Expanding the Pool of Stem Cell Therapy for Lung Growth and Repair

Running title: Kourembanas; ECFC Function and BPD

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Key words: Bronchopulmonary dysplasia, mesenchymal stem cells, paracrine mechanisms, Editorial
The lung’s regenerative capacity resides within long-lived stem cells that can divide, self-renew, and differentiate to repair injured tissue or cell loss and maintain normal homeostasis. The ability to enhance endogenous stem cell capacity to regenerate lung tissue is the key to the treatment of a multitude of debilitating lung diseases such as bronchopulmonary dysplasia (BPD), idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, pulmonary arterial hypertension (PAH), and other acute and chronic ailments of the lung. The challenge lies in identifying the progenitors of a tissue and, in the case of the lung, understanding the complex interactions lung progenitors have with the unique environment of an air-liquid interphase, proximal and distal airways, the intricate vascular tree, and the innate immune response. Indeed, many different lung stem/progenitor cells have been described, and their identity and role in lung regeneration continue to be debated.

In the last decade, endothelial progenitor cells (EPCs) have been isolated from the lungs of animals and shown to have vasculogenic activity. Vasculogenesis was thought to occur only in the yolk sac of the developing embryo but this dogma was challenged in 1997 by Asahara and coworkers who first reported the isolation and characterization of putative endothelial progenitor cells from human peripheral blood, showing they can differentiate into mature endothelial cells and be incorporated in the vessels of animal models of disease. Lung vascular development is closely linked with and may drive lung growth and airway development through the release of endothelial derived angiogenic factors that induce proliferation of epithelial progenitor cells to support lung alveolization. Hence, lung EPCs may play a critical role not only in normal lung development but also in lung injury and repair to restore endothelial cell function and maintain homeostasis. By extension, inability of lung EPCs to maintain lung vascular integrity and repair endothelial cell dysfunction would allow injury to evolve and lead
Several studies in recent years have revealed the biology of EPCs to be quite complex with the existence of more than one cellular phenotype, with cell surface marker expression varying with time in culture, and with the *in vivo* vasculogenic potential being indirect and not necessarily limited to or even to include cell differentiation and incorporation into the vasculature. The first level of complexity stems from the specific EPC phenotype under study and the precise definition of an EPC. Most studies use flow cytometry to select cells expressing CD34, CD133, or vascular endothelial growth factor receptor-2 (VEGFR-2). These cells do not adhere to fibronectin and form colonies within five days in culture that have low proliferative capacity and exhibit macrophage-like phagocytic activity. Unlike these cells, an alternative population of putative EPCs has been isolated from peripheral blood that adheres to collagen, is highly proliferative, and generates colonies within 14-21 days ("late out-growth" EPCs).\(^3\,4\) It is these endothelial colony-forming cells (ECFCs) rather than the "early out-growth," hematopoietic-like, angiogenic progenitors that form chimeric vessels in *in vivo* models of angiogenesis. Both EPC types have been widely investigated as biomarkers of human disease and tested as therapeutic agents in preclinical models.

The early-outgrowth angiogenic EPCs have been reported to correlate inversely with disease in some studies, but the number and vasculogenic potential of ECFCs may be the strongest biomarker of endothelial dysfunction in pulmonary and systemic vascular disease. Dysfunction of ECFCs is linked to many pathological states such as diabetes or preeclampsia. ECFC dysfunction has been reported in infants of diabetic and pre-eclamptic mothers as well as in low birth weight infants, and may underlie intrauterine growth restriction.\(^5\) In diseases of the lung, ECFC dysfunction has been proposed to play a pathogenic role in patients with familial...
PAH that have mutations in bone morphogenetic protein type II receptor and in infants who develop BPD.

The angiogenic EPCs have been extensively tested in several preclinical models as well as in patients with cardiovascular, pulmonary, or peripheral vascular disease, and, while shown to be safe, have had limited clinical benefit. ECFCs have been used as therapeutic agents in multiple preclinical models of systemic vascular disease and have been shown to have protective effects (reviewed by ). Although ECFCs have in vivo vasculogenic activity in animal models and can incorporate into blood vessels, it is not clear if they promote vessel growth, whereas angiogenic EPCs do not incorporate into vessels but better stimulate vessel growth suggesting perhaps a combination of these two cell types may be required to optimize regenerative therapy.

ECFCs have been previously isolated from rat and mouse lung and in this issue of Circulation, Alphonse and colleagues report the successful isolation of ECFCs from human fetal lung. They demonstrate that human fetal lung ECFCs are susceptible to hyperoxia in culture and lose their vasculogenic potential with decreased capillary-like network formation and decreased proliferation compared with normoxic cells. Similar ECFC dysfunction was noted in ECFCs isolated from the lungs of neonatal rats that had suffered alveolar growth arrest from exposure to hyperoxia. Interestingly, these authors further showed that intravenous delivery of human umbilical cord blood-derived ECFCs to immunocompromised mice prevented alveolar injury from hyperoxia exposure as well as pulmonary hypertension and right ventricular hypertrophy, features of severe BPD. These results are particularly meaningful when put in the context of the underlying pathophysiology of BPD, a disease that is characterized by an arrest in lung growth with reduced alveolar number and blood vessels. Multiple reports support the notion
that arrest in vascular growth drives alveolar arrest in BPD (reviewed by ¹², ¹³). Of interest, despite the known vasculogenic properties of ECFCs described above, engraftment of cells in the lung vasculature was observed to be minimal in this model and the reparative ECFC effect was recapitulated with the daily administration of cell-free media. This paracrine mechanism of action is reminiscent of the mechanisms by which mesenchymal stem cells (MSCs) are thought to exert their therapeutic effects¹⁴ and are similar to those of the angiogenic EPCs. The fact that these authors demonstrated dysfunction of the endogenous lung ECFCs from hyperoxia exposure suggests that exogenous stem cell therapy with healthy, nondysfunctional ECFCs is able to restore endogenous stem cell function, and that this could be achieved with either cell or cell-free treatment. This is supported by their findings that following infusion of human cord blood-derived ECFCs, recipient rat lung-derived ECFCs manifest restored vasculogenic function in ex vivo experiments. Although not addressed in this study, it would be informative to determine whether ECFCs isolated from the injured hyperoxic rat lung are unable to rescue BPD injury when compared with normal lung-derived progenitor cells, thus lending credence to the hypothesis that ECFC dysfunction plays a key role in BPD pathophysiology and that stem cell treatments should be targeted towards restoring endogenous ECFC function.

A previous study by Baker et al, reported that both ECFCs and ECFC conditioned media prevented PAH associated with BPD in a neonatal bleomycin model, but neither had a protective effect on the architectural alveolar injury.¹⁵ Differences in the experimental models used (hyperoxia vs. bleomycin) as well as differences in dosing regimens may explain the disparate results. Nonetheless, these findings highlight the need for further work on ECFC characterization, identification of specific cell markers, and a better understanding of ECFC biology to decipher the putative reparative role of these EPCs in BPD.
Many questions remain regarding the best choice of stem cell type(s) to be applied in regenerative medicine, not only for the treatment of lung disease but also for many other human conditions that pose a therapeutic challenge to modern medicine. An ever increasing body of literature reports on the use of MSCs to treat various diseases of inflammation and hypoxic-ischemic injury in the cardiovascular and nervous systems, among others. These cells are thought to have low immunogenicity\(^\text{16, 17}\) and can be readily isolated from healthy donor bone marrow or umbilical cord blood, among other sources, expanded in vitro and banked to be tested in an allogeneic manner for patients with various ailments. Indeed, in 2012, MSCs were approved in Canada for the treatment of children with steroid resistant graft vs. host disease, and more recently a phase I clinical trial of stored cord blood-derived MSCs was reported for BPD in preterm infants.\(^\text{18}\) The mechanism of MSC action remains elusive but is known to occur in a paracrine manner, perhaps by the release of immunomodulatory mediators, some of which are packaged in exosomes, microvesicles that carry nucleic acid material, proteins and lipids.\(^\text{19}\) Similar microvesicles may be the carriers of ECFC action reported in the current study, potentially in cooperation with other mediators which ultimately act on the immune system to dampen inflammation, cell injury, and apoptosis, or restore the function of endogenous progenitor cells by enhancing their ability to proliferate and differentiate in order to repair the lung. The present study provides support for this mechanism of repair, and builds on previous work implicating vasculogenesis as a critical partner to lung growth.

For their ultimate use in the treatment of vascular diseases including BPD, a great deal needs to be learned about the biology of ECFCs in both normal and diseased states where accumulating literature suggests there is ECFC dysfunction. This attribute of ECFCs is a drawback to using autologous cells to regenerate the injured vasculature. Heterologous ECFCs
will induce an immune response and hence immunosuppressive therapy would be required. Recent studies investigate methods to restore ECFC function that could result in healthy EPCs for effective cell therapy. In addition, as the field of iPS technology advances, an alternative therapeutic option may be to differentiate the patient’s somatic cells into ECFCs with restored vasculogenic function; these cells can then be administered to the same patient, theoretically avoiding immune surveillance, although not proven at this time. Given that each progenitor cell may have varying capacity to repair tissue or modulate inflammation, testing the combination of more than one stem cell type in preclinical models and eventually in human clinical trials with the parallel testing of cell free components will identify the best therapeutic option with the least risk of adverse consequences.

This is an exciting time in the field of regenerative medicine where the study of stem cell biology is exploding in parallel with intervention studies in preclinical models and in human trials to treat a wide spectrum of otherwise incurable diseases. At the same time, extreme caution must be exercised to avoid causing harm in the long run given the potential tumorigenic property of stem cell therapy and its particular relevance to the human newborn infant whose immune system is not fully developed. This requires the careful design of long-term follow-up studies to evaluate for such potential adverse complications. The authors in the present study should be commended for evaluating the long-term outcome of the treated animals, and it is encouraging to see that none of the treated animals developed tumors at 10-month follow-up. A better understanding of stem cell mechanism of action is critically-important and should be aggressively pursued as we carefully advance forward with clinical testing in humans.

**Funding Sources:** This work was supported by NIH grants R01 HL055454, R01 HL085446, T32 HD007466
Conflict of Interest Disclosures: None.

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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

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