Video Article

Isolation and Large Scale Expansion of Adult Human Endothelial Colony Forming Progenitor Cells

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Abstract

This paper introduces a novel recovery strategy for endothelial colony forming progenitor cells (ECFCs) from heparinized but otherwise unmanipulated adult human peripheral blood within a mean of 12 days. After large scale expansion >1x10^8 ECFCs can be obtained for further tests. Advantageously by using pHPL the contact of human cells with bovine serum antigens can be excluded. By flow cytometry and immunohistochemistry the isolated cells can be characterized as ECFC and their *in vitro* functionality to form vascular like structures can be tested in a matrigel assay. Further these cells can be subcutaneously injected in a mouse model to form functional, perfused vessels *in vivo*. After long term expansion and cryopreservation proliferation, function and genomic stability appear to be preserved. [3,4]

This animal-protein free isolation and expansion method is easily applicable to generate a large quantity of ECFCs.

Video Link

The video component of this article can be found at https://www.jove.com/video/1524/

Protocol

A.) ECFC ISOLATON

DAY 1:

1. Before you start with the experiment prepare the cell culture medium Endothelial Growth Medium-2 (EGM-2). Supplement 500ml Endothelial Basal Medium-2 with heparin (1ml of 1000 U/ml), 5ml 100x solution of penicillin/ streptomycin (all Sigma, St. Louis, MO; www.sigmaaldrich.com), L-glutamine (2 mM final concentration), and 50 ml pooled human platelet lysate (pHPL). Then add 0.2 ml hydrocortisone, 2 ml hFGF-B, 0.5 ml VEGF, 0.5 ml EGF, 0.5 ml IGF-1 and 0.5 ml ascorbic acid (provided as SingleQuots, all Lonza, Walkersville, MD; http://www.lonzabioscience.com/). Sterile filtrate the medium through a 0.2 m-pore size 500 ml Millipore vacuum filter. Since pHPL forms small clots you may need two filters for one medium. Pre-warm filtered EGM-2 to 37°C in a water bath
2. Collect the blood samples by drawing 5 ml of adult human peripheral blood from the cubital vein into a 6ml preservative free sodium-heparin blood vacutainer tube. You should process the blood samples within a maximum of two hours.
3. Start by preparing one 75 cm^2 (T75; Costar) flask with a vented cap, one 25 ml pipette and two 5 ml pipettes in a laminar flow bench.
4. Pre-fill 15 ml pre-warmed supplemented EGM-2 into the flask with the 25 ml pipette. Now open the blood vial and sample all 5 ml with the 5 ml pipette into the flask. Rinse the empty blood vial with 5 ml of additional EGM-2 with the second 5 ml pipette. The total volume within the T75 is 25 ml. Close the vented-cap of the flask and place in an incubator at 37°C, 5 % CO_2 and 95 % humidity for 24 hours.

DAY 2:

1. After approximately 24 hours (over night) you should remove the medium-blood mixture to get rid of non adherent cells. For this prepare two 25 ml pipettes and three 5 ml pipettes in the laminar flow, pre-warm 20 ml of supplemented EGM-2 (prepared on the previous day) and 30 ml PBS to 37°C in a water bath.
2. Remove the flask with the sample from the incubator and remove all the supernatant with a 25 ml pipette. Take care not to scratch the flask surface to avoid damaging any potential colony. Wash the inner flask cell adherence surface three times by gently adding 10 ml of pre-warmed PBS and tilting the flask from one side to the other. Remove the PBS immediately each time and discard.
3. Add 20 ml of fresh pre-warmed EGM-2 to the flask and place it back into the incubator. This process should be performed as fast and gentle as possible for the attached cells not to detach. ECFCs detach easily if they are kept in PBS or at room temperature for to long.

4. Screen through the flask systemically every second day starting at day two using a low magnification of a light microscope looking for ECFC outgrowth. When a colony is found mark on top at the outer side of the flask indicating the position of the colony. Note that the flaks should not be outside of the incubator for more than 5 minutes.

DAY 5:

1. On the fifth day after seeding perform a complete medium change to remove non-adherent cells. For this purpose pre-warm 20 ml of EGM-2 to 37°C in a water bath and prepare two 25 ml pipettes.
2. Remove the complete medium using a 25 ml pipette and add fresh, pre-warmed supplemented EGM-2 as soon as possible. Cells should not be left without medium for more than a minute to avoid drying. Place back into the incubator and repeat screening the flask daily.
3. Feed the cells by replacing 30 % of medium by fresh supplemented EGM-2 twice weekly.

DAY 14-28:

1. Typical colonies with cobble stone morphology should appear around day 12. Mark the top outer side of the flask to indicate the position of each colony. Allow the colonies to grow until day 28 unless single cells start to detach. Once, primary colonies have been identified they can be harvested between days 14 and 28, depending on their size.

B.) ECFC HARVEST

1. Pre-warm 5 ml trypsin 0.05%/EDTA, 20 ml PBS and 5ml fresh supplemented EGM-2.
2. Completely remove medium from the flask into a Falcon tube (take care not to touch the surface of the flask to avoid damaging any potential colony) and wash cells carefully with 10 ml preheated PBS.
3. Add 5 ml trypsin/EDTA and incubate for 5 minutes in the incubator. The cells should not be exposed to trypsin for more than 7 min since it becomes toxic. Thoroughly tapping the sides of the flask helps cells to detach. Quench trypsin protease activity by adding approximately 10 ml of the preserved used culture medium.
4. Remove cell suspension into a 50 ml Falcon tube. Wash the flask once with 10 ml PBS which is thereafter added to the harvested cell suspension. Centrifuge the cell suspension at 300 g for 7 min at 4°C to collect the cells and remove the trypsin. Discard the supernatant and resuspend the cell pellet immediately in 1 ml PBS. Keep on ice.
5. Determine cell number as described in a JoVE protocol by R. Ricardo and K. Phelani.[5]

C.) ECFC EXPANSION

1. Supplement 500 ml EGM-2 as described in A 1.1). Pre-warm to 37°C in a water bath.
2. For approximately 2500 cm^2 culture space prepare a either 11 T220 (225 cm^2) or 15 T175 (175 cm^2) or one four-layered cell factory (CF-4; Nunc, Naperville, IL; http://www.nuncbrand.com). Additionally you will need two fresh vent-caps and a special sterile funnel in the laminar flow bench.
3. To seed ECFCs at a starting cell density of 100 c/cm^2 calculation of the cell number is necessary. For one CF-4 252,800 cells are resuspended in 500 ml fresh EGM-2 and poured into the CF-4 using the sterile funnel. Close one vent with the vent-cap and tilt the CF-4 to one longitude side, enabling an equal distribution of the medium between all 4 flours. Then tilt the chamber back and open the lid of the vent-cap. Place CF-4 in incubator.
4. Change 20 % of the medium at least once weekly until CF-4 is confluent.
5. Harvest cells as described in B.) by using 70 ml of trypsin/EDTA and approximately 200 ml PBS.

REPRESENTATIVE RESULTS

Using this isolation method we observed a primary colony formation at day 12. In a previous study were able to recover a mean of 4.0 ± 0,8 ECFC-colonies derived from each ml of peripheral blood.[4] ECFCs showed a typical cobble stone appearance. After large scale expansion, with a seeding density of 100 cells/cm^2, we obtained> 1 x 10^8 cells in a total of approximately 30 days from 3 male and 1 female volunteers (age between 26 and 50 years).

ECFC cultured under these conditions were shown to be proliferative, functional and genomically stable.[4] Furthermore cells can be stored (in 10 % DMSO) in liquid nitrogen for further use. [3,4]

Discussion

This novel isolation and expansion method is a simple and efficient process that is more efficient and less time consuming by avoiding any cell separation (no density gradient needed) and less expensive than conventional techniques. By using pHPL contact to bovine antigens and putative subsequent xenoimmunization is excluded. During the culture period small clots can form, caused by the pHPL. Based on our experience these clots do not effect colony formation and cell proliferation or function.

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