Endothelial Colony-forming Cells from Preterm Infants Are Increased and More Susceptible to Hyperoxia

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Rationale: Preterm birth and hyperoxic exposure increase the risk for bronchopulmonary dysplasia (BPD), a chronic lung disease characterized by impaired vascular and alveolar growth. Endothelial progenitor cells, such as self-renewing highly proliferative endothelial colony-forming cells (ECFCs), may participate in vascular repair. The effect of hyperoxia on ECFC growth is unknown.

Objectives: We hypothesize that umbilical cord blood (CB) from premature infants contains more ECFCs with greater growth potential than term CB. However, preterm ECFCs may be more susceptible to hyperoxia.

Methods: ECFC colonies were quantified by established methods and characterized by immunohistochemistry and flow cytometry. Growth kinetics were assessed in room air and hyperoxia ($FiO_2 = 0.4$).

Measurements and Main Results: Preterm CB (28–35 wk gestation) yielded significantly more ECFC colonies than term CB. Importantly, we found that $CD45^-/CD34^+/CD133^+/VEGFR-2^+$ cell number did not correlate with ECFC colony count. Preterm ECFCs demonstrated increased growth compared with term ECFCs. Hyperoxia impaired growth of preterm but not term ECFCs. Treatment with superoxide dismutase and catalase enhanced preterm ECFC growth during hyperoxia.

Conclusions: Preterm ECFCs appear in increased numbers and proliferate more rapidly but have an increased susceptibility to hyperoxia compared with term ECFCs. Antioxidants protect preterm ECFCs from hyperoxia.

Keywords: endothelial progenitor cells; bronchopulmonary dysplasia; umbilical cord blood; reactive oxygen species; antioxidants

An exponential increase in lung vascular and alveolar growth occurs during the third trimester of gestation in humans (1). Premature birth often results in neonatal respiratory distress syndrome (RDS). Treatment of RDS with improved ventilatory strategies, supplemental oxygen, antenatal steroids, and post-natal surfactant now permits the consistent survival of smaller more immature infants (2). However, the overall prevalence of

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Endothelial progenitor cells (EPCs) are mobilized in response to lung injury. Umbilical cord blood is a rich source of circulating EPCs, but the role of EPCs during prenatal vascular development is unknown.

What This Study Adds to the Field

A specific subgroup of EPCs, endothelial colony-forming cells (ECFCs) are increased in preterm cord blood. Compared to term ECFCs, preterm ECFCs proliferate more rapidly but are more sensitive to hyperoxia, a known contributor to bronchopulmonary dysplasia.

bronchopulmonary dysplasia (BPD), a chronic lung disease of infancy that follows preterm birth, has changed little (3–5). BPD is characterized by disrupted pulmonary vascular growth and impaired alveolarization (6). A better understanding of the mechanisms that promote and preserve lung vascular growth in premature infants may lead to new strategies to reduce the incidence of BPD.

Vascular growth is achieved through three processes: vasculogenesis (formation of vessels from primitive hemangioblasts), angiogenesis (the direct extension of existing vessels), and arteriogenesis (collateral vessel growth) (7–10). Bone marrow-derived circulating endothelial progenitor cells (EPCs) home to the peripheral microvasculature where they contribute to post-natal vasculogenesis (11). After vascular injury, increased EPC levels are associated with neovascularization of the heart (12, 13), brain (14), retina (15), and ischemic hindlimb (11, 16) in adult animal models. Clinically, EPCs acutely increase with acute lung injury (17), sepsis (18), and exercised-induced ischemia (19). EPC levels correlate with increased survival in critically ill patients with acute lung injury (17), and changes in EPC number are inversely proportional to cardiovascular disease risk (20, 21). Decreased EPC levels may contribute to persistent vascular dysfunction resulting in adverse clinical outcomes. EPCs have been isolated from umbilical cord blood (CB) (22), yet the role of these cells in normal vascular growth during prenatal development is unclear.

Multiple methodologies have been developed to isolate and quantify EPCs (11, 20, 23). There is a distinction between minimally proliferative early outgrowth EPCs (also known as colony-forming unit endothelial cells or CFU-ECs) and highly proliferative late-outgrowth EPCs (known as endothelial colony-forming cells or ECFCs) (24, 25). ECFCs demonstrate an endothelial phenotype (by light microscopy, flow cytometry, and immunohistochemistry), a capacity for self-renewal, and a high proliferative potential (22). CFU-ECs are not highly proliferative and have characteristics of both angiogenic

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macrophages and hematopoietic cells (26). This suggests that ECFCs may be the target cell population that participates in vasculogenesis.

The exposure of the preterm lung to increased oxygen, which is often necessary for survival, contributes to the risk for developing BPD (3). Exposure of neonatal animals to hyperoxia results in a disruption of alveolar and vascular growth similar to that of BPD (27, 28). In addition, hyperoxia leads to a decrease in bone marrow, circulating, and lung EPCs in neonatal mice (29). Oxidative stress and reactive oxygen species (ROS) such as superoxide contribute to endothelial dysfunction in pulmonary vascular disease (30–32). Treatment of human ECFCs with hydrogen peroxide decreases their clonogenic capacity and increases apoptosis (33). Perturbations in EPC redox control in the presence of oxidative stress may contribute to vascular injury (34). However, the effect of hyperoxia on ECFC growth has not been studied.

Given the rapid increase in lung vascular growth during the third trimester, we hypothesize that the number of circulating ECFCs will increase in CB from preterm infants compared with term newborns, and that preterm ECFCs will demonstrate accelerated growth kinetics. In light of the association of oxygen treatment with impaired lung vascular growth in infants with BPD, we hypothesize that preterm ECFCs will be more sensitive to hyperoxia in terms of growth and function. In this study, we report an increase in ECFC colonies in the CB of preterm neonates. Additionally, we demonstrate that preterm ECFCs have an increased susceptibility to hyperoxia *in vitro*. Some of the results of these studies have been previously reported in the form of abstracts (35, 36).

METHODS

Full protocol details, including product catalog numbers, can be found in the online supplement.

Cord Blood Samples and ECFC Culture

CB samples ($n = 50$) were collected with the informed consent and approval of the Institutional Review Board and maintained at room temperature until analysis (within 24 h). Blood was diluted in phosphate buffered saline and then underlaid with Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ) for gradient centrifugation. The mononuclear cell (MNC) buffy coat was washed with complete EGM-2 media (Lonza, Mapleton, IL) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), and 2% antibiotic/antimycotic (Invitrogen). MNCs were plated on type 1 collagen (BD Biosciences, San Jose, CA) and ECFC colonies were identified daily and enumerated on Day 14 (23).

Flow Cytometry Analysis

We analyzed MNCs and cultured ECFCs using fluorescence activated cell sorting (FACS). Cells ($0.5\text{--}1.0 \times 10^6$) were washed and stained with antibodies to Glycophorin-A, CD45, CD133, VEGFR-2, CD31, CD34, CD38, CD90, CD105, and/or CD146 (*see* details in online supplement). CompBeads (BD Biosciences) and fluorescence minus-one controls enhanced multicolor compensation and gating. In accordance with current recommendations (37), we examined CD45⁺/CD34⁺/CD133⁺/VEGFR-2⁺ MNCs as well as other combinations of these markers. Cultured ECFCs were stained with a single antibody or the appropriate isotype control. Cells were analyzed using a Beckman Coulter FC500 flow cytometer and reanalyzed using Summit Software, version 4.3 (Dako Cytomation, Glostrup, Denmark).

Immunohistochemistry

Early-passage (p3) ECFCs were plated on type-1 collagen at a density of 2,500 cells per well. At 90% confluence, cells were stained with antibodies to eNOS, CD31, VE-Cadherin, and vWF (*see* online supplement). Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI). Images were obtained using an Olympus IX71 fluorescence microscope.

Tube Formation Assay

Vascular tube formation was assayed by mixing 1×10^5 ECFCs in 7.5% Cultrex type I bovine collagen (R&D Systems, Minneapolis, MN) and EBM-2 medium. The center of each well was imaged at 48 hours. The number of branch points, closed loops, and the total line length were measured to quantify tube formation. Each cell type was studied in four separate wells.

ECFC Growth Assay

In preliminary studies, we discovered that ECFCs proliferate equally in 10% FBS and 2.5% FBS (data not shown). To minimize the effect of FBS, we used the lower serum concentration. Low-passage (p3) ECFCs were plated in complete EGM-2 medium on collagen at a density of 2.5×10^4 cells/well and incubated in room air. The next day (Day 0), cells were placed in 2.5% FBS-complete EGM-2 medium and incubated in either room air or 40% oxygen. Cells were counted using either a hemacytometer or a Vi-CELL cell counter (Beckman Coulter, Fullerton, CA). Fold-increase (FI) was determined by dividing Day n count by Day 0 count.

Antioxidant Treatment During Hyperoxic Exposure

Growth assays were performed as described above. However, hyperoxia-exposed preterm ECFCs received 500 ng/ml of copper-zinc superoxide dismutase (SOD; Biotechnology General, Rehovot, Israel), 2,000 units/ml of catalase (Worthington, Lakewood, NJ), or both. Concentrations were determined by preliminary *in vitro* studies and in consultation with Dr. Carl White (private communication) (38). Growth of treated ECFCs was compared with untreated controls in both room air and 40% oxygen.

Statistical Analysis

Data are presented as means $\pm$ SE. Statistical analysis was performed with the Prism software package, Version 4 (GraphPad, La Jolla, CA). Unpaired t tests were used to compare ECFC colony number, antigen profiles from flow cytometry, and mean decreases in cell number. Growth kinetics were compared using two-way analysis of variance (ANOVA) with Bonferroni post-test analysis. P values less than 0.05 were considered significant.

RESULTS

Preterm CB Contains a Greater Number of Late Outgrowth ECFC Colonies

Preterm CB ($n = 26$; gestation age [GA], 28–35 wk) yielded four-fold more ECFC colonies than term CB ($n = 24$) on Day 14 (5.1 ± 0.7 vs. 1.2 ± 0.4 colonies per 10^7 MNCs plated; $P < 0.0001$; Figure 1A). Colonies varied in size, but all had an endothelial-like morphology (Figure 1B insert). Both groups of ECFCs expressed CD31, CD105, and CD146 by flow cytometry (Figure 2A) and VE-cadherin and von Willebrand Factor by immunohistochemistry (Figure 2B). ECFCs were weakly positive for CD34, but negative for CD133 (surface markers of immaturity; Figure 2A). They did not express hematopoietic (CD38 or CD45) or fibroblastic (CD90) markers (Figure 2A). Both term and preterm ECFCs spontaneously formed vascular networks in the tube formation assay (Figure 2C). There was no quantifiable difference in tube formation between term and preterm ECFCs (data not shown).

CD45[−]/CD34⁺/CD133⁺/VEGFR-2⁺ Cell Frequency in CB Does Not Correlate with the Number of ECFC Colonies

When we analyzed MNC isolates from term ($n = 11$) and preterm ($n = 10$; GA, 31–35 wk) cord blood by FACS, there was not a statistically significant difference in the percentage of triple-positive (CD34⁺/CD133⁺/VEGFR-2⁺) cells in term and preterm CB ($0.014 \pm 0.007\%$ vs. $0.006 \pm 0.003\%$, respectively; $P = 0.33$). Additionally, there was not a significant correlation

Figure 1. (A) Comparison of outgrowth ECFC colonies from preterm and term cord blood (CB) samples. Preterm CB yields more outgrowth ECFC colonies per 10^7 MNCs plated than term CB. Error bars represent SEM ($*P < 0.0001$). (B) Low-power ($40\times$) image revealing two typical late-outgrowth ECFC colonies (arrows) on Day 14. Insert: high-power image ($100\times$) demonstrating the typical endothelial-like morphology of these cells.

between triple-positive cell number by FACS and ECFC colony number ($r^2=0.09$; $P = 0.18$; Figure 3A). Term CB did demonstrate a significantly higher percentage of $CD34^+$ cells ($1.48 \pm 0.33\%$ vs. $0.58 \pm 0.13\%$; $P < 0.03$), $CD34^+/CD133^+$ cells ($1.25 \pm 0.27\%$ vs. $0.36 \pm 0.10\%$; $P < 0.01$), and $VEGFR-2^+$ cells ($1.62\% \pm 0.41\%$ vs. $0.59 \pm 0.21\%$; $P < 0.05$). However, these trends inversely correlate with the number of ECFCs. There was no significant difference in the number of $CD133^+$, $CD34^+/VEGFR-2^+$, or $CD133^+/VEGFR-2^+$ cells (data not shown). Term and preterm ECFCs showed no difference in the $CD45^-/CD34^+$ cell fraction ($P = 0.83$). Additionally, there was no correlation between a sample's ECFC colony number and the percentage of $CD45^-/CD34^+$ MNCs before culture ($r^2=0.006$; $P = 0.75$; Figure 3B).

Preterm ECFCs Show Increased Growth Compared with Term ECFCs

In comparison with term ECFCs, preterm ECFCs proliferated at a greater rate. The FI from Day 0 was 19.2 ± 2.4 vs. 8.7 ± 1.0 at Day 6 ($n = 5$ in each group; $P < 0.01$ by two-way ANOVA; Figure 4). The rate of proliferation of term ECFCs decreased

upon reaching confluence. In contrast, preterm ECFCs continued to increase in number despite reaching confluence.

Exposure to Ambient Hyperoxia Decreases the Growth Potential of Preterm ECFCs

Term ECFCs were exposed to high concentrations of oxygen ($FI_{O_2} = 0.80$ and 0.60) in serial experiments. Cell proliferation was not only inhibited, but cell detachment and death occurred as demonstrated by decreasing cell counts in preliminary studies (data not shown). When exposed to mild hyperoxia ($FI_{O_2} = 0.40$), the growth potential of ECFCs from term infants was not significantly different from that of room air controls ($FI = 7.30 \pm 0.61$ vs. 7.71 ± 0.55 ; $P = 0.70$; Figure 5A; results representative of ECFCs from four patients). However, preterm ECFC cell number was significantly decreased after exposure to hyperoxia ($FI = 58.09 \pm 4.19$ vs. 34.71 ± 2.29 ; $P < 0.01$; Figure 5B; result representative of ECFCs from four patients). Yet even in hyperoxia, preterm ECFC growth still exceeded that of many term ECFCs. When the results of all eight experiments were compiled, the difference in cell number due to hyperoxia on Day 6 was significantly larger in preterm ECFCs ($28.9 \pm 6.8 \times 10^4$ cells; $n = 4$) than term EPCs ($2.0 \pm 7.1 \times 10^4$ cells; $n = 4$; $P < 0.05$; Figure 5C).

Treatment with Antioxidants Rescues Preterm ECFCs from the Effect of Hyperoxia

To assess the contribution of oxidant stress on impaired ECFC growth, we treated preterm ECFCs with the antioxidants SOD and catalase. When preterm ECFCs were treated with SOD during the exposure to hyperoxia ($FI_{O_2} = 0.4$), growth was improved at Day 4 compared with hyperoxia control subjects ($FI = 12.71 \pm 1.66$ vs. 5.39 ± 1.16 ; $P < 0.01$) but not Day 6 ($FI = 13.15 \pm 0.14$ vs. 12.79 ± 0.31 ; $P = 0.35$; Figure 6A). Treatment with catalase during hyperoxia restored the growth of preterm ECFCs to a level similar to room air controls ($FI = 19.98 \pm 0.72$ vs. 21.99 ± 0.77 ; $P = 0.13$), well above that of hyperoxia-exposed control subjects ($FI = 19.98 \pm 0.72$ vs. 12.79 ± 0.31 ; $P < 0.01$; Figure 6B). Combined treatment with both SOD and catalase during hyperoxia resulted in Day-6 growth greater than that of both hyperoxia-exposed controls (27.64 ± 2.51 vs. 12.79 ± 0.31 ; $P < 0.001$) and room air controls (27.64 ± 2.51 vs. 21.99 ± 0.77 ; $P < 0.01$; Figure 6C). We repeated this experiment with ECFCs from four different patients and saw similar results. Combined treatment with SOD and catalase consistently resulted in significantly improved growth over that of hyperoxia-exposed controls.

DISCUSSION

In this study, we show that preterm CB samples have greater numbers of ECFC progenitor cells than CB from term newborns as evidenced by the appearance of ECFC colonies in culture. We report that the number of ECFC colonies does not correlate with the traditionally defined "triple-positive" EPC population by FACS. We also found that preterm ECFCs proliferate at a greater rate than term ECFCs. However, mild hyperoxia slows the growth of preterm ECFCs, whereas term ECFCs are not affected by similar levels of hyperoxia. Treatment with SOD and catalase preserves the growth of preterm ECFCs exposed to hyperoxia.

The precise definition of an EPC has been the subject of much debate during the past decade (24). The colonies originally identified by Asahara in 1997 demonstrate an endothelial-like morphology and stain positively for both endothelial and immature hematopoietic cell surface antigens (11). These early

Figure 2. (A) Flow cytometry of term and preterm ECFCs at passage 3. Each single-stain result (gray) is compared with the appropriate isotype control (white). Both term and preterm ECFCs stain were strongly positive for CD31, CD105, and CD146, but negative for CD38, CD45, and CD90. ECFCs were negative for CD133, a marker of cell immaturity. ECFCs are weakly positive for CD34. All antibodies were preconjugated with fluorescein isothiocyanate (FITC) except for CD133-phycoerythrin (PE). (B) Immunohistochemistry of term (left) and preterm (right) ECFCs stained for DAPI-positive nuclei (blue), VE-cadherin (red), and von Willebrand factor (green). (C) Tube formation assay. Both term (left) and preterm (right) ECFCs form tube-like structures when suspended in a mixture of collagen and EBM-2 media.

EPCs (CFU-ECs) are derived from the initially nonadherent fraction of mononuclear cells (MNCs) and appear after 5 to 7 days when cultured on fibronectin (24). CFU-ECs consist of central clusters of round cells with sprouts of spindle-shaped cells at the periphery. The authors noted that the colonies resemble the island-like character of the quail epiblast, a previously described cluster of hemangioblasts (39). In contrast, ECFCs are obtained by plating MNCs on collagen and following the adherent fraction for 14 to 21 days until endothelial-like colonies appear (23). Unlike highly proliferative ECFCs, early-EPCs do not proliferate significantly *in vitro* and have the characteristics of a cell type better described as an angiogenic macrophage (26, 40). However, both cell types may be involved

in normal vascular development and the physiologic response to vascular injury (26, 41, 42).

The cells we studied (ECFCs) are not a homogeneous population. They are more accurately defined as colonies of cells that contain subpopulations of lineage-committed endothelial progenitors. These endothelial progenitors are cells with high proliferative potential that have the capacity for self-renewal and will differentiate into terminally differentiated endothelial cells (22). The characterization of these subpopulations of cells is the subject of ongoing experiments. However, in this study, for convenience, we refer to all of the cells in these colonies as ECFCs.

Although both CFU-ECs and ECFCs can be consistently isolated by the assays described above, attempts to identify the

Figure 3. ECFC colony number (on Day 14) plotted against flow cytometry results for both preterm samples (solid circles) and term samples (open circles). (A) There is no correlation between ECFC colony number and the percentage of CD45^{dim}/CD34⁺/CD133⁺/VEGFR-2⁺ MNCs before culture ($r^2 = 0.09$; $P = 0.18$). (B) There is also no correlation between ECFC colony number and the percentage of CD45⁻/CD34⁺ MNCs before culture ($r^2 = 0.006$; $P = 0.75$).

phenotype of these cells by FACS have proven to be more difficult. Using FACS, EPCs are classically identified as rare “triple-positive” cells that express CD34 and CD133 (surface markers associated with hematopoietic progenitors) as well as the vascular endothelial growth factor receptor-2 (VEGFR-2, an endothelial marker) (11, 43–45). Further, the EPC is thought not to express the hematopoietic marker CD45. The notion that EPCs coexpress these three classic markers has not been confirmed by attempts to culture endothelial cells from MNCs sorted for these markers by FACS (40). Numerous surface antigens have been associated with EPCs: CD14, CD31, CD105,

CD144, CD146, von Willebrand factor (vWF), c-Kit, Tie-2, E-selectin, and others (11, 46–48). However, studies have not been successful in culturing endothelial cells from circulating cells isolated by FACS irrespective of the chosen surface antigen profile. In our present study, we found a four-fold increase in the number of ECFC colonies in preterm cord blood, yet we did not find a significant difference in the number of triple-positive cells identified by FACS between term and preterm cord blood samples. This suggests that triple-positive cells may not reflect the subpopulation of circulating cells defined as ECFCs. There was also no correlation between ECFC colony number and the fraction of the CD34⁺ population that is completely CD45⁻ (not dim). Previous studies suggested that this population may contain ECFCs (40). Clearly, the surface antigen profile of ECFCs is not fully understood. Based on the fact that ECFCs appear in culture at a frequency of 1 to 5 colonies per 10⁷ MNCs plated, it follows that even if a cell surface phenotype was known, the isolation of these cells by FACS would require analysis of 10⁷ to 10⁸ events for reproducible and reliable enumeration, whereas in current protocols, 10⁵ to 10⁶ cells are typically analyzed (49–51).

We report that preterm ECFCs proliferate more rapidly than term ECFCs, which themselves proliferate more rapidly than ECFCs derived from adult peripheral blood (22). These findings suggest that the function of ECFCs may change as an organism matures. Significant expansion of the pulmonary microvasculature occurs during late-gestation fetal development. This appears to correlate with the rapid degree of proliferation we have seen in this study. It is no surprise that ECFC proliferation remains increased, to a lesser degree, at term (compared with adult ECFCs) given the fact that lung vascular and alveolar development continues during the first several years of life. Microvascular expansion also contributes to organogenesis in the retina, the renal glomerulus, and elsewhere during late gestation. Further *in vitro* and *in vivo* studies are needed to better understand the impact of oxidant stress on progenitor cells in specific organ systems as well as their role in normal vascular development. In healthy mature adults, where little vasculogenesis occurs, the circulating progenitor is thought to primarily play a role in vascular homeostasis and the response to injury. Adult ECFCs, from healthy individuals, proliferate much more slowly than ECFCs from either term or preterm cord blood (22). A better understanding of the mechanisms influencing circulating ECFC levels in preterm infants may lead to the ability to mobilize adult ECFCs to aid in the repair of the vasculature after injury.

Whereas previous studies have shown that ECFC colonies are increased in umbilical cord blood compared with adult peripheral blood (22), we report a distinct difference in ECFC colony number and function between cord blood at two distinct

Figure 4. Comparison of growth of ECFCs harvested from preterm versus term CB samples. Growth of preterm ECFCs (solid line; $n = 5$) is greater than term ECFCs (dashed line; $n = 5$). ECFCs were plated in low-serum medium and counted in triplicate over 6 days. Error bars represent SEM (* $P < 0.01$). The right graph shows ECFC growth by Day 6 for the five independent experiments from each group, expressed as fold-increase versus baseline values.

Figure 5. (A) Term ECFC growth is not affected by exposure to hyperoxia (solid line, room air; dotted line, 40% O₂; $P =$ not significant). (B) Hyperoxia decreases growth of preterm ECFCs (solid line, room air; dotted line, 40%; $*P < 0.001$). Figures are representative of four independent experiments. (C) Mean difference in Day 6 ECFC number. Hyperoxia decreases the number of preterm ECFCs ($*P < 0.05$ vs. term controls). Error bars represent SEM.

maturation time points. Before this study, ECFC growth had not been evaluated in hyperoxic conditions. In this study, we show that the *in vitro* susceptibility of cord blood-derived ECFCs to hyperoxia is greater when an infant is born prematurely. The growth of preterm ECFCs is preserved with antioxidant therapy. We suspect that exposure to hyperoxia results in the increased generation of superoxide and that extracellular recombinant human SOD acts to scavenge these free radicals. Furthermore, because SOD converts superoxide into hydrogen peroxide (H₂O₂), catalase treatment provides further protection from hyperoxia by decomposing H₂O₂ to water and oxygen gas, thus reducing cellular exposure to ROS (52). The fact that preterm ECFCs are more susceptible to oxidative stress correlates with the clinical observation that postnatal oxygen therapy contributes to the development of BPD in preterm newborns. Further studies are needed to determine if exposure to hyperoxia contributes to the patho-

Figure 6. (A) Treatment with superoxide dismutase (SOD) partially rescues preterm ECFCs from hyperoxia-induced growth attenuation (solid line, room air; dotted line, 40% O₂; dashed line, SOD; $*P < 0.001$). (B) Catalase therapy (Cat) more effectively preserves growth potential in preterm ECFCs exposed to hyperoxia (solid line, room air; dotted line, 40% O₂; dashed line, Cat; $*P < 0.01$; $\#P < 0.001$). (C) Combined treatment with both SOD and Cat more effectively protects from hyperoxia than either therapy alone (solid line, room air; dotted line, 40% O₂; dashed line, SOD + Cat; $*P < 0.001$; $\#P < 0.001$; $\dagger P < 0.01$). Results are representative of four independent experiments. Error bars represent SEM.

genesis of BPD via impairment of circulating progenitor cell proliferation.

Potential limitations of this study include the fact that EPC level (as reflected by the number of colonies in cord blood) may be varied throughout gestation and this study was not sufficiently powered to detect differences within preterm populations. Additionally, we did not obtain samples from extremely preterm infants (GA < 28 wk), certainly an important group of patients. The causes of preterm birth are numerous and may affect both the number of circulating EPCs and their *in vitro* functionality. It is possible that the etiologies of preterm birth (e.g., chorioamnionitis, infection, and maternal drug use) influence the observed changes in ECFC colony number. In this

study, we did not collect prospective data on the outcomes of these preterm infants, but studies are ongoing to determine if cord blood ECFC levels correlate with the development of BPD.

We conclude that cord blood-derived ECFC colonies appear in greater number in preterm newborns. We emphasize that CD45⁻/CD34⁺/CD133⁺/VEGFR-2⁺ cells characterized by FACS do not correlate with the number of ECFC colonies cultured from cord blood. The increased rate of proliferation of preterm ECFCs may reflect the rapid microvascular growth that occurs during this stage of prenatal development in the lung and other organ systems. We speculate that exposure to hyperoxia contributes to the development of BPD in preterm newborns by impairing the *in vivo* functionality of circulating EPCs. This may contribute to the disruption in vascular growth and homeostasis that results in the changes in lung structure seen in infants with BPD. Further investigation is needed to understand the mechanism by which oxidative stress impairs ECFC growth and function. Improved methods to identify patients at risk for dysmorphic vascular development and therapies that promote vascular growth may serve as potential targets for reducing the incidence of BPD in preterm infants.

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