Measuring Colony Forming Potential of Human iPSC-derived ECFCs *in vitro*

Introduction

Human iPSC-derived Endothelial Colony Forming Cells (hiPSC-ECFCs) (Axol Bioscience) are highly expandable and show comparable expression and functionality to primary cells, providing a robust and physiologically relevant tool for use in numerous applications.

ECFCs are rare circulating endothelial cells that have a high proliferative potential and a capacity for self-renewal making them key players in angiogenesis and the repair of damaged blood vessels. ECFCs circulating in the vascular endothelium have been identified to have a wide range of clonal growth potentials[1]. Professor Mervin Yoder's laboratory at Indiana University, identified that ECFCs display a complete hierarchy of proliferative potential. By plating single cells into a well and scoring the growth of the colonies after 14 days, Prof .Yoder observed four different colony forming capabilities; a single cell (non-dividing endothelial cell), small colonies (endothelial clusters), larger colonies (ECFCs with low proliferative potential (LPP-ECFCs)) or very large colonies (ECFCs with high proliferative potential (HPP-ECFCs)). The largest colonies were able to give rise to colonies of the same size or greater highlighting the potential of self-renewal. The hierarchy of proliferative potentials of ECFCs was presented at ISSCR 2017[2] by Prof. Yoder, this talk can be downloaded from our website.

http://www.axolbio.com/page/presentations

The heterogeneous population of ECFCs can be segregated by conducting a clonogenic assay. This application note gives technical information on identifying endothelial colonies and their proliferation potential.

Clonal assays are useful for screening for toxic compounds that either enrich or diminish one particular proliferative population of ECFCs, as an increase in HPP-ECFCs may be beneficial for promoting vascular repair whereas this increase can aid the vascularisation of tumours enhancing the progression and nourishment of cancer cells[3].

Research has shown that, mycophenolate, a drug used in the treatment of vasculitis, reduces the most proliferative population of ECFCs (HPP-ECFCs) and has also been shown to decrease the formation of new blood vessels[4]. In this guide, the effect of mycophenolate on ECFCs is shown along with the protocol on how to apply this toxicity screen in your lab.

Key points

- Human iPSC-derived ECFCs show a hierarchy of proliferative cell types equivalent to human primary cells, making them an excellent translatable human model for studying vascular repair and tumour vascularisation.
- Axol Human iPSC-derived ECFCs are a pure population of ECFCs from one donor as such they are a reliable reproducible source of hiPSC-ECFCs.
- This study shows that the hierarchy of proliferative potential in the Human iPSC-derived ECFCs population are applicable for toxicology screening of agents that target proliferating ECFCS or screening of potential drugs for cancer therapy.

Aims & Objectives

- This study investigated whether Axol Human iPSC-derived ECFCs were able to form colonies *in vitro* that hold a hierarchy of proliferative potentials.
- To compare the in vitro clonogenic activity of hiPSC-ECFCs with human primary umbilical cord blood (CB) ECFC.
- Showcase the application of the clonogenic assay in toxicology screening, to identify the susceptible ECFC population.

Materials and Methods

ECFC preparation

CB-derived ECFC were isolated from citrate phosphate dextrose anti-coagulated human umbilical cord blood mononuclear cells (MNC). The MNC were obtained by diluting 1:1 fresh cord blood with Hanks balanced salt solution (HBSS; Invitrogen, Grand Island, NY), then overlaying the suspension onto an equivalent volume of Histopaque 1077 (ICN, Costa Mesa, CA) and using 740 x *g* for 30 minutes to obtain a buffy coat of MNC. The isolated MNC were assessed for viability and then plated at 10 X 10^6 cells per well of a 6-well plate in Endothelial Growth Medium BulletKit (EGM-2) (Lonza, Walkersville, MD). The culture medium was replaced on days 3, 7, and 10 of culture. CB-ECFC emerged between days 7 and 10 of culture. The adherent colonies were released from the tissue culture wells using trypLE Express (Invitrogen) and the cell suspension was replated into T25 flasks in EGM-2 medium.

Axol Human iPSC-Derived ECFCs (ax2019) (1 X10^6) were removed from liquid nitrogen storage and swirled constantly in a 37ºC water bath to thaw the cells (until a small ice clump was evident). The contents of the vial were transferred to a sterile centrifuge tube containing 3 mL of pre-warmed EGM-2 medium, a cell viability test was performed, and the cells were added to one well of a 6-well plate (previously coated with 10 µg/mL human plasma fibronectin). The cell culture medium was replaced the following morning.

Single cell clonogenic assay

CB-ECFCs or hiPSC-ECFCs were tested in a single-cell assay to evaluate clonogenic proliferative potential. Briefly, endothelial cells were treated with trypLE Express (Invitrogen) to obtain a single-cell suspension. Cell counts and serial dilutions were performed to obtain a concentration of 0.68 cells per well in individual wells of 96-well culture plates. Wells were examined the day after plating to ensure the presence of a single cell per well. Culture medium was changed on days 4, 8 and 12. On day 14 of culture, cells were stained with Sytox reagent (Invitrogen), and each well was examined to quantitate the number of cells using a fluorescent microscope. Those wells containing two or more cells were identified as positive for proliferation under a fluorescent microscope at 10x magnification using a Zeiss Axiovert 25 CFL inverted microscope with a 10x CP-ACHROMAT/0.12 NA objective. Wells with endothelial cell counts of 2–50, 51–500, 501–2,000 and ≥2,001 were labelled as endothelial cell clusters (cluster), low proliferative potential ECFCs (LPP-ECFC) and high proliferative potential ECFCs (HPP-ECFC), respectively. Cloning efficiencies were similar with the CB-ECFC and hiPSC-ECFC with colonies in approximately 25-35% of the wells.

Mycophenolate treatment

Mycophenolate stock solution was made by dissolving mycophenolic acid powder (Selleck Chemical, Houston, TX) in 0.6% methanol before diluting in EGM-2 culture medium to make final concentrations of 1 µM and 2.5 µM. Single endothelial cells (EC) were sorted and placed into each well of a 96-well tissue culture plate pre-coated with collagen using a FACSAria Sorter (Becton Dickinson, San Jose). The medium was replaced with fresh medium in the absence or presence of drug at various concentrations after 24 hours. Cells were incubated at 37°C with 5% CO_2 for 13 days with a change of medium every 5 days. On day 14, each well was then examined for colony formation using an inverted microscope under 100x magnification.

Results

The single cell clonogenic activity of the Axol hiPSC-ECFCs was equivalent to human primary CB-ECFC (**Figure 1**) with most of the single cells giving rise to LPP-ECFC or HPP-ECFC. The utility of this clonogenic assay can be observed when the drug mycophenolate was added at concentrations (known to inhibit human umbilical vein endothelial cell proliferation) to the CB-ECFC and cells were plated in the single cell assay (**Figure 2**). The mycophenolate at 1 µM concentration abolished growth of HPP-ECFC and significantly diminished LPP-ECFC. At a higher concentration of 2.5 µM mycophenolate treatment completely suppressed growth of LPP- and HPP-ECFC and only ECFC clusters emerged in culture. In addition, the treatment of CB-ECFC with mycophenolate hampered colony forming efficiency by 21% and 11% for cells treated with 1 µM or 2.5 µM mycophenolate, respectively.

Conclusions

Axol hiPSC-ECFCs display single cell clonogenic activity similar to human primary CB-ECFC. Given the known variability in CB-ECFC isolation and culture (due to variability in obtaining fresh anti-coagulated umbilical cord blood at delivery), the hiPSC-ECFCs represent an easily obtained commercial product that can be utilized in screening chemicals or drugs to examine anti- or pro-proliferative effects on endothelial cells.

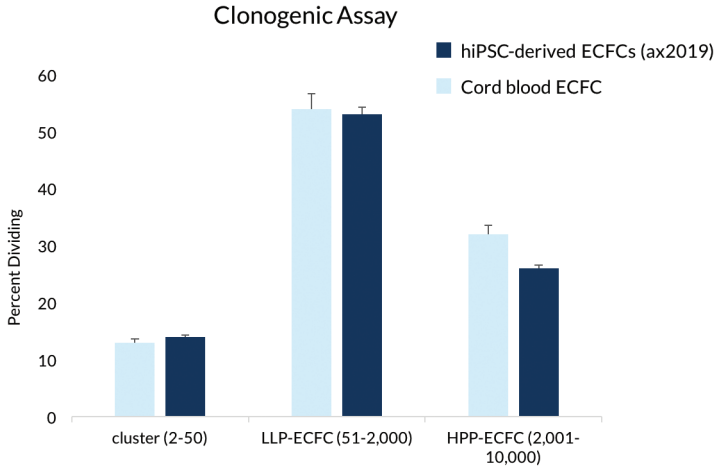

Figure 1: Mycophenolate treatment inhibited the formation of moderate (51-2000 cells; LPP-ECFC) and high proliferative (2001-10,000; HPP-ECFC) ECFCs.

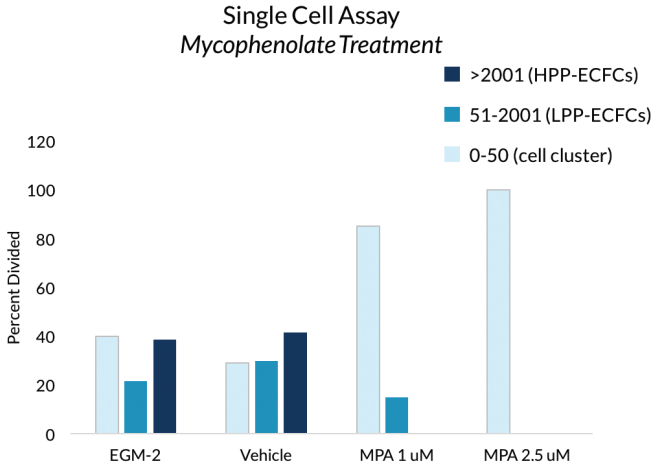

Figure 2: Single cell clonogenic assay shows the percentage of dividing cells that gave rise to endothelial colonies of various sizes.

References

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