Acellular Embryonic Stem Cell-Derived Matrices for Vascular Regeneration

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Statement of Purpose: Stem cell therapies typically stimulate a robust angiogenic response despite minimal repopulation of the host vasculature, suggesting that stem cells may provide paracrine factors that transiently induce endogenous angiogenesis of tissues undergoing regeneration. Early differentiating embryonic stem cell (ESC) aggregates, referred to as embryoid bodies (EBs), can undergo vasculogenic differentiation, and also produce extracellular matrix and growth factors that induce proliferation, differentiation, and tissue morphogenesis. The objective of this project is to harness extracellular angiogenic growth factors by deriving a novel acellular stem cell-derived matrix from EBs differentiating towards vascular cell types to stimulate re-vascularization of ischemic tissues.

Methods: D3 mouse ESCs (4 x 10^5 cells/ml; 10 ml per 100 mm plate) were cultured without LIF in rotary suspension culture (40 rpm) to induce EB differentiation. Gene expression analysis was performed via qRT-PCR using EBs cultured for 4, 7, and 10 days of differentiation to compare to undifferentiated ESCs. Using an RT^2 Profiler PCR Array (SA Biosciences), 84 genes for growth factors were assessed simultaneously, in addition to traditional RT-PCR for endothelial cell markers. In order to examine specific growth factor protein production and secretion by EBs into the environment, EB-conditioned media (EB-CM) and acellular EBs were collected and analyzed by ELISA. EB-CM (serum-free, 0.1% bovine serum albumin) was collected following 2 days conditioning by EBs formed for 4, 7, and 10 days with serum. EBs were decellularized using mechanical methods, previously described by our lab (1). *In vivo* studies were performed in 9-week old male athymic mice induced with hindlimb ischemia to analyze the angiogenic potential of acellular EBs, either lyophilized or lyophilized and treated with DNase (L+D). The femoral artery was ligated and cauterized in the left hindlimb to induce severe ischemia. Immediately following ischemic induction, 2.59±0.47 mg of lyophilized matrix or 1.22±0.19 mg of L+D matrix (from equal numbers of EBs) was placed into the ischemic hindlimb for treated animals, while untreated controls were sutured without implantation of matrix. Re-vascularization of the hindlimb was observed for up to 4 weeks and analyzed by laser Doppler perfusion imaging (LDPI) and histology.

Results: After 10 days of EB differentiation, the gene expression of endothelial cell markers - vascular endothelial cadherin and platelet endothelial cell adhesion molecule-1, was increased, and concurrently, the gene expression of 13 angiogenic growth factors, including fibroblast growth factor 2 and vascular endothelial growth factor (VEGF), was increased at least 3-fold compared to undifferentiated ESCs. Even though the EB-CM contained similar levels of total protein content at different time points of differentiation, there was increased protein expression of both VEGF and insulin-like growth factor-2 (IGF-2), a factor implicated in improved skeletal muscle regeneration, with increased EB differentiation time. Furthermore, these two growth factors were retained and significantly increased in content within the lyophilized EBs at later time points of differentiation (Figure 1, top). The initial results of the *in vivo* studies demonstrated that the acellular EB matrix was introduced into the ischemic hindlimb without the need for a delivery vehicle and stably retained for up to 2 weeks post-surgery (Figure 1, bottom). The frequency of limb salvage was higher in both lyophilized and L+D matrix treatment groups, compared to untreated mice, demonstrating that the implanted matrix did not inhibit re-perfusion of the hindlimb. Additionally, LDPI results confirmed the re-perfusion of the salvaged hindlimbs of both treatment groups, with a perfusion ratio of ischemic (left):non-ischemic (right) of ~1, only 2 weeks following ischemia induction. On-going animal studies are being performed to quantify re-vascularization using micro-computed tomography (microCT) analysis.

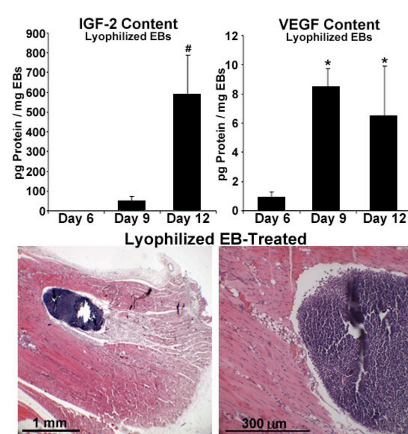

Figure 1. (Top) Retained growth factors, IGF-2 (left) and VEGF (right) in acellular EBs quantified by ELISA. # $p < 0.05$ compared to Days 6 and 9 samples. * $p < 0.05$ compared to Day 6 samples. (Bottom) Hematoxylin & eosin staining of ischemic hindlimb muscle. Lyophilized EBs present 2 weeks after implantation.

Conclusions: These studies demonstrate that the endogenous expression of angiogenic growth factors by EBs increases as endothelial differentiation progresses. Matrix-associated growth factors secreted by early differentiating ESCs retained in lyophilized EBs may affect exogenous cell types involved in regenerating vasculature within an ischemic hindlimb. Acellular matrices derived from vasculogenic EBs could be utilized to stimulate angiogenic responses for a variety of tissue regeneration applications.

References:

(1) Ngangan AV. Biomaterials 2009;30:1143-1149