A Xeno-Free Culture System for Efficient Derivation and Amplification of Human Endothelial Colony-Forming Cells From Umbilical Cord Blood

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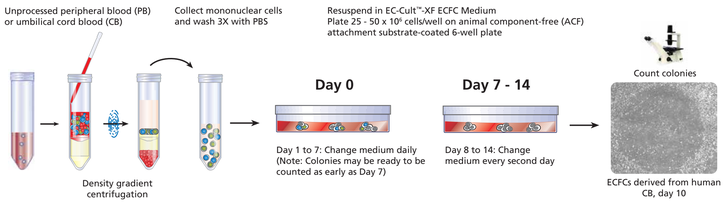
Introduction

Endothelial colony-forming cells (ECFCs) have been identified as endothelial progenitor cells with robust proliferative potential in vitro and for their ability to form new blood vessels in vivo when transplanted in immunodeficient mice [(1) (2) (3)]. Thus, ECFCs have emerged as a favorable target for vascular regenerative therapies for a variety of diseases, including ischemia, diabetic retinopathy, bone repair and cancer. However, the therapeutic study of ECFCs is compromised by low frequency in both peripheral blood (PB) and umbilical cord blood (UCB) and therefore samples must be expanded in vitro prior to use. Current formulations for expanding ECFCs in vitro contain fetal bovine serum (FBS), and as such exhibit experimental variability. Furthermore, animal proteins or serum in the medium raise concern when ECFCs are considered for therapeutic applications. To minimize these risks associated with exposure to animal serum, new culture media are required. Here we characterized ECFCs derived and expanded in EC Cult™-XF ECFC Medium, a novel xeno-free (XF) medium and matrix system to isolate and expand ECFCs from primary CB without the use of FBS. Our data shows that EC Cult™-XF ECFC Medium is highly efficient at supporting cell attachment, clonogenic growth and long-term expansion of ECFCs directly from CB under xeno free culture conditions without any serum requirement.

Materials & Methods



Derivation of ECFC Colonies

Primary UCB was processed by Lymphoprep™ density separation and mononuclear cells (MNCs) were isolated. For outgrowth of ECFC colonies, MNCs were either resuspended in EC Cult™-XF ECFC or serum-containing (SC) media. MNCs (2.5 -5 × 10^7/well) were seeded onto 6- well pre-coated plates with animal component-free attachment substrate. The frequency of ECFC colonies was determined by measuring the total number of colonies in the primary culture on day 10.

Expansion of ECFCs

ECFCs were expanded by passaging at 10,000 cells/cm² in the medium in which they were derived. The number of population doublings (PD) occurring between passages (P) was calculated as log2 (# viable cells at harvest/# cells seeded). The population doubling time (PDT) was derived using the time interval between cell seeding and harvest divided by the number of PDs for that passage.

Single Cell Clonogenic Assays

Early passaged (1 – 2) ECFC-derived ECs were plated at one cell per well into 96-well plates pre-coated with animal component-free (ACF) attachment substrate. Cells were cultured in either EC Cult™-XF ECFC or SC media for 14 days. Cells were fixed with 4% paraformaldehyde, washed and stained with 1.5 µg/mL DAPI, and examined for the growth of ECs. For those wells with more than 50 cells, colonies were imaged and cell number quantified using an Image J1.36v program (Wayne Rasband, NIH).

Immunophenotyping of ECFCs

Early passaged ECFCs (p4-p5) cultured in EC Cult™-XF ECFC or SC media, were analyzed by flow cytometry for expression of endothelial markers CD31, CD144 and hematopoietic marker CD45. To assess the ability of endothelial cells to incorporate DiI-acetylated–low-density lipoprotein (DiI-Ac-LDL), attached cells were incubated with 10 µg/mL DiI-Ac-LDL for 4 hours at 37°C. Cells were washed 3 times and stained with 1.5 µg/mL of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma). Cells were examined for uptake of DiI-Ac-LDL using a Zeiss microscope.

In Vitro Functional Assay

In vitro vascular network formation was tested by seeding 5,000, 7,500, 10,000 and 20,000 cells ECFCs on Matrigel® coated wells of a 96-well plate. The vascular network formation was documented by acquiring images at 2 hour intervals covering a time period of 24 hours using an Olympus TH4-100

In Vivo Functional Assay

To evaluate vascular repair ECFCs derived in EC Cult™-XF ECFC or SC media were used in a mouse model of oxygen-induced retinopathy (OIR). Oxygen-induced retinopathy was induced in C57/BL6 wild-type mice, as previously described[(4)]. Briefly, postnatal day (P) 7 newborn mice received a 0.5 µL intravitreal injection containing EC Cult™-XF ECFC or SC derived ECFCs and were exposed to 75% oxygen for 5 days. DPBS was used as vehicle and injected in the left eye of each pup as a control. At P12 they were transferred back to room air. All pups were euthanized at P17 and retinal flat mounts were stained with isolectin B4-594. Stained retinas were visualized and imaged using a confocal microscope. Obliteration area and neovascularization were quantified using Adobe Photoshop software as described[(4)].

Results

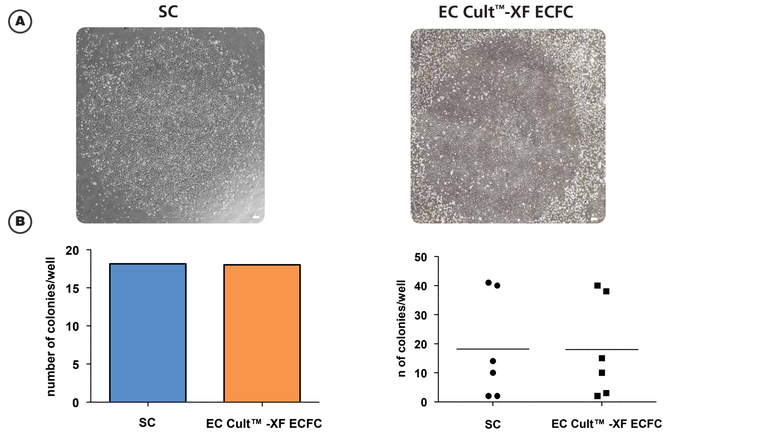


Figure 1: Isolation of ECFCs and number of colonies obtained in SC and EC Cult™-XF ECFC media. A) Colonies derived in either media displayed a cobblestone appearance with variations in size, consistent with heterogeneous proliferative rates previously reported. **B)** No difference was observed in the frequency of ECFCs recovered on day 10 under EC Cult™-XF ECFC and SC media (n = 3 donors).

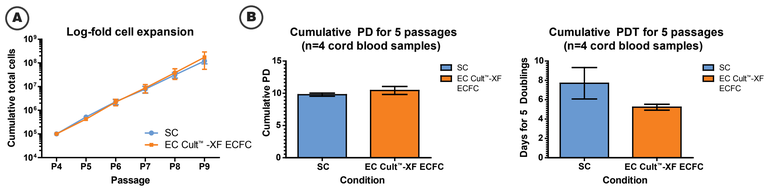


Figure 2: ECFC expansion in SC and EC Cult™-XF ECFC Media. A) Average cell expansion of cultured ECFCs from P4 to P9 in EC Cult™-XF ECFC and SC media. **B)** To quantitate and compare the proliferative kinetics of ECFCs expanded in both media cumulative PD and PDTs were calculated. There was no significant difference in the PD and PDT of EC Cult™-XF ECFC derived ECFC compared with SC derived ones.

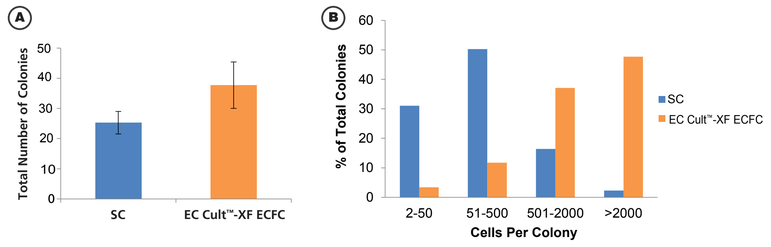


Figure 3: Clonogenic ability is maintained in human CB ECFCs derived and expanded in SC and EC Cult™-XF ECFC Media. A) A significantly higher number of colonies formed from ECFCs isolated in EC Cult™-XF ECFC than in SC medium (37.7 ± 3.5 versus 25.3 ± 6.4, mean ± SEM; $p<0.05$, paired t test). **B)** The entire hierarchy of ECFCs, composed of high proliferative potential (HPP)-, low proliferative potential (LPP)-ECFC, endothelial-cluster, and non-dividing mature ECs was derived from ECFCs cultured in either EC Cult™-XF ECFC or SC media. Remarkably 40% of single-plated EC Cult™-XF ECFC derived ECFCs that divided formed well-circumscribed colonies containing from 2,000 to 10,000 cells in the 14-day culture period.

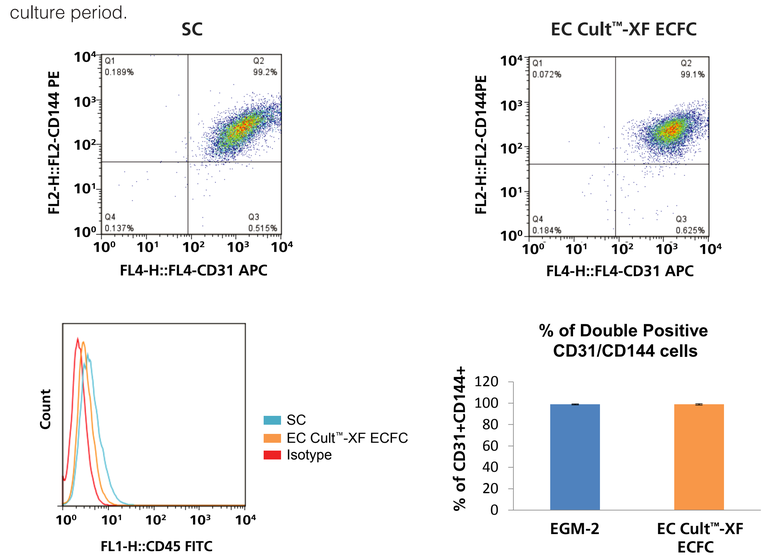


Figure 4: Phenotypic characterization of human CB ECFCs derived and expanded in SC and EC Cult™-XF ECFC Media. Immunophenotyping of the endothelial monolayer revealed that ECs cultured in EC Cult™-XF ECFC medium expressed endothelial cell surface antigens CD31 and CD144, similar with those cultured in SC medium. Most importantly, ECFC colonies cultured in EC Cult™-XF ECFC medium did not express the hematopoietic cell surface antigen CD45.

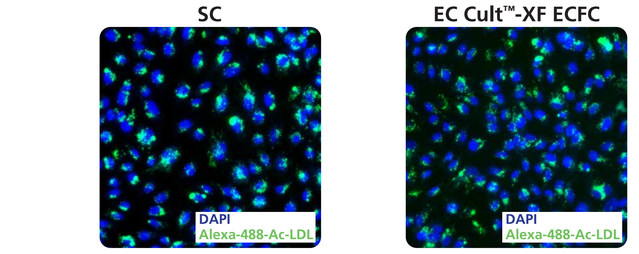


Figure 5: EC Cult™-XF ECFC and SC derived ECFCs subcultured from adherent colonies uniformly incorporated DiI-Ac-LDL. Blue = DAPI; Green = Alexa-488-Ac-LDL.

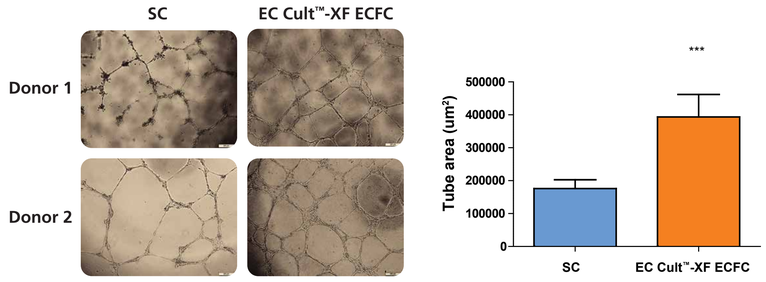


Figure 6: EC Cult™ XF ECFC and SC derived ECFCs form tube-like structures in vitro. Representative images of vascular network formation from 2 donors of ECFCs cultured in SC and EC Cult™ XF ECFC on Matrigel®. Representative pictures are taken at 18 hrs. ECFCs grown EC Cult™ XF ECFC form much bigger tubes than ECFCs grown in SC media ($p>0.0001$, Incucyte™ software).

Oxygen Induced Retinopathy: Animal Model of Neovascularization

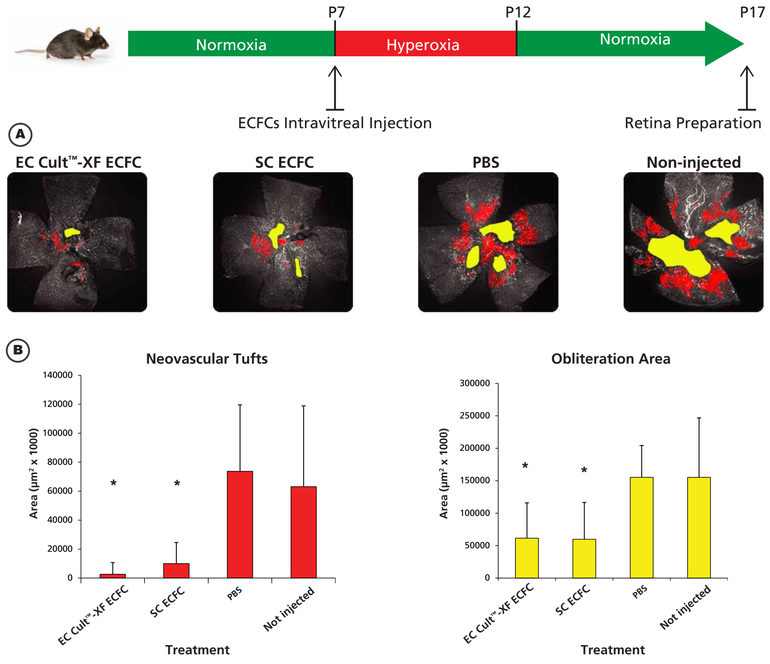


Figure 7: Contribution of ECFCs derived in EC Cult™ XF ECFC and SC media to vascular repair of ischemic retina. In the OIR model, ECFCs derived in both media decreased the areas of vascular obliteration and neovascular tufts compared to control (PBS) ($p = 0.02$ and $p = 0.02$ respectively; n = 10). **A)** Representative flat mounted retinas of mice injected with EC Cult™-XF ECFC, SC ECFC, PBS, and not injected. Avascular areas are indicated in yellow and neovascular tufts are indicated in red. **B)** Graphs represent quantification of neovascular area (red) and Obliteration area (yellow).

Summary

- EC Cult™-XF ECFC is a new medium able to efficiently support attachment, isolation, clonal growth and expansion of ECFCs directly from primary human CB.
- ECFCs cultured in EC Cult™-XF ECFC Medium showed similar expansion to ECFCs cultured in serum control medium.
- For the first time, it is possible to derive and expand ECFCs from human CB in a defined xeno- free culture system facilitating their use in translational research.

References

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4. Medina, R.J., *et al.* Invest. Ophthalmol. Vis. Sci. 51, 5906-5913, 2010.

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