Video Article

Generation and Culture of Blood Outgrowth Endothelial Cells from Human Peripheral Blood

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Abstract

Historically, the limited availability of primary endothelial cells from patients with vascular disorders has hindered the study of the molecular mechanisms underlying endothelial dysfunction in these individuals. However, the recent identification of blood outgrowth endothelial cells (BOECs), generated from circulating endothelial progenitors in adult peripheral blood, may circumvent this limitation by offering an endothelial-like, primary cell surrogate for patient-derived endothelial cells. Beyond their value to understanding endothelial biology and disease modeling, BOECs have potential uses in endothelial cell transplantation therapies. They are also a suitable cellular substrate for the generation of induced pluripotent stem cells (iPSCs) via nuclear reprogramming, offering a number of advantages over other cell types. We describe a method for the reliable generation, culture and characterization of BOECs from adult peripheral blood for use in these and other applications. This approach (i) allows for the generation of patient-specific endothelial cells from a relatively small volume of adult peripheral blood and (ii) produces cells that are highly similar to primary endothelial cells in morphology, cell signaling and gene expression.

Video Link

The video component of this article can be found at https://www.jove.com/video/53384/

Introduction

Until recently, the post-natal generation of new blood vessels was believed to occur exclusively through a process known as angiogenesis, defined as the sprouting of new vessels from the endothelial cells of pre-existing vessels.[1] This process contrasts from the vasculogenesis, or the *de novo* formation of blood vessels from endothelial progenitors, which was thought to occur exclusively during embryogenesis.[2] However, more recent studies have identified and isolated circulating endothelial progenitor cells (EPCs) in the peripheral blood of adults. These cells possess the capacity to differentiate into mature endothelial cells in culture and are believed to participate in postnatal vasculogenesis.[3,4]

Protocols for the isolation and expansion of these EPCs typically involve the culture of peripheral blood mononuclear cells (PBMNCs) in media containing endothelial growth factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor-2.[5-8] EPC cultures produce a variety of dramatically different cell types. Initial cultures (<7 days) are dominated by a monocytic cell type, known in the literature as "early" EPCs. Despite their name, these cells express the monocyte marker CD14, are negative for the progenitor marker CD34 and express only minimal levels of the classical endothelial markers CD31 and VEGF receptor 2 (VEGFR2).[5] Continued culture gives rise to a secondary population of cells, known as late outgrowth EPCs or blood outgrowth endothelial cells (BOECs), which appear as discreet colonies of endothelial-like cells. Unlike the monocytic early EPCs, BOECs, which have also been called endothelial colony forming cells (ECFCs), outgrowth endothelial cells or late-outgrowth endothelial cells, exhibit the cobblestone morphology that is typical of endothelial cell monolayers and are highly similar in surface marker[5] and gene expression[9] to mature endothelial cells.

The generation of endothelial-like cells from peripheral blood offers several advantages, particularly for the study of the endothelial cell dysfunction associated with vascular disorders such as pulmonary arterial hypertension (PAH)[10] or von Willebrand disease.[11] Prior to the availability of BOECs, endothelial cells could only be derived from explanted organs at time of death or organ transplantation, or isolated from the umbilical vein at birth. This reduced availability represented a serious limitation to understanding the biology of endothelial cells from patients with cardiovascular disorders, as well as the interactions between endothelial cells and either blood cells or mural cells. Furthermore, isolating and culturing a pure population of endothelial cells from these sources is technically challenging and the cells derived by these methods exhibit only a limited proliferative capacity. BOECs therefore offer a valuable surrogate for the isolation and culture of patient-derived primary endothelial cells.

In addition to their *in vitro* applications, BOECs are also potentially useful in autologous cell transplantation therapies. These applications include both endothelial cell transplantation to promote neovascularization (see [12] and references therein), as well as the generation of induced pluripotent stem cells (iPSCs).[13] BOEC-derived iPSCs can be used for disease modeling and offer immense potential as the starting material

for autologous cell therapies. BOECs reprogram faster and with a higher efficiency than skin fibroblasts. Furthermore, BOECs also allow for the generation of iPSCs that are free of karyotypic abnormalities, which is an essential feature of any technology that will be suitable for translational applications. The ability to generate iPSCs from a patient blood sample also eliminates the need for a skin biopsy and the generation of skin fibroblasts, thereby facilitating the generation of cells from patients with wound healing disorders, or the very young.

The protocol detailed below, approved by and conducted in accordance with guidelines of the National Research Ethics Service Committee (East of England), provides a simple and reliable method for the generation of BOECs with greater than 90% efficiency from a relatively small volume (60 ml) of peripheral blood. These cells are highly proliferative and can be passaged repeatedly, allowing for the generation of hundreds of millions of cells from a single blood sample.

Protocol

A schematic of the BOEC generation protocol is shown in **Figure 1**.

1. Blood Collection and Density Gradient Centrifugation

1. For each donor, add 3 ml of sodium citrate to each of two 50 ml conical centrifuge tubes. Collect 60 ml of blood by venipuncture and add 30 ml to each tube. Invert gently 2-3 times to mix. Use as little as 40 ml of blood in the protocol, with citrate volumes adjusted accordingly.
   NOTE: It is essential that blood samples are processed within 2 hr of collection. Delayed processing of blood results in a marked reduction in outgrowth colony yield.
2. Prepare new conical tubes containing density gradient centrifugation medium by first inverting the bottle several times to mix. In a sterile hood, add 15 ml of density gradient centrifugation medium per 50 ml conical tube (1 tube for every 10 ml of blood, 6 tubes per donor).
3. Dilute blood 1:1 with Dulbecco's Phosphate-Buffered Saline (DPBS). Tilt the tube containing the density gradient centrifugation medium to an angle of 20° with the work surface. Using a pipette aid and a 25 ml serological pipette, slowly layer 21 ml of diluted blood on top of the medium by gradually adding blood down the wall of the tilted tube.
   NOTE: Doing so will minimize mixing of the blood with the density gradient layer and will produce a tighter buffy coat, as well as an enhanced yield.
4. Centrifuge samples at 400 x g for 35 min at RT with the accelerator and the brake off.
5. During this centrifugation period, begin the collagen coating detailed in steps 2.1 - 2.4. Ensure these steps have been completed before proceeding to step 3.1.

2. Collagen Coating of T-75 Flask and Preparation of Culture Medium

NOTE: Carry out the following steps in a cell culture hood.

1. Prepare a 50 µg/ml collagen solution by diluting stock Type I collagen in 10 ml of 0.02M acetic acid. **Example:** for collagen stock at a concentration of 4.05 mg/ml, add 123.5 µL of collagen to 10 ml of 0.02 M acetic acid. Sterile filter collagen suspension prior to use.
2. Using a serological pipette, add 7.5 ml of collagen solution to a T-75 cell culture flask. This volume gives 5 µg collagen/cm^2 (or 375 µg/flask). Coat flask for 1 hr at RT.
3. Prepare the endothelial growth medium used for BOEC generation by supplementing endothelial basal medium with the growth factor supplements provided by the manufacturer. Add all supplements, but do not add serum. To prepare 15 ml of BOEC generation medium, add 2.5 ml of defined fetal bovine serum (FBS) to 12.5 ml of endothelial growth medium containing the growth factor supplements.
4. Proceed with the steps outlined in Section 3. After the 1 hr coating period, aspirate the collagen solution and wash away residual acetic acid by pipetting in 10 ml DPBS. Aspirate off the DPBS and repeat this wash step once more. If the cell suspension obtained during step 3.3 is not ready for plating at this time, add 5mL of BOEC generation medium to the flask to keep the collagen coating from drying-out.

3. Collection and Plating of PBMNCs

1. Following the density gradient centrifugation outlined in step 1.4, carefully collect the buffy coat layer using a sterile plastic transfer pipette. Collection of the buffy coat should yield approximately 20 ml of cell suspension and plasma from each tube. Avoid transferring the density gradient medium.
2. Dilute the mononuclear cell suspension 1:1 in DPBS and invert to mix. Centrifuge at RT for 20 min at 300 x g with brake and accelerator at maximum.
3. Following centrifugation, aspirate the supernatant and resuspend cells by adding 1 ml of BOEC generation medium to each pellet and pipetting up and down repeatedly. Pool cell suspensions and top up total volume to 10 ml with the remaining medium.
4. To get an estimation of total cell number, sample 10 µl of cell suspension and dilute 50x with 490 µl of Turk's solution, which will lyse red blood cells. Count a 10 µl sample of the diluted cell suspension using a haemocytometer.
   NOTE: 60mL of blood should yield approximately 100-150 x 10^6 white blood cells for plating.
5. Plate entire cell suspension into a single, collagen-coated T-75 flask, top-up medium volume to 15mL/flask and culture at 37 °C in a humidified atmosphere containing 5% CO_2. Cells plated at this time represent passage 0.
   NOTE: It is possible to freeze the isolated PBMNCs prior to BOEC isolation in order to derive the BOECs at a later time or in order to transport the PBMNCs to a different laboratory where the BOECs can be generated. However, it is important to note that freezing PBMNCs could reduce the efficiency of BOEC isolation. See section 8 below for method.

4. Long-term Cell Culture

1. Change medium every 2 days by adding 15 ml of fresh BOEC generation medium (5:1 mixture of endothelial cell growth medium and defined FBS). For weekends, medium changes can be delayed to every 3 days.
   NOTE: Outgrowth colonies should appear between 7 and 14 days of culture.
2. Monitor the BOEC culture flask on days 7-14 for the appearance of outgrowth colonies. Identify colonies as circular groups of cells exhibiting a classic endothelial cobblestone morphology.
3. Once a colony or multiple colonies are identified in the flask, continue with medium changes and allow colonies to grow to approximately 1000 to 2000 cells per colony before passaging. Determine the cell number per colony through a rough visual estimate.
   NOTE: As a guide, it is customary to passage initial outgrowth colonies one week after the identification of the first outgrowth colony.
4. Passage cells by rinsing flasks twice with 10 ml of DPBS. Add 5 ml of 1X trypsin-EDTA and incubate in a 37 °C incubator for 5 min. After 5 min, neutralize trypsin with 10 ml of medium (containing FBS) and bring cells into suspension with repeated pipetting.
5. Centrifuge suspension at 300 x g for 5 min, resuspend in 15 ml of fresh BOEC generation medium and plate entire cell suspension into a new T-75 flask (representing passage 1, P1). No collagen coating is required.
6. Continue medium changes as described above until cells are confluent (roughly 3-5 x 10^6 cells per flask). Once confluent, passage cells as described in step 4.3.
   NOTE: From passage 2 onwards, cells no longer require BOEC generation medium and can be cultured in endothelial cell growth medium and 10% standard heat-inactivated FBS. Collagen coating can be used as an optional step going forward, as there is evidence to suggest that the presence of collagen can enhance BOEC proliferation rates.
7. For continued passaging, plate no fewer than 750,000 cells per T-75 flask as low cell densities can cause the BOECs to stop proliferating.

5. Freezing and Thawing BOEC Cultures

1. Trypsinize cells as described in section 4.3 and centrifuge at 300 x g for 5 min. Aspirate the supernatant and resuspend in 10 ml of medium. This does not require endothelial cell growth medium; DMEM with 10% standard FBS will be sufficient. Collect a 10 µl sample of the cell suspension to perform a manual cell count using a haemocytometer.
2. Centrifuge again and resuspend at 2x10^6 cells/ml in ice-cold cryopreservation medium (40% DMEM with 50% FBS and 10% DMSO). Add 0.5 ml (10^6 cells) to each vial, place vials in an ice cold isopropanol cryopreservation vessel and place in a -80 °C freezer for at least 2 hr before transferring to liquid nitrogen. Do not leave cells at -80 °C for more than 24 hr.
3. To thaw cells, add 10 ml of pre-warmed medium to a 15 ml conical centrifuge tube.
   NOTE: When thawing out passage 1 cells, thaw into endothelial cell growth medium supplemented with 20% defined FBS for the first 2 days after thawing. This leads to a more stable cell isolation going forward.
4. Remove cells from liquid nitrogen and thaw in a 37 °C water bath with gentle agitation until only a small ice crystal is left in the vial. Add the contents of the vial drop-wise to the conical centrifuge tube and spin down at 300 x g for 5 min.
5. Aspirate supernatant and resuspend cell pellet in 10 ml EGM-2MV+10%FBS. Add to T-75 flask and top up medium to 15 ml.

6. Characterization of BOECs by Flow Cytometry

1. Once the BOEC colonies described in Section 4 have been allowed to expand, trypsinize cells as described in section 4.4 and resuspend at a concentration of 10^6 cells per ml in staining buffer (DPBS with 2% FBS and 2 mM EDTA).
2. Combine 10^5 cells with fluorochrome-conjugated antibodies directed against CD45, CD14, CD34, CD31 (use all at 1:20 dilution) or VEGFR2 (dilute 1:10) (see **Table 1** for full details). Incubate for 30 min at 4 °C in the dark.
3. Wash cells with 1 ml staining buffer and centrifuge at 300 x g for 5 min. Aspirate supernatant and resuspend in 400 µl staining buffer for analysis. Keep cells at 4 °C and protected from light prior to analysis.
4. Perform cytometric analysis on a cytometer equipped to detect the fluorescently conjugated antibodies used in the assay.
5. Use an unstained BOEC sample to identify the cell population on the cytometer by adjusting forward and side scatter voltages, as well as the voltages for the FITC and APC channels.
6. Determine gating thresholds using isotype stained cells for each fluorochrome. Assess positivity for each cell surface marker based on the presence of a peak shift in fluorescence intensity versus isotype control for the fluorochrome being analysed.

7. Characterization of BOECs by Immunofluorescent Microscopy

1. Plate BOECs in a 24-well, flat-bottom tissue culture plate without coverslips at a density of 5 x 10^4 cells in 500 µl of endothelial cell growth medium + 10% heat-inactivated FBS per well and leave to adhere and grow at 37 °C, 5% CO_2 until cells cover at least 70-80% of the surface area of each well (estimate confluency by visual inspection under a light microsope).
2. Wash cells in each well for 5 min with 1 ml of DPBS.
3. Fix cells O/N at 4 °C with 500 µl of 4% paraformaldehyde solution in DPBS.
4. Wash cells for 3 x 5 min with 1 ml of DPBS per well. Add and remove the DPBS using a pipette and an aspirator, respectively.
5. Permeabilize cells for 3 x 10 min with 0.2% polysorbate 20 in DPBS.
6. Incubate cells for 1 hr at RT in blocking buffer containing 10% FBS in DPBS.
7. Stain cells at 4 °C O/N in 300 µl of antibody dilution buffer (0.1% bovine serum albumin in DPBS) containing primary antibodies directed against CD34 (10 µg/ml), CD144 (1:300) or vWF (1:250). See **Table 1** for full details.
8. Wash off unbound primary antibodies for 5 x 10 min with DPBS.
9. Incubate cells for 1 hr at RT with the appropriate fluorescently-labelled secondary antibody, as detailed in **Table 1** (all at 1:200).
10. Wash off unbound secondary antibodies for 5 x 10 min with DPBS.
11. Incubate cells with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) in DPBS for 10 min at RT.

12. Wash cells with DPBS for a further 3 x 5 min.
13. Image cells at 100x magnification using an inverted microscope equipped with an external light source for fluorescence excitation, and capture images using an attached digital camera for subsequent analysis offline.

8. Freezing and Thawing PBMNCs

1. At the end of stage 3.2, following centrifugation, aspirate the medium and manually disrupt the cell pellet with repeated pipetting up and down using a P1000 pipette tip. Add 1 ml of freezing medium, 90% serum and 10% DMSO, making sure to mix gently to produce a cell suspension. Transfer the cell suspension to a cryovial and freeze and thaw as described in Section 5.

Representative Results

Isolation of buffy coat mononuclear cells from 60 ml of blood typically yields 100-150x10^6 total white blood cells. When plated into a single T-75 flask, the large number of cells in the unlysed cell suspension makes it difficult to resolve individual adherent cells using brightfield microscopy. Repeated medium changes result in the clearance of non-adherent cells and allow for the visualization of the adherent population of monocytic "early" EPCs. At day 7, the T-75 will contain approximately 7x10^6 of these elongated adherent cells. From day 7 to 14, colonies of BOECs should appear within the flask (**Figure 2A**). Colonies first appear as 3 to 5 adjacent cells. Outgrowth colony number can vary from 1 to 10 colonies per 60 ml of blood. Generally, younger donors (*i.e.*, 20-25 years old) tend to produce a greater number of outgrowth colonies, which also appear earlier in culture. BOECs proliferate from this central group of cells to form circular colonies consisting of several hundred cells. Cells within these colonies exhibit a classic endothelial cobblestone morphology.

It is preferable to passage initial colonies when they contain approximately 1000 cells per colony. Outgrowth colonies typically reach this size 7 days after their original appearance in culture. Once the original colonies are passaged into a new T-75 flask, they should proliferate to form a confluent monolayer (3-5x10^6 cells per flask) within 5 days or less (**Figure 2B**). In a small percentage of isolations (<10%), outgrowth colonies fail to appear, or do not proliferate sufficiently following this initial passaging step. BOECs that exhibit low proliferation after their first passaging rarely go on to become stable BOEC isolations and often stop proliferating within two to three passages. The monocytic early EPCs contained within the BOEC cultures are non-proliferative and are typically cleared from the cultures within 1-2 passages. Clearance of these early cells is due to cell death, failure of the early cells to re-adhere after passaging and dilution of the non-proliferative early EPCs with repeated passaging.

Passage 1 cells can either be passaged further in culture or frozen down for later use. Once a stable isolation is generated, cells can be passaged at a rate of 1 confluent flask to 3-5 new T-75 flasks up until passage 8 or 9 before becoming quiescent. Again, cell density is critical throughout culture. In our experience, no fewer than 750,000 cells should be plated in a T-75 flask, as lower cell densities can cause growth arrest. Despite the high proliferative potential of BOECs, cells should also not be allowed to become overconfluent (*i.e.*, >5x10^6 cells per flask) as this can cause conversion of BOECs to a non-proliferative phenotype.

We propose that any cell being labeled as a BOEC should display appropriate cell surface and intracellular staining for endothelial markers. Characterization of BOECs can be achieved by flow cytometry (**Figure 3**) or fluorescence microscopy (**Figure 4**), following staining for typical endothelial cell markers. BOECs are positive for the endothelial surface markers CD31 and VEGFR2 and are negative for the monocyte marker CD14 and the pan-leukocyte marker CD45. BOECs also posess Weibel-Palade bodies and thus express Von Willebrand Factor (vWF) as discrete, punctate cytoplasmic staining. Unlike other mature endothelial cell types, such as pulmonary artery or aortic endothelial cells, BOECs also express the progenitor and activation marker CD34 on their surface.

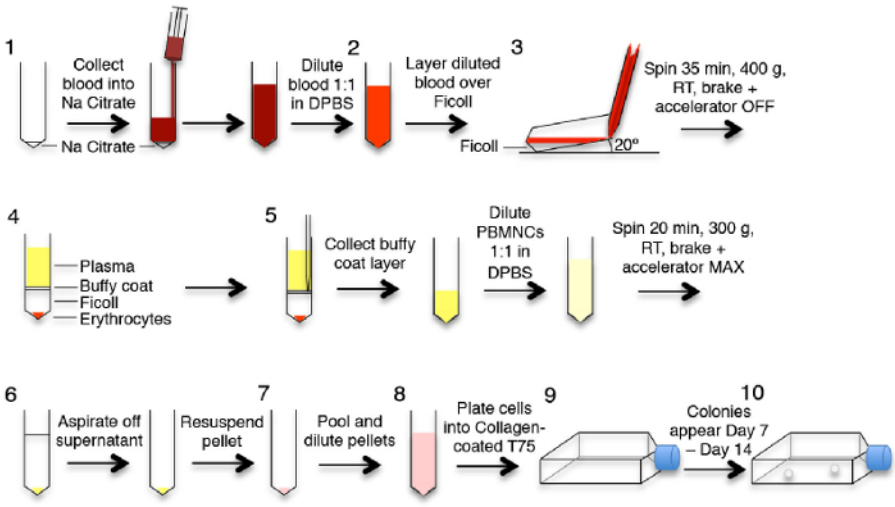

Figure 1. Schematic diagram of BOEC generation protocol. Peripheral blood mononuclear cells are isolated from venous blood by density gradient centrifugation and cultured on collagen-coated plates in endothelial growth medium containing defined FBS. BOEC colonies appear within 7-14 days of culture. Please click here to view a larger version of this figure.

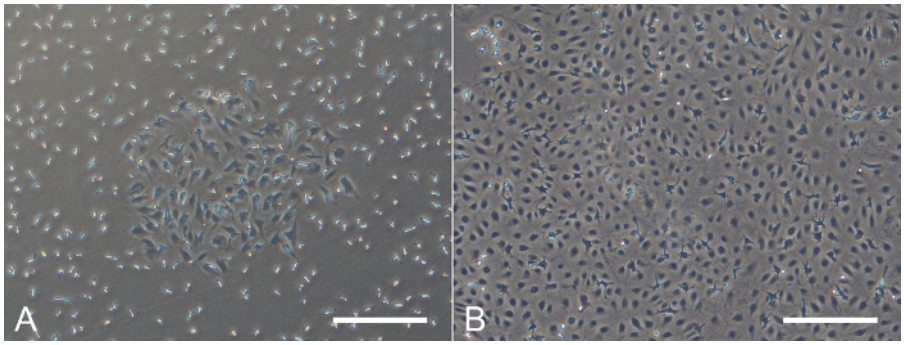

Figure 2. Brightfield images of representative BOEC cultures. (A) Outgrowth colonies appear in cultures between days 7 and 14. Colonies present as collections of endothelial-like cells, which are arranged in a cobblestone monolayer and proliferate radially out from a central point. Surrounding the outgrowth colonies are the adherent monocytic cells that make up the vast majority of cells in early cultures. These cells, previously described as "early" endothelial progenitor cells, have a spindle-like morphology and express the monocytic marker CD14. (B) Following passaging, the highly proliferative BOECs take over cultures, as the non-proliferative monocytic cells either die off or fail to re-attach after passaging. Scale bar 250μm. Please click here to view a larger version of this figure.

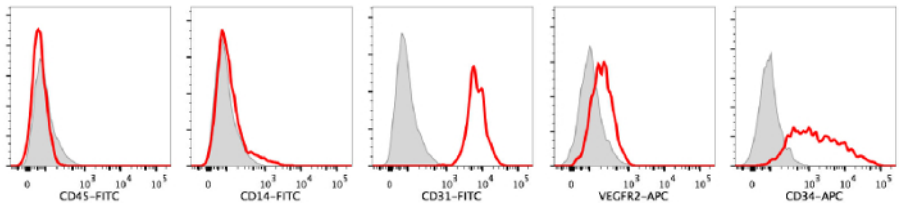

Figure 3. Characterization of BOECs by flow cytometry. BOECs were trypsinized and stained with fluorescently-conjugated isotype control antibodies (grey filled peak) or antibodies directed against specific surface markers (red line). Surface markers for cytometric characterization include the hematopoietic markers CD45 and CD14, the endothelial markers CD31 and VEGFR2 and the progentior and endothelial activation marker CD34. Please click here to view a larger version of this figure.

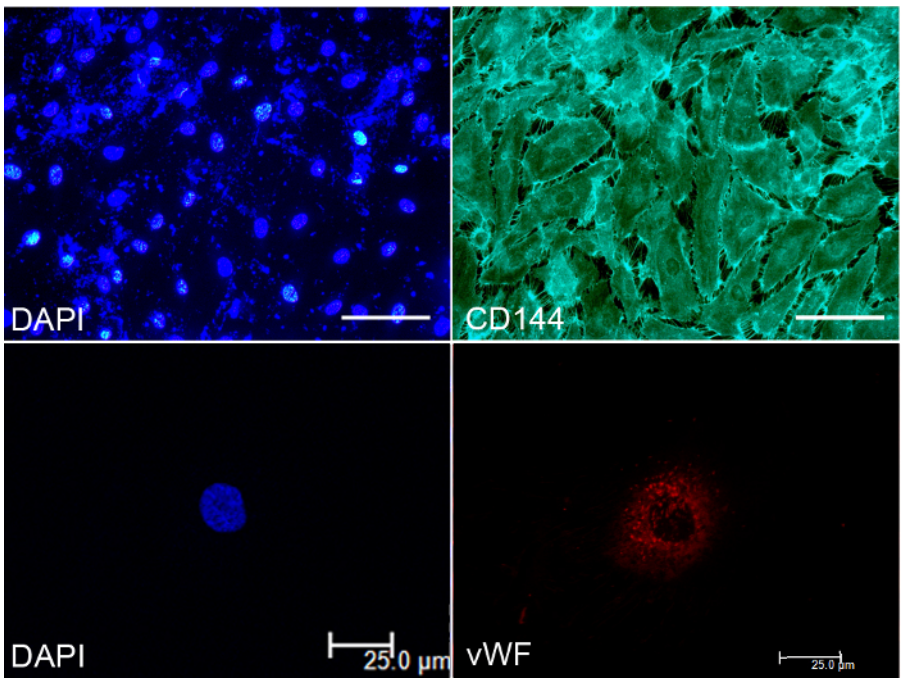

Figure 4. Immunofluorescent staining of BOECs for endothelial cell markers. Representative immunofluorescence images of BOECs immunostained with antibodies directed against endothelial cell surface marker CD144 (VE-cadherin, top right panel) and the blood glycoprotein von Willebrand Factor (vWF, bottom right panel). Corresponding panels showing nuclear DAPI staining are shown to the left. Scale bar 50μm. for CD144. Please click here to view a larger version of this figure.

Discussion

We present a detailed protocol that allows for the robust and efficient derivation of BOECs from adult peripheral blood mononuclear cells (PBMNCs). Our protocol includes two important refinements that represent advances on previous methods of BOEC isolation.[14-16] These include

the absence of heparin in the initial PBMNC culture medium and the use of defined, embryonic stem cell-qualified serum. This latter refinement is of particular importance. Embryonic stem cell (ESC)-qualified serum is a more consistent grade of serum and, although it is not known yet what component(s) are enriched in the serum that benefit BOEC isolation, the impact of this defined serum on the efficiency of BOEC generation is clear in our hands. In addition, we have also had success in isolating BOECs using human serum, thereby allowing for the generation of BOECs for clinical translation. In our hands, this refined protocol results in the successful isolation of stable BOEC cultures from greater than 90% of donors, making it one of the most reliable BOEC generation methods reported thus far. Although the use of particular sera is critical to BOEC generation, it also represents a primary limitation of the current protocol. Future improvements to the technique could include the generation of these cells in serum-free, defined culture conditions.

Critical Steps in the protocol include processing blood samples as soon as possible after collection, complete harvesting of the buffy coat cells after density gradient centrifugation and the timely passaging of initial colonies from P0 to P1. This passaging step is critical to establishment of a stable isolation. Like other endothelial cells, BOECs appear to be very sensitive to plating density. If the plating density after passaging is too low, the BOECs will not proliferate. Conversely, if the colonies are allowed to become overconfluent before passaging, the cells will also cease to proliferate and have the tendency to convert into an elongated, mesenchymal cell phenotype. If few colonies appear from days 7 to 14, or if the colonies are small in size, troubleshooting can include increasing cell density by passaging P0 colonies into a T-25 flask instead of a T-75.

Once the technique is mastered, the resultant BOECs can be used in several applications, including *in vitro* studies of endothelial cell biology, disease modeling and drug screening, as well as *in vivo* cell transplantation therapies. An important consideration for the development of any cell therapy process is to use cells that are free from pathogenic mutations. We have previously shown that BOECs isolated using our protocol possess genomes that are free from copy number variations and are thus representative of the individual from which they were collected. In addition, we have also demonstrated that the majority of BOEC-derived iPSC lines are free from copy number variations.[13] This contrasts with previous reports of copy number variation in fibroblast-derived iPSCs. To date, these cells remain the only iPSCs for which this degree of genomic fidelity has been reported. This feature is important for the field of iPSC biology and the use of iPSCs in disease modeling, drug screening and future cell transplantation therapies.

Disclosures

The authors have nothing to disclose.

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