2730 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

Human endothelial colony-forming cells (ECFCs) are endothelial progenitors that can be isolated from umbilical cord blood (UCB) and peripheral blood (PB) (1) (2). These highly proliferative cells possess the unique property of forming functional blood vessels in vivo upon transplantation (3) (4) (5).

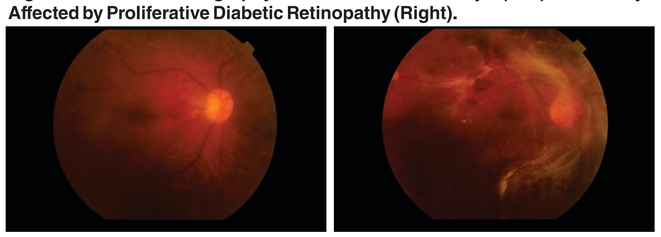
Cell Therapy Applications of ECFCs

ECFCs are particularly attractive for cell therapy for enhanced vascular repair in ischemic diseases as demonstrated by preclinical studies in:

- Peripheral arterial disease (6)
- Ischemic retinopathy (2)
- Myocardial infarction (7)
- Stroke (8)

Ischemic retinopathies such as diabetic retinopathy, retinopathy of prematurity, and retinal vein occlusion are major causes of visual impairment and it has recently been suggested that the common underlying vascular insufficiency of these diseases could be treated using ECFCs (9). ECFC cell therapy has also been shown to have impressive efficacy in murine models of retinal disease (2, 10), and therefore, there is a growing basis for using these cells in patients.

Figure 1. Fundus Photography of a Human Normal Eye (Left) and an Eye Affected by Proliferative Diabetic Retinopathy (Right).



Images provided by Dr. Susumu Sakimoto.

Barriers to Research and Clinical Applications

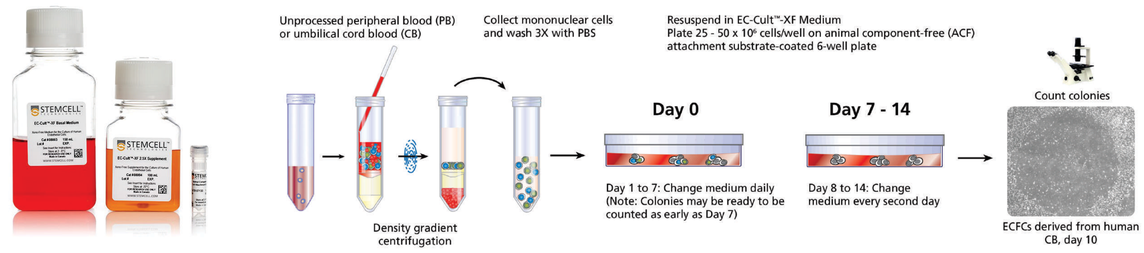
The utility of ECFCs for therapeutic applications is compromised by low frequency in both PB and UCB, and therefore samples must be expanded in vitro prior to use. Culture media currently used for expanding ECFCs in vitro contain fetal bovine serum (FBS) and often exhibit variable performance. Furthermore, animal proteins or serum in such media raise concern due to their undefined chemical compositions and/or potential immunogenicity. To minimize the risks associated with exposure to animal serum, a xeno-free culture medium is required.

Rationale and Results

Here we characterized ECFCs derived and expanded in EC-Cult™-XF Medium, a novel xeno-free (XF) medium and matrix system for isolation and expansion of ECFCs from primary UCB without the use of FBS. Our data show that EC-Cult™-XF Medium is highly efficient at supporting cell attachment, clonogenic cell growth, and long-term expansion of ECFCs directly from UCB. Moreover, intravitreally injected ECFCs, derived and amplified in EC-Cult™-XF Medium, can reside in the vitreous and accelerate retinal vascular repair both morphologically and functionally in a mouse model of ischemic retinopathy (10).

PROTOCOL

Figure 2. Product Format and Protocol



Derivation of ECFC Colonies

Mononuclear cells (MNCs) were isolated from primary UCB by Lymphoprep™ density centrifugation. For outgrowth of ECFC colonies, MNCs were resuspended in either EC-Cult™-XF Medium or serum-containing (SC) medium and seeded (2.5 - 5 x 10^7/well) into 6-well plates coated with animal component-free (ACF) attachment substrate. The frequency of ECFC colonies was determined by measuring the total number of colonies in the primary culture on day 10.

Sample Source	Minimum Suggested Blood Volume For Processing	Time Of Colony Appearance
Fresh cord blood (cells plated < 6 hours of delivery)	35-50 mL	Day 4 onwards
Older cord blood (> 18 hours post delivery)	60-70 mL	Days 7-15
Adult peripheral blood	100 mL	Days 14-28

Expansion of ECFCs

ECFCs were expanded by passaging 10,000 cells/cm^2 in the medium from which they were derived for 10 passages. The number of population doublings (PD) occurring between passages (p) was calculated as $\log_2$ (No. viable cells at harvest / No. 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.

Clonogenic Cell Assays

Early passaged (p1 - p2) ECFCs were plated at one cell per well into 96-well plates (Corning #3603) coated with ACF attachment substrate. Cells were cultured in either EC-Cult™-XF Medium or SC medium for 14 days. Cells were fixed with 4% paraformaldehyde, washed and stained with 1.5 µg/mL of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), and examined for the growth of ECs. Cells were imaged on the ImageXpress Micro 4 high-content imaging system (Molecular Devices) and cell number was quantified using the "count nuclei" application on the MetaXpress 6 (Molecular Devices).

Immunophenotyping of ECFCs

ECFCs (p4 - p5) cultured in EC-Cult™-XF Medium or SC medium were analyzed by flow cytometry for expression of endothelial markers CD31 and CD144, and the hematopoietic marker CD45. To assess the ability of endothelial cells to incorporate acetylated–low-density lipoprotein (Ac-LDL), attached cells were incubated with 10 µg/mL Alexa Fluor® 488-Ac-LDL (Molecular Probes) for 4 hours at 37°C. Cells were washed and stained with 1.5 µg/mL DAPI. Cells were examined for uptake of Alexa Fluor® 488-Ac-LDL using a Zeiss microscope.

In Vitro Functional Assay

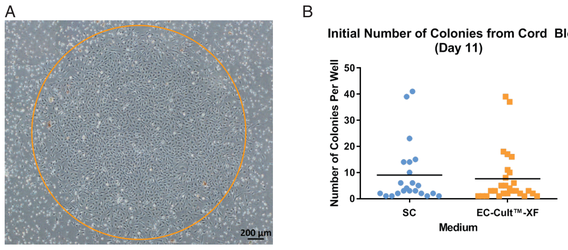
In vitro vascular network formation was tested by seeding 5000, 7500, 10,000, and 20,000 ECFCs on Matrigel™- coated wells of a 96-well plate. Vascular network formation was documented by acquiring images at 2-hour intervals covering a time period of 24 hours using an Olympus TH4-100.

In Vivo Functional Assay

To evaluate vascular repair potential, ECFCs derived in EC-Cult™-XF Medium or SC medium were tested in a mouse model of oxygen-induced retinopathy (OIR). OIR was induced in C57BL/6 wild-type mice as previously described (10-11). Briefly, postnatal day 7 (P7) newborn mice received a 0.5 µL intravitreal injection containing EC-Cult™-XF-derived or SC-derived ECFCs and were exposed to 75% oxygen for 5 days. D-PBS was used as the vehicle and injected in the left eye of each pup as a control. At P12, mice 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 (11) .

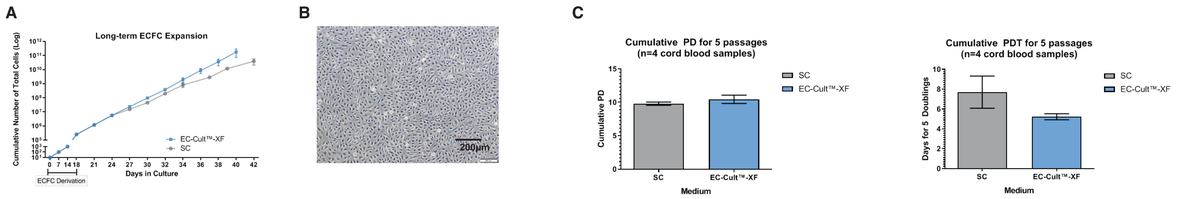
RESULTS:

Figure 3. EC-Cult™-XF Medium Facilitates Isolation of Similar Numbers of ECFC Colonies as SC Medium



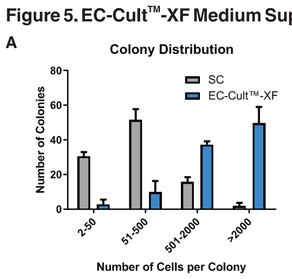
A) A representative phase contrast image of an ECFC colony at day 11 derived from human UCB in EC-Cult™-XF Medium.
B) No difference was observed in the frequency of ECFCs recovered on day 11 from EC-Cult™-XF and SC media (n = 13 donors).

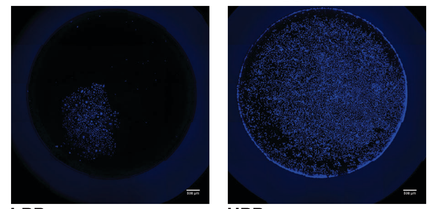
Figure 4. Superior Expansion of ECFCs Cultured in EC-Cult™-XF Compared to SC Media



A) Human CB-derived ECFCs expanded in EC-Cult™-XF Medium demonstrate a greater expansion rate compared to ECFCs expanded in SC medium. At late passages, cells cultured in EC-Cult™-XF Medium continue to expand at the same rate as in early passages, while the expansion rate of ECFC cultured in SC medium started to decline as early as p6 (n = 4).
B) A representative phase contrast image of ECFCs derived and expanded in EC-Cult™-XF Medium at p3. These cells grow as monolayers and retain the typical cobblestone-like morphology of endothelial cells.
C) To compare the proliferative kinetics of ECFCs expanded in both media, cumulative PD and PDTs were calculated.

Figure 5. EC-Cult™-XF Medium Supports the Derivation and Expansion of More Primitive Human CB ECFCs That Generate Large Colonies





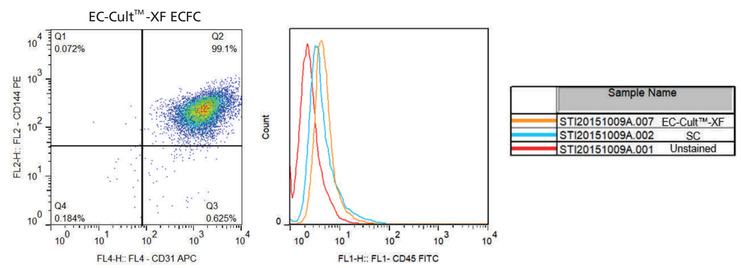
Clonogenic Assay:

LPP: Low **P**roliferative **P**otential Colonies
HPP: High **P**roliferative **P**otential Colonies (with Self-renewal potential)

Colony Type	% in SC Medium	% EC-Cult™-XF
LPP	82	13
HPP	18	87

A) EC-Cult™-XF Medium supports and maintains (HPP)-ECFCs
B) Percentage of LPP-ECFCs and HPP-ECFCs obtained with SC medium and with EC-Cult™-XF medium (Table 1).

Figure 6. Human CB ECFCs Derived and Expanded in EC-Cult™-XF Media Express ECFC Markers



Immunophenotyping of the endothelial monolayer revealed that ECs cultured in EC-Cult™-XF expressed endothelial cell surface antigens CD31 and CD144. Most importantly, ECFC colonies cultured in EC-Cult™-XF did not express the hematopoietic cell surface antigen CD45.

Figure 7. EC-Cult™-XF-Derived ECFCs Subcultured from Adherent Colonies Are Functional and Incorporate Ac-LDL

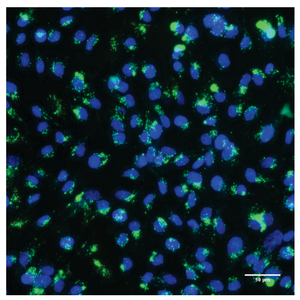
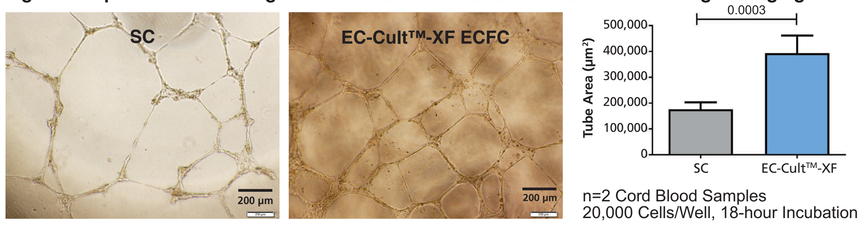


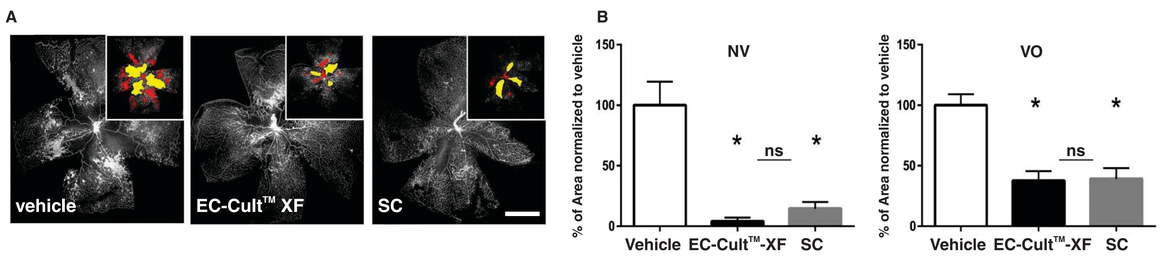
Figure 8. Superior Tube-Forming Potential of EC-Cult™-XF-Derived ECFCs in a Matrigel® Angiogenic Assay



n=2 Cord Blood Samples
20,000 Cells/Well, 18-hour Incubation

Representative images of vascular network formation from one donor of ECFCs cultured in SC or EC-Cult™-XF media on Corning® Matrigel®. Representative pictures are taken after 18 hrs in each medium. ECFCs cultured in EC-Cult™-XF form larger tubes than cells cultured in SC medium ($p > 0.0001$, Incucyte™ software).

Figure 9. ECFCs Derived in EC-Cult™-XF Medium Mediate Vascular Repair of Ischemic Retina



A) Representative flat-mounted retinas of mice injected with vehicle, EC-Cult™-XF ECFCs, and SC ECFCs. Retinal vasculature stained with Isolectin B4. Avascular areas are indicated in yellow and neovascular tufts are indicated in red.

B) Quantification of neovascular area (NV) and vascular obliteration area (VO). In the OIR model, ECFCs derived in both media decreased the areas of vascular obliteration and neovascular tufts compared to vehicle (D-PBS) (*p = 0.02 for ECFCs cultured in either medium vs. control, n = 10).

SUMMARY

ECFCs possess outstanding vessel-forming potential in vivo that can be further augmented upon culturing in EC-Cult™-XF Medium.

- EC-Cult™-XF is the first xeno-free medium for deriving, expanding, and maintaining human ECFCs
- EC-Cult™-XF maintains the entire ECFC hierarchy, supporting particularly the survival and growth of HPP ECFCs
- ECFCs show greater expansion (> 10 passages) in EC-Cult™-XF Medium than in current commercially available serum-containing media
- Cultured ECFCs demonstrate a strong reparative effect in an animal model of ischemic retinopathy

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