufficiency, hypoxemia, exercise intolerance, wheezing, severe developmental delay, and in some cases, death (8). Thus therapeutic interventions that promote endothelial cell growth and angiogenesis may decrease the subsequent risk for BPD and late respiratory disease.

The precise mechanisms that govern pulmonary vascular growth are unclear. Both angiogenesis, the direct extension of existing vessels, and vasculogenesis, the formation of vessels from endothelial precursors or angioblasts, contribute to growth of the pulmonary microvascular bed (14). It has been speculated that circulating bone marrow-derived endothelial progenitor cells (EPCs) contribute to vasculogenesis during development (2). EPCs consist of diverse progenitor-like cell populations including early-outgrowth EPCs and late-outgrowth endothelial colony-forming cells (ECFCs). Unlike early EPCs, ECFCs have an endothelial morphology, express endothelial markers, are highly proliferative, and demonstrate a progenitor-like capacity for self-renewal (2, 6, 23). ECFCs are increased in umbilical cord blood compared with adult peripheral blood (23). Furthermore, ECFCs are generally increased in the cord blood of preterm infants but are decreased in infants who subsequently develop BPD (5, 6, 10). Although oxygen tension appears to have little to no effect on term ECFC growth, mild hyperoxia decreases the growth of highly proliferative preterm ECFCs, a process that may be mediated by disruption in the vascular endothelial growth factor-nitric oxide signaling axis (6, 17). However, although ECFC levels appear to be protective, ECFCs and other bone marrow-derived progenitors have not been shown to engraft into the lung vascular endothelial layer (32).

More recent studies have shown that bone marrow-derived mesenchymal stromal cells (MSCs) are therapeutically beneficial in numerous animal models of lung injury (21, 29, 34). Interestingly, similar benefit occurs when rodents with hyperoxia-induced lung injury are treated with MSC-conditioned medium (CM) in lieu of the cells themselves (4, 40). Whether

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CM from ECFCs is protective in experimental BPD is unknown. Therefore, we hypothesized that treatment with ECFC-CM promotes fetal pulmonary artery endothelial cell (PAEC) growth and angiogenesis in both room air (RA) and hyperoxia. However, since we previously showed that hyperoxia impairs preterm ECFC growth (6), we suspected that preterm ECFC-CM would have a lesser effect when collected during hyperoxia. In addition to its effects on endothelial cells, we hypothesized that ECFC-CM promotes the growth of alveolar type 2 (AT2) epithelial cells.

In this study, we treated neonatal rats with systemic bleomycin. In adult rodents, bleomycin treatment induces oxidative stress and inflammation that leads to pulmonary fibrosis (12, 22, 39). However, in the newborn rat, daily intraperitoneal bleomycin impairs vascular and alveolar growth and causes secondary pulmonary hypertension without inducing pulmonary fibrosis (38). Therefore, this animal model of BPD serves as an in vivo parallel to the cell culture hyperoxia experiments. We demonstrate that treatment with ECFC-CM decreases pulmonary fibrosis. Therefore, this animal model of BPD serves as an in vivo parallel to the cell culture hyperoxia experiments. We demonstrate that treatment with ECFC-CM decreases pulmonary hypertension, but does not restore lung structure, in bleomycin-treated newborn rats.

MATERIALS AND METHODS

All animal studies were performed with the approval of the Animal Care and Use Committee at the University of Colorado Anschutz Medical Campus. The Colorado Multiple Institutional Review Board approved the clinical protocol for umbilical cord blood collection.

Cord blood collection and ECFC isolation. After informed consent was obtained, cord blood was collected from pregnant mothers during delivery. Samples were maintained at room temperature and processed within 24 h as previously described (6). By using gradient centrifugation (Ficoll-Paque PLUS), the mononuclear cell (MNC) buffy coat was isolated and washed with complete EGM-2 medium (Lonza) with 10% fetal bovine serum [FBS; Hyclone], and 2% antibiotic/antimycotic (Invitrogen). MNCs were cultured in complete EGM-2 medium on type 1 collagen and late-outgrowth ECFC colonies were identified over 21 days by previously described methods (6). ECFCs were then expanded and cryopreserved for additional study and analysis. Low-passage ECFCs (p2–3) were thoroughly characterized by polychromatic flow cytometry, immunohistochemistry, in vitro angiogenesis, and single-cell assay to demonstrate self-renewal (5). All of the studied ECFCs were of low passage (less than p5) and previously cryopreserved in liquid nitrogen in a suspension of 5% dimethyl sulfoxide-95% FBS.

ECFC conditioned medium preparation. To obtain CM, ECFCs were plated at 10% confluence and cultured for 5 days on type I collagen in RA or hyperoxia (50% oxygen) in complete EGM-2 medium (Fig. 1). On day 5, cells were rinsed twice with phosphate-buffered saline (PBS) and medium was replaced with Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, with 2.5% FBS and no antibiotic/antimycotic). MNCs were cultured in complete EGM-2 medium (Fig. 1). On day 5, cells were rinsed twice with phosphate-buffered saline (PBS) and medium was replaced with Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, with 2.5% FBS and no other supplements). Since serum-free medium caused ECFC death (not shown), DMEM with 2.5% FBS was used in these experiments. After 24 h, ECFC-CM was collected from cells in either RA (ECFC-CMRa) or 50% oxygen (ECFC-CMSo) conditions. CM was filtered with 0.2 μm filters (Corning) and frozen at −70°C until use. The unconditioned control medium was handled similarly, but never exposed to ECFCs. For the animal treatment studies, CM was concentrated 10× with Amicon Ultra 3,000 MWCO filters (Millipore). CM was obtained from both term and preterm ECFCs, resulting in the following four types of CM: term ECFC-CMRA, term ECFC-CMSo, preterm ECFC-CMRA, and preterm ECFC-CMSo.

Fetal pulmonary artery endothelial cell isolation. PAECs were harvested from the proximal pulmonary arteries of late-gestation fetal sheep at day 135 (day 147 is term), as previously described (19, 28). Immunohistochemistry with standard endothelial markers confirmed the cell phenotype. Low-passage PAECs (p4–5) were then exposed to ECFC-CM in the experiments below.

Growth of PAECs while exposed to ECFC-conditioned medium. Fetal PAECs were plated in triplicate at 200,000 cells/well in DMEM with 10% FBS on 0.1% gelatin-coated plates and allowed to adhere overnight. PAECs were rinsed twice with PBS and the medium was replaced with one of the four types of ECFC-CM. PAECs were placed in either RA or 50% oxygen for 3 days (with daily replacement of the ECFC-CM). On day 3, PAECs were removed by use of 0.25% trypsin and counted with a cell counter (Beckman Coulter; Fullerton, CA).

Term and preterm ECFCs proliferate at different rates in RA and hyperoxia (6). To ensure that observed differences in ECFC-CM were not due to differences in cell number, we performed an additional control experiment. We collected term ECFC-CM from plates seeded at two different concentrations (1 and 0.5 × 10⁶ cells/plate). After collection of term ECFC-CM, we confirmed that cell number remained disproportionate (4.7 × 10⁶ ± 3.8 × 10⁵ vs. 2.6 × 10⁶ ± 1.3 × 10⁵ cells/plate, P < 0.001). We then compared the effect of “term ECFC-CM” and “term ECFC-CM1/2” (ECFC-CM from half as many plated cells) on PAEC growth. All cells (ECFCs and PAECs) were studied in RA. We found that both term ECFC-CM and term ECFC-CM1/2 significantly augmented PAEC growth but were not different from each other (fold increase 1.8 ± 0.1 vs. 1.9 ± 0.1, respectively, over control PAEC growth: P = NS).

PAEC tube formation assay. To assay in vitro angiogenesis, we cross-linked rat-tail collagen using 0.2% flavin mononucleotide and a UV Stratalinker 1800 (Stratagene), and 50,000 cells/well were added in 1.25% FBS DMEM medium. Cells were then incubated in either control (3% oxygen) or 50% oxygen for 18 h to allow angiogenic networks to form. Wells were imaged with an Olympus IX71 fluorescence microscope (Olympus). The number of branch points was measured to quantify tube formation. Each cell type was studied in four separate wells.

Human umbilical vein endothelial cell isolation. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cord segments of healthy full-term infants, and 0.1% collagenase was used to detach endothelial cells from the vessel lumen. These cells were collected and resuspended in e-EGM2 with 10% FBS on type I collagen. HUVEC colonies typically appeared within 5 days and were expanded and cryopreserved at passage 2. HUVEC-CMRA was prepared by the method used for ECFC-CM preparation (described above).

Fig. 1. Study design for endothelial colony-forming cell (ECFC)-conditioned medium (CM). Subconfluent umbilical cord blood (UCB)-derived ECFCs are cultured in room air (RA) or hyperoxia for 5 days. On day 5, cells are incubated with low-serum DMEM for 24 h. ECFC-CM is then collected for use in all CM experiments. Human umbilical vein endothelial cell (HUVEC)-CM collected in a similar fashion. ECFC-CMRA and ECFC-CMSo, ECFC cultured in either RA or 50% oxygen conditions.
ECFC-CM augments fetal PAEC growth in RA. Treating PAECs with either term ECFC-CMRA or term ECFC-CM50 augmented their growth in RA over controls (1.93 ± 0.13 vs. 1.0 ± 0.03, P < 0.001; 1.95 ± 0.12 vs. 1.0 ± 0.03, P < 0.001, respectively; Fig. 2). Preterm ECFC-CMRA also significantly increased PAEC growth in RA (1.88 ± 0.11 vs. 1.0 ± 0.03, P < 0.001; Fig. 2). However, in contrast with term ECFC-CM50, preterm ECFC-CM50 did not increase PAEC growth. As a control, HUVEC-CMRA did not affect fetal PAEC growth in RA.

RESULTS

Preterm ECFC-CMRA or ECFC-CM50 restore PAEC growth in hyperoxia. Hyperoxia (50% oxygen) impaired the growth of fetal PAECs in the 3-day growth assay compared with RA controls (0.72 ± 0.03 vs. 1.0 ± 0.03, P < 0.01; Fig. 3). However, when PAECs in hyperoxia were treated with term ECFC-CMRA, PAEC growth exceeded that of the RA controls (1.5 ± 0.19 vs. 1.0 ± 0.03, P < 0.01; Fig. 3). When PAECs in hyperoxia were treated with term ECFC-CM50, fetal PAEC growth again exceeded RA control growth (1.45 ± 0.19 vs. 1.0 ± 0.03, P < 0.01; Fig. 3). PAECs in hyperoxia were also treated with CM from preterm ECFCs. Preterm ECFC-CMRA and preterm ECFC-CM50 both significantly increased PAEC growth in hyperoxia (1.15 ± 0.09 vs. 0.72 ± 0.03, P < 0.01; 0.91 ± 0.08 vs. 0.72 ± 0.03, P < 0.05, respectively; Fig. 3). HUVEC-CMRA had no effect in hyperoxia.

Alveolar type 2 cell isolation. Distal lung was dissected free, avoiding large airways and blood vessels. Distal lung was further minced to 3- to 5-mm3 sections and incubated with 0.1% collagenase and 0.025% trypsin for 30 min at 37°C. Trypsin inhibitor solution was added to the lung digest and then subjected to low-speed homogenization for 60 s. Lung suspension was filtered through a 10-μm Nitex filter to remove undigested tissue. Cells were centrifuged at 300 relative centrifugal force for 10 min, resuspended in wash medium, and plated on IgG-coated dishes for 1 h at 37°C.

Nonadherent cells were recovered, centrifuged, and plated at a density of 1 × 105 cells/well. We confirmed the AT2 cell phenotype by positive immunostaining for pro-SPC and negative staining for T1α (an alveolar type 1 cell marker), von Willebrand factor (an endothelial marker), and desmin (a smooth muscle cell marker).

Alveolar type 2 cell growth assay. Fetal lamb AT2 cells were plated at 1 × 105 cells/well and allowed to adhere overnight. Cells were plated in DMEM/F12 medium supplemented with 10% FBS for 24 h to allow cells to adhere. Cells were washed with PBS. Then, either CM or DMEM with 2.5% FBS was added to the AT2 cells. The cells were placed in RA or 50% O2 ECFC-CM50 and medium was changed daily. After 4 days of treatment, AT2 cells were washed twice with PBS and incubated with 0.25% trypsin until all cells were detached. Cells were assayed for viability by trypan blue exclusion and counted on a hemocytometer (viability >90%).

Bleomycin newborn lung injury model. Timed-pregnant Sprague-Dawley rats were purchased (Charles River) and remained in Denver, CO for at least 1 wk before pups were delivered (altitude 1,600 m, barometric pressure 630 mmHg, PIO2 122 mmHg). At 24 h after natural birth, litters of 10–12 were treated with daily intraperitoneal bleomycin (1 mg/kg) for 14 days.

Animals were treated by one of two methods. First, on day of life (DOL) 8, animals (6–9 pups per study group) were anesthetized with inhaled isoflurane (1–2%) and treated with 2 × 105 ECFCs/100 μl DMEM with 2.5% FBS, 100 μl 10 × ECFC-CM (all four types), or HUVEC-CM, or 100 μl 10 × DMEM with 2.5% FBS by right jugular vein injection. Separate animals were treated with daily intraperitoneal injections (3 μl/g body wt) of 10 × term ECFC-CM, 10 × HUVEC-CM, or 10 × control medium (2.5% FBS DMEM) from DOL 1 to 15. On DOL 15, lungs were prepared for tissue histology as previously described (7). Briefly, after pentobarbital euthanasia, lungs were inflated in situ with intratracheal 4% paraformaldehyde in PBS at 30 cmH2O for 1 h. Lungs were embedded in paraffin, and 5-μm sections were cut with a microtome and stained with hematoxylin and eosin. Additional sections were stained with a von Willebrand factor antibody for vessel density measurements. Blinded investigators performed morphometric analysis. Radial alveolar counts (RACs) were measured to quantify alveolarization (15). Vessel density was measured as the number of vessels per high-power field at ×200 magnification. Pulmonary artery wall thickness was measured as previously described (38). Briefly, 30- to 80-μm arteries were identified to be associated with terminal bronchi and distal air spaces. Wall thickness (%) was determined by this formula: [external diameter – internal diameter/external diameter] × 100. At least 10 pulmonary arteries were measured for each animal. Right ventricular hypertrophy (RVH) was measured by weighing hearts at autopsy and calculating the Fulton’s index, which is the ratio of the weight of the right ventricle to that of the left ventricle plus intraventricular septum [RV/(LV+S)] (18).

Statistical analysis. Data are presented as means ± SE. Statistical analysis was performed with the Prism software package (v. 5.0a, GraphPad). One-way ANOVA with Bonferroni posttest analysis was performed. P values less than 0.05 were considered significant.
ECFC-CM increases fetal PAEC angiogenesis in control and hyperoxic conditions. As shown in Fig. 4A, PAECs formed angiogenic networks in RA and to a markedly lesser degree in hyperoxia. Treatment with all four types of ECFC-CM resulted in increased branch points per high-power field (Fig. 4B). However, preterm ECFC-CMRA caused a significantly smaller increase in branch point number than term ECFC-CMRA, term ECFC-CM50, and preterm ECFC-CMRA ($P < 0.05$, Fig. 4B). When the PAEC angiogenic assay was performed in hyperoxia, treatment with all four types of ECFC-CM restored branch point number to that of RA controls ($P < 0.05$, Fig. 4C). In contrast, HUVEC-CMRA did not affect branch point number in either condition.

Fig. 4. Effect of ECFC-CM on PAEC in vitro angiogenesis. 

A: light microscopy shows that exposure to hyperoxia significantly decreases tube formation. Treatment with either term ECFC-CMRA or preterm ECFC-CM50 causes a striking increase in angiogenesis. 

B: quantification of tube formation by branch point in RA. All 4 types of ECFC-CM augmented tube formation. However, preterm ECFC-CM50 stimulated angiogenesis to a lesser degree than preterm ECFC-CMRA. 

C: branch point assessment of tube formation in hyperoxia. All 4 types of ECFC-CM augment in vitro angiogenesis. HUVEC-CMRA had no effect on tube formation. 

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 

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ECFC-CM RA but not HUVEC-CM RA increases fetal alveolar type 2 cell growth during hyperoxia. When AT2 cells were studied in RA with either term ECFC-CM RA or preterm ECFC-CM RA, AT2 proliferation was no different from controls (Fig. 5A). For this reason, the effect of ECFC-CM 30 on AT2 growth was not studied. In hyperoxia, term ECFC-CM RA (3.95 ± 0.40 vs. 1.52 ± 0.05, P < 0.05) and preterm ECFC-CM RA (3.52 ± 0.19 vs. 1.52 ± 0.05, P < 0.05) significantly increased AT2 growth, but not to that seen in the RA controls (Fig. 5A). The control HUVEC-CM RA had no effect on AT2 growth in RA or hyperoxia.

Term ECFC-CM RA prevents pulmonary arterial hypertension after bleomycin lung injury. Bleomycin-exposed new born rats developed significant RVH compared with controls (0.46 ± 0.03 vs. 0.28 ± 0.03, P < 0.001, Fig. 6A). RVH was decreased in bleomycin-exposed pups treated with a single dose of term ECFC-CM RA (0.32 ± 0.02 vs. 0.46 ± 0.03, P < 0.001, Fig. 6A). Similarly, treatment with term ECFCs significantly decreased RVH (0.33 ± 0.03 vs. 0.46 ± 0.03, P < 0.01, Fig. 6A). HUVEC-CM RA also had a similar effect on RVH (0.30 ± 0.03 vs. 0.46 ± 0.03, P < 0.01, Fig. 6A). As shown in Fig. 6, B–F, bleomycin treatment caused alveolar simplification as demonstrated by decreased RAC (7.11 ± 0.42 vs. 9.68 ± 0.61, P < 0.05). Daily intraperitoneal bleomycin also significantly reduced vessel density (5.23 ± 0.27 vs. 11.37 ± 0.43 vessels per high-power field, P < 0.001, Fig. 6, B–E and G). However, none of the treatment groups showed improved alveolarization or vessel density over the bleomycin-exposed pups. We also treated bleomycin-exposed pups with daily intraperitoneal doses of ECFC- or HUVEC-CM RA. As observed with the single intravenous interventions, alveolarization was not different from controls in the CM-treated animals (not shown).

Since treatment with intravenous term ECFC-CM RA decreased RVH without improving lung structure, we repeated the experiment above treating the bleomycin-exposed rat pups with each of the four types of ECFC-CM. We again showed that daily intraperitoneal bleomycin resulted in RVH compared with controls (0.57 ± 0.04 vs. 0.34 ± 0.03, P < 0.05; Fig. 7A) and that term ECFC-CM RA decreased RVH (0.31 ± 0.02 vs. 0.57 ± 0.04, P < 0.05; Fig. 7A). Term ECFC-CM 30 and preterm ECFC-CM RA also decreased RVH (0.39 ± 0.09 and 0.39 ± 0.06 vs. 0.57 ± 0.04, respectively, both P < 0.05, Fig. 7A). However, treatment with preterm ECFC-CM 30 did not significantly decrease RVH. In this experiment, bleomycin again caused decreased RAC (4.5 ± 0.4 vs. 10.0 ± 0.4, P < 0.001, Fig. 7B), but none of the ECFC-CM improved lung structure. Bleomycin decreased vessel density (4.0 ± 0.4 vs. 13 ± 1.0, P < 0.001, Fig. 7C) and ECFC-CM had no effect on this process.

Given the improvement in RVH observed with ECFC-CM without increased vessel density, we also measured distal pulmonary arterial vessel wall thickness. Bleomycin significantly increased vessel wall thickness compared with untreated controls (29.1 ± 1.0 vs. 15.0 ± 0.7%, P < 0.001), but ECFC-CM did result in significantly decreased wall thickness (Fig. 8).

**DISCUSSION**

In this study, we report that ECFC-CM from both term and preterm infants augmented the growth of fetal PAECs. However, the CM from hyperoxia-exposed preterm ECFCs did not augment fetal PAEC growth. Hyperoxia suppressed fetal PAEC growth, but ECFC-CM protected against this effect. Term and preterm ECFC-CM augments in vitro angiogenesis. AT2 cell growth was not affected by treatment with ECFC-CM in RA, but ECFC-CM protected AT2 cells from hyperoxia-mediated growth impairment. Treatment with ECFC-CM (except that collected from hyperoxia-exposed preterm ECFCs) as well as term ECFCs and HUVEC-CM prevented RVH in bleomycin-treated newborn rats, but it had no effect on alveolar septation.

These findings suggest that ECFCs have a paracrine influence on fetal PAEC growth and function. Since their first description, bone marrow-derived EPCs have been thought to respond to vascular injury by homing to injured peripheral vasculature, in the lung and elsewhere (2, 3, 11, 16, 26, 37, 42, 43). However, the mechanisms by which EPCs promote microvascular healing are not known. The ongoing presence of ECFCs in the ischemic hind limb has recently been shown to be necessary for restored perfusion, suggesting that ECFC engraftment may occur in this model (35). However, other studies have failed to identify EPCs in injured vascular beds, suggesting that engraftment may not be the primary mechanism by which bone marrow-derived angiogenic progenitors contribute to all types of vascular repair (32, 33, 36, 41). Voswinckel et al. (41) studied a pneumonectomy model of compensatory lung growth and found that mice that underwent transgenic bone marrow transplantation (containing endothelial cell-specific lacZ reporters) had no lacZ expression in either large vessels or pulmonary capillaries 3 wk after pneu-

**Fig. 5.** ECFC-CM augments AT2 cell growth in hyperoxia. A: treatment of AT2 cells in RA with term ECFC-CM RA, preterm ECFC-CM RA, or HUVEC-CM RA for 4 days had no effect on AT2 growth. B: in hyperoxia, AT2 growth was significantly decreased. Term and preterm ECFC-CM RA, but not HUVEC-CM RA restored AT2 growth to RA levels. *P < 0.05.
Fig. 6. Bleomycin (Bleo)-induced lung injury and right ventricular hypertrophy (RVH). One-day-old Sprague-Dawley rats are treated with daily bleomycin for 14 days. A: animals are treated with intravenous ECFCs, term ECFC-CMRA, or HUVEC-CMRA on day 8. Bleomycin caused RVH as demonstrated by Fulton’s index, i.e., ratio of the weight of the right ventricle to that of the left ventricle plus intraventricular septum [RV/(LV+S)]. Treatment with ECFCs, term ECFC-CMRA, and HUVEC-CMRA resulted in resolution of RVH. Lung histology (left, hematoxylin and eosin; right, von Willebrand factor vessel staining; x200; scale bar = 100 μm) from control rat pups (n = 7; B), after bleomycin injury (n = 6; C), bleomycin + term ECFCs (n = 6; D), and bleomycin + term ECFC-CMRA (n = 9; E). Bleomycin results in alveolar simplification quantified by radial alveolar counts (RAC; F) and decreased vessel density (G). No treatment group showed improved RAC or vessel density over controls. *P < 0.05, **P < 0.01, ***P < 0.001.
Whether or not sustained engraftment of ECFCs occurs, the CM studies described in this manuscript demonstrate that ECFCs augment the growth and function of pulmonary endothelial and AT2 cells by indirect mechanisms.

We previously showed that hyperoxia has a variable effect on term and preterm ECFC growth (6). Although they proliferate more rapidly in RA, preterm ECFC growth is significantly impaired by hyperoxia whereas term ECFC growth is not. This led us to expose term and preterm ECFCs to hyperoxia during the collection of CM anticipating that hyperoxia would variably modulate which soluble factors are secreted into the CM. Our results support this hypothesis in that treatment with preterm ECFC-CM50 does not increase PAEC growth. Control experiments, using medium conditioned by different numbers of cells, showed that the observed effect was independent of cell number (data not shown).

Although hyperoxia decreased PAEC tube formation, all four types of ECFC-CM caused increased tube formation in both RA and hyperoxia. We previously reported that term and preterm ECFCs do not differ in their ability to form vascular networks in vitro (6). It is thus consistent with these previous findings that term and preterm ECFC-CM do not differ in their ability to promote angiogenesis in the mature PAECs.

In this study, we utilize a novel method for the isolation and culture of ovine pulmonary AT2 cells. AT2 cells also demonstrate decreased proliferation in hyperoxia that was rescued by treatment with either term or preterm ECFC-CM. This AT2 culture assay will be useful in future coculture studies aimed at furthering our understanding of the interactions between endothelial and epithelial cells in the pulmonary microcapillary bed.

Our findings that treatment with ECFC-CM or term ECFCs decreased RVH are similar to those previously described in the murine monocrotaline model of pulmonary hypertension (43). Of note, preterm ECFC-CM50 did not significantly decrease RVH. Interestingly, HUVEC-CM also decreased RVH suggesting that mature EC may also secrete beneficial factors to have a local paracrine or autocrine effect. We expected that any improvement in RVH would be accompanied by a similar restoration of lung architecture. However, neither ECFCs nor ECFC-CM had any apparent benefit on alveolarization, vessel density, or vessel wall thickness. Although HUVEC-CM was effective in treating RVH in vivo, it did not replicate the proangiogenic effect of ECFC-CM in any of the in vitro studies.
experiments. This may suggest that both ECFC-CM and HUVEC-CM contain vasodilatory factors or act directly on the right heart as our experiments were not designed to measure these variables. The study of ECFCs was limited to intravenous treatment at a single time point. However, daily intraperitoneal injections of ECFC-CM also failed to restore lung structure. Importantly, in this model, we continued to treat animals with daily bleomycin until the time of tissue harvest rather than permit a time of posttreatment recovery. This may have limited the therapeutic benefit regardless of the route and timing of the CM and cellular therapies.

The ECFCs used to generate ECFC-CM and those used to treat experimental BPD were human derived. To date, technical limitations and a lack of species-specific reagents prevent us from isolating and characterizing ECFCs in both small and large animal models of BPD. The cross-species nature of these studies is a potential limitation and may in part explain why lung architecture was not restored. We did not, however, detect any adverse response that could be characterized as rejection of the treated cells or conditioned medium.

We conclude that ECFC-CM from term and preterm ECFCs significantly enhances the growth and angiogenesis of mature PAECs and protects AT2 cells from hyperoxia. ECFC-CM treats RVH in an animal model of pulmonary hypertension. The beneficial effect of preterm ECFC-CM is attenuated when ECFCs are exposed to hyperoxia during the CM preparation process. Further study is needed to characterize ECFC-CM to specifically identify which soluble factors mediate its beneficial effects on other cell types. Both focused study toward quantifying known pro- and antiangiogenic factors in CM and advanced analyses such as liquid chromatography-tandem mass spectrometry will help describe the ECFC “secretome” and how it is altered by exposure to hyperoxia. This will better our understanding of the role of ECFCs in normal pulmonary vascular growth and clarify how postnatal hyperoxia impairs ECFC function after preterm birth.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


