Bone Marrow-Derived Endothelial Progenitor Cells: Isolation and Characterization for Myocardial Repair

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Abstract

Vascular regeneration with bone marrow (BM) stem/progenitor cells is one of the promising therapeutic strategies for myocardial repair in cardiovascular diseases. Endothelial progenitor cells (EPCs) have demonstrated the beneficial effects on ischemia-induced myocardial damage promoting angiogenesis in ischemic tissue. Isolation of EPCs from BM or peripheral blood is required for the cell-based therapeutic approach. Among a variety of EPC isolation methods, specifically in the case of experimental animal models, we have shown our recently developed mouse-cultured EPC isolation protocol, which is viable for obtaining enough number of viable cells for not only in vitro but also in vivo experiments, and the protocol for human CD34+ cell (EPC rich cell population) isolation from peripheral blood and characterization by fluorescence-activated sorting (FACS) system.

Key words: Bone marrow, Stem/progenitor cell, Regeneration, Angiogenesis, Cell therapy, Cytokine, Growth factor, Ischemia, Myocardium

1. Introduction

Ischemic heart diseases are one of the major causes of death or hospitalization that are limiting our daily life. Therefore, a certain promising therapeutic approach for myocardial repair needs to be developed rather than conventional therapy such as medication alone. Revascularization is well known to be a crucial event during the recovery process in myocardium with ischemic injury. In the traditional view, revascularization of ischemic tissue was thought to occur through the migration and proliferation of mature endothelial cells in nearby tissues – a process called “angiogenesis”; however, recent evidences have indicated that the peripheral blood (PB) of adults contains bone marrow (BM)-derived cells
with properties similar to those of embryonic angioblasts (1–4). These precursor cells have the potential to differentiate into mature endothelial cells and are collectively referred to as endothelial progenitor cells (EPCs). Emerging evidences indicate that the so-called EPC-participating vasculogenesis contributes significantly to postnatal neovascularization. These novel insights into molecular processes that contribute to the formation of blood vessels suggest a potential strategy for treatment of ischemic heart disease, namely, the transplantation of EPCs to induce neoangiogenesis in ischemic myocardium which also plays a critical role for myocardial repair including cardiac functional recovery.

The remainder of this chapter describes detailed protocols for EPC isolation from mouse BM and CD34+ cell (EPC rich cell population) isolation from human peripheral blood with the characterization not only as a distinct progenitor of endothelial cell (EC), which directly contributes to vasculogenesis (5,6), but also as a heterogeneous EC-like cell population which can promote angiogenesis indirectly (7,8).

## 2. Materials

### 2.1. Mouse BM Mononuclear Cell Isolation

1. 2,2,2-Tribromoethanol (AvertinTR, Sigma, St. Louis, MO) is dissolved in tissue-culture water at 20 mg/mL with heat and stored in aliquots at 4°C protecting from light.
2. Chemical hair remover (NairTR, Church & Dwight Co., Inc., Lakewood, NJ).
3. 10% Povidone/Iodine prep pad (Dynarex PVP Iodine Prep Pads, Fisher Scientific, Pittsburgh, PA).
5. Forceps (Tissue Forceps Slim – 1 x 2 Teeth, FST).
6. Disposable 1 mL syringe with 23G needle (Terumo, Tokyo, Japan).
7. Disposable 3 mL syringe with 18G needle (Terumo).
8. 10 cm cell culture dishes (Corning, Lowell, MA).
11. 40 µm Cell Strainer (BD Falcon, Tokyo, Japan).
12. 5 mM EDTA/DPBS: Solution of 5 mM ethylenediamine tetraacetic acid (EDTA) in Dulbecco’s Phosphate Buffered Saline (DPBS) (Invitrogen, Tokyo, Japan).
13. 50 mL and 15 mL conical tubes (BD Falcon).
14. Histopaque 1083\textsuperscript{TR} (Sigma) stored at 4°C protecting from light.
15. 0.8% (w/v) NH\textsubscript{4}Cl with 0.1 mM EDTA: Ammonium chloride solution (StemCell Technologies, Vancouver, Canada).

2.2. Mouse BM Mononuclear Cell Culture

1. RepCell\textsuperscript{TR}: Temperature-responsive culture ware (CellSeed, Tokyo, Japan) (see Note 1).
2. Rat vitronectin (rVN, Sigma) is dissolved in 5 µg/mL (1 V/10 mL) of tissue-culture water and stored in 15 mL conical tube (BD Falcon) at 4°C.
3. Cell culture medium: EGM-2 medium (500 mL EBM-2\textsuperscript{TR} plus SingleQuots\textsuperscript{TR} of growth supplements without hydrocortisone, Lonza, Tokyo, Japan) stored at 4°C. Growth supplements (aqueous solution): 50 mL fetal bovine serum (FBS) (see Note 2), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), GA-1000 (Gentamicin Sulfate and Amphotericin-B).

2.3. Mouse EPC Characterization

2.3.1. EPC Culture Assay

1. Dulbecco’s Phosphate Buffered Saline with calcium and magnesium (DPBS w/\textit{Ca&\textit{Mg}}, Invitrogen, Tokyo, Japan).
2. Acetylated Low Density Lipoprotein, labeled with 1,1’-diodotadecyl – 3,3’,3’-tetramethyl-indocarbocyanine perchlorate (200 µg/mL DiI-ac-LDL, Biological Technologies, Stoughton, MA) stored at 4°C.
3. Fluorescein-labeled GSL I isolectin B4 (ILB4) (1 µg/mL, Vector Laboratories, Burlingame, CA) stored at 4°C.
4. Two percent paraformaldehyde (PFA)/DBPS solution is made by diluting 4% PFA/DPBS (Wako, Osaka, Japan) with DPBS and stored at 4°C.

2.3.2. Fluorescent Immunocytochemistry

1. Microscope cover slips (24×40 mm) (Matsunami Glass, Osaka, Japan) and Lab-Tek 8-well chamber glass slides (Nalge Nunc, Naperville, IL).
2. Two percent PFA/DBPS solution is made by diluting 4% PFA/DPBS with DPBS (1:1) and stored at 4°C.
3. Antibody dilution buffer: 2% BSA in DPBS.
4. Quench solution: 50 mM NH\textsubscript{4}Cl in DPBS.
5. Primary antibodies: rabbit antimouse endothelial nitric oxide synthase (eNOS) antibody (Sigma), rat antimouse Flk-1/vascular endothelial growth factor-receptor 2 (VEGF-R2) antibody (CHEMICON/Millipore, Billerica, MA), rabbit antimouse vascular endothelial cadherin (VE-cadherin) antibody.
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(Santa Cruz Biotechnology, Santa Cruz, CA), rabbit antivon Willebrand factor (vWF) antibody (CHEMICON), rat antimouse CD31 antibody (Santa Cruz Biotechnology), rat antimouse CD105 antibody (BD Bioscience, San Jose, CA), biotinylated goat ant-Tie2 antibody (R&D).

6. Secondary antibodies: Cy2 or Cy3 conjugated goat antirabbit IgG antibody, Cy2 or Cy3 conjugated goat antirat IgG antibody, Cy2 or Cy3 conjugated rabbit antigoat IgG antibody, FITC conjugated Streptavidin (Jackson ImmunoResearch, West Grove, PA).

7. Permeabilization solution: 0.1% (v/v) Triton X-100 (Sigma) in DPBS.

8. Nuclear stain: 300 nM 4,6-diamidino-2-phenylindole (DAPI, Sigma) in DPBS.

9. Mounting medium: (Gel/Mount\textsuperscript{TR}, GeneTex, San Antonio, TX) stored at room temperature.

2.3.3. Tube Formation Assay

1. 24-well flat bottom clear cell culture plate (Corning).
2. Growth factor reduced (GFr) Matrigel\textsuperscript{TR} (BD Biosciences) stored at −20°C.
3. Cell culture medium: 500 mL EGM-2 medium (Lonza) (Subheading 2.2).

2.4. Human PB Mononuclear Cell Isolation

1. 50 mL and 15 mL conical tubes (BD Falcon).
2. 10 mL disposable syringe with 18G needle (Terumo).
3. Drip infusion kit with 18G i.v. catheter (Terumo).
4. Tourniquet (Velket\textsuperscript{TR} Tourniquets, VWR, West Chester, PA) and alcohol prep pad (VWR).
5. Solution of 5 mM EDTA in Ca and Mg-free DPBS (Invitrogen).
6. Histopaque 1077\textsuperscript{TR} (Sigma) stored at 4°C protecting from light.
7. NH\textsubscript{4}Cl: Ammonium chloride solution (StemCell Technologies) stored at 4°C.

2.5. Human CD34\textsuperscript{+} Cell Isolation by MACS System

1. CD34 MultiSort Kit (# 130-056-701, Miltenyi Biotec, Tokyo, Japan) containing:
   - MultiSort Release Reagent for enzymatic release of MultiSort MicroBeads bound to the cell surface.
   - MultiSort Stop Reagent to inhibit the release reaction for further separations.
2. FcR-Blocking Reagent (Miltenyi Biotec).
3. MiniMACSTM Separator with MS Column Adaptor and MS Column (Miltenyi Biotec).
4. 30 µm nylon mesh (Preseparation Filter # 130-041-407, Miltenyi Biotec).
5. DPBS supplemented with 0.5% (w/v) bovine serum albumin (BSA) and 2 mM EDTA by diluting MACSTR BSA Stock Solution with Ca and Mg free DPBS at 1:20 ratio (Miltenyi Biotec).
6. 15 mL conical tube (BD Falcon).

2.6. Human CD34+ Cell Characterization by FACS Analysis

1. FACS CaliburTR 4A (BD Biosciences).
2. 1.5 mL micro tubes: (Eppendorf 3180, Fisher).
3. Buffer: Hanks Balanced Salt Solution (HBSS, Invitrogen) supplemented with 3% FBS.
4. PI buffer: HBSS supplemented with 2 µg/mL Propidium Iodine (PI Solution, Sigma) and 3% FBS.
5. FcR Blocking Reagent, human (Miltenyi Biotec).
6. PE-conjugated antimouse IgG1k (BD Pharmingen).
7. APC-conjugated antimouse IgG1k (BD Pharmingen).
8. FITC-conjugated antimouse IgG1k (BD Pharmingen).
9. PE-conjugated antihuman CD34 mouse IgG1k antibody (BD Pharmingen).
10. APC-conjugated antihuman CD133 mouse IgG1k antibody (Miltenyi Biotec).
11. APC-conjugated antihuman CD117/c-kit mouse IgG1k antibody (BD Pharmingen).
12. FITC-conjugated antihuman CD31 mouse IgG1k antibody (BD Pharmingen).
13. Purified antihuman VEGF-R2/KDR mouse IgG1k antibody (Sigma).
14. Biotin antimouse IgG1k (BD Biosciences).
15. APC-conjugated Streptavidin (BD Pharmingen).
16. APC-conjugated antihuman Tie2 (DAKO, Tokyo, Japan).
17. Purified antihuman VE-cadherin mouse IgG2ak (HyCult Biotechnology, Uden, Netherlands).
18. Biotin antimouse IgG2ak (BD Biosciences).
19. FACS Calibration beads (Calibrite 3 beads, BD Calibrite).
20. FACS washing solutions: BD FACS Flow solution, BD FACS Clean solution, BD FACS Rinse solution (BD FACS).
3. Methods

Since the discovery of BM-derived circulating EPCs as a CD34+ cell fraction in adult human peripheral mononuclear cells (1), we and others have been using CD34+ or CD133+ cells alone or the combination of KDR+ as a EPC-rich cell fraction (9) in peripheral blood in clinical studies for chronic ischemic limb and heart diseases. On the other hand, a distinct definition of mouse EPCs by cell surface markers has not been determined, and investigators currently isolate mouse EPCs with their own criteria, i.e., any combination of stem/progenitor markers, sca-1, c-kit, CXCR4, or CD34 and endothelial markers, CD31, VE-cadherin, Tie2, or Flk-1, etc. in Lineage, CD11b, or CD45 negative cell population by FACS or MACS sorting method. (9) Moreover, one of the disadvantages in the freshly isolated mouse EPCs is the difficulty in obtaining enough number of cells for any experiment, specifically in vivo study. In contrast, although mouse-cultured EPCs isolated from BM are quite heterogeneous, enough number of EPCs can be obtained by this method and are practical for any experiment.

In this method, since the definition of mouse EPCs by cell surface markers is controversial, the author focuses on cultured EPCs for mouse cells. The author also focuses on CD34+ cells for human EPCs that are currently used as a cell source of autologous BM-derived EPC transplantation therapy in patients with not only chronic hindlimb ischemia in our institute but also chronic ischemic cardiovascular diseases in other institutes. The author particularly introduces recently developed culturing technique for mouse EPC isolation by which most of the other differentiated cell types, i.e., monocyte/macrophage, mature endothelial cell and fibroblast can be eliminated comparing with an originally developed method (1,7,10), and a sensitive characterization method for human EPCs (CD34+ cells) by FACS analysis.

3.1. Mouse BM Mononuclear Cell Isolation

1. After anesthetizing a mouse by intraperitoneal injection of 0.5 mL AvertinTR solution, take as much blood as possible with a 23G needle-1 mL disposable syringe from the heart percutaneously; remove all back hair by shaving and using NairTR followed by putting povidone/iodine on the back.

2. Fix the mouse on dissecting board in abdominal position and remove all bones (all limbs, hip, sternum, and back bone) with the muscle using sterile forceps and scissors roughly and soak in 5-mM EDTA/DPBS solution with a 10-cm cell culture dish.

3. Remove muscle/spinal cord from all bones including spine and soak the cleaned bones in new 5-mM EDTA/DPBS solution with a 10-cm cell culture dish.
4. Transfer all bones to sterile mortar and mince them with sterile scissors into 3–4 mm sized pieces.

5. Grind the minced bones by stone stick in 5-mM EDTA/DPBS solution (see Note 3).

6. Collect supernatant (BM cell suspension) with a 18G needle-5-mL disposable syringe, and transfer to 40-µm cell strainer with a 50-mL conical tube.

7. Repeat step 6 with fresh 5-mM EDTA/DPBS solution until the supernatant becomes clear (see Note 4).

8. Divide the cell suspension into two 15-mL conical tubes with 4 mL of Histopaque1083 gently overlaying cell suspension on Histopaque1083 (see Note 5).

9. Centrifuge samples at $1,100 \times g$ speed for 20 min at room temperature (RT) without brake.

10. Collect cloudy mononuclear cell layer with 18G needle-3 mL disposable syringe (see Note 6) and transfer to new 15 mL conical tubes, and fill the tubes with 5-mM EDTA/DPBS solution.

11. Centrifuge samples at $1,150 \times g$ speed for 5 min at 4°C with low brake.

12. Discard the supernatant and add 1 mL of 5-mM EDTA/DPBS solution suspending cell pellet by pipetting.

13. Add 13 mL of 5-mM EDTA/DPBS solution and centrifuge at $240 \times g$ speed for 5 min at 4°C with low brake.

14. Discard (platelet containing) supernatant and add 1 mL of 5 mM EDTA/DPBS solution suspending pellet by pipetting.

15. Add 4 mL of NH$_4$Cl mixing well and incubate for 10 min at 4°C (on ice).

16. Add 9 mL of 5 mM EDTA/DPBS solution and centrifuge at $350 \times g$ speed for 15 min at 4°C with low brake.

17. Discard supernatant and add 2 mL of EGM-2 medium in each tube.

18. Combine two samples into one 50 mL conical tube (total 4 mL), and count the cell number.

1. Add 3 mL of 5 µg/mL rVN solution in RepCell dish covering all surface and quickly return the solution to original 15 mL conical tube, and incubate the rVN-coated dish at RT in clean bench until the dish dries up.

2. Add appropriate amount of DMEM supplemented with 10% FBS in mouse BM mononuclear cell suspension (4 mL in 50 mL conical tube, Subheading 3.1) and seed the cells dividing into rVN-coated RepCell dishes as indicated in Table 1.
3. After 24 hours in culture in CO₂ incubator (5% CO₂ at 37°C), transfer all floating cells with whole culture medium to new 50 mL tube, and leave the RepCell dish at RT for 15–20 min (see Note 7).

4. After centrifugation of the 50 mL tube (step 3) at 300 × g speed for 5 min, the pelleted cells are suspended with EGM-2 medium.

5. Remove the attached cells by washing/pipetting with DPBS three times, and put floating cell suspension (step 4) into original RepCell dish.

6. After 3 days in culture, change the medium with fresh EGM-2 medium removing floating cells and keep culturing in CO₂ incubator for further 3 days.

7. On day 7, after replacing medium with DPBS, incubate the RepCell dishes with attached cells at RT for 15–20 min followed by washing/pipetting with the left DPBS, and collect all detached cell solution in 15 mL conical tubes.

8. After centrifugation at 300 × g speed for 5 min, pelleted cells can be used for any experiment as EPC-rich cell population.

3.3. Mouse EPC Characterization

3.3.1. EPC Culture Assay

1. Seed mouse cultured EPCs obtained from BM mononuclear cells according to the above protocol (Subheading 3.2) onto 8-well chamber glass slide at a density of 2 × 10⁴/well with 250 µL of EGM-2 medium, and further culture until the cells attach on the bottom (approximately 4–6 h).

2. Add 4 µL of DiI-ac-LDL solution into each well and shake the slide gently and incubate for 4 h.

3. Wash the samples with DPBS w/Ca&Mg three times and fix the cells with 100 µL of 2% PFA/DPBS solution for 15 min at RT.

4. Remove 2% PFA/DPBS solution and wash the samples with DPBS w/Ca&Mg three times, and then incubate the fixed cells with 100 µL of FITC-ILB4 solution for 1 h at 37°C.

<table>
<thead>
<tr>
<th>Dish size</th>
<th>Cell number</th>
<th>Medium (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 cm diameter</td>
<td>60–80 × 10⁶</td>
<td>16–20</td>
</tr>
<tr>
<td>6 cm diameter</td>
<td>20–30 × 10⁶</td>
<td>4–6</td>
</tr>
</tbody>
</table>
5. Remove FITC-ILB4 solution and wash the samples with DPBS w/Ca&Mg three times, and then incubate with 100 µL of DAPI solution for 10 min at RT for nuclear staining.

6. Rinse samples with DPBS w/Ca&Mg and remove gaskets carefully from the glass slides using a razor blade.

7. Mounting medium and a cover-slip are added to the glass slide followed by sealing the sample with nail varnish. The sample slides can be viewed immediately after the varnish is dry, or be stored in the dark at 4°C for up to a month.

8. Slides are viewed under fluorescent microscopy. Excitation at 549 nm includes the DiI fluorescence (red emission) for ac-LDL uptake, excitation at 490 nm includes the FITC fluorescence (green emission), and excitation at 350 nm includes the DAPI fluorescence (blue emission). Software (Adobe PhotoshopTR CS3, Adobe Systems, San Jose, CA) can be used to overlay fluorescent images to detect double positivity of DiI and FITC signals, which indicates one of the EPC characteristics. An example result is shown in Fig. 1a.

3.3.2. Fluorescent Immunocytochemistry

1. Seed mouse cultured EPCs obtained from BM mononuclear cells according to the above protocol (Subheading 3.2) onto 8-well chamber glass slide at a density of 2 × 10⁴/well with 200 µL of EGM-2 medium, and further culture until the cells attach on the bottom (It takes approximately 4–6 h).

2. Wash samples with DPBS three times and replace DPBS with 100 µL of 2% PFA/DPBS solution for 15 min at RT to fix the cells.

3. Remove 2% PFA/DPBS solution and wash the samples with DPBS three times.

4. Residual PFA is quenched by incubation in 50 mM NH₄Cl solution for 10 min at RT, followed by further washing with DPBS three times.

5. Cells are permeabilized by incubation in Triton X-100 solution for 5 min at RT (see Note 8), and then rinsed with DPBS three times.

6. Samples are blocked by incubation in antibody dilution buffer for 1 h at RT, and the plastic chambers should be removed leaving gasket on the slide.

7. Remove blocking solution and replace with primary antibodies: anti-eNOS antibody (1:200), anti-vWF antibody (1:500), anti-VE-cadherin(1:50), anti-Tie2 antibody (1:50), anti-Flk-1 antibody (1:500), anti-CD31 antibody (1:50), and anti-CD105 antibody (1:20) in antibody dilution buffer at 4°C over night.

8. Remove primary antibodies and wash samples three times for 5 min each with DPBS.
Fig. 1. EPC characteristics by EPC culture assay and immunocytochemistry with mouse BM mononuclear cells. (a) Endothelial characteristics are assessed by EPC culture assay. Dil-acLDL uptake cells are observed in red and FITC-ILB4 binding cells are observed in green under fluorescent microscope. Double positive (yellow in merged images) cells are considered as BM-derived endothelial lineage cells, namely, EPCs. (b) Immunocytostaining with a series of endothelial markers confirms endothelial phenotype of EPCs. CD31, CD105, eNOS, Flk-1, Tie2, VE-cadherin, and vWF are stained in red with DAPI (blue). Over 80% of total adherent cells are positive for each marker except for Flk-1 (40%) compared with IgG staining control (Ctrl). Original magnification: ×400.
9. Secondary antibodies are prepared at 1:500 in antibody dilution buffer and added to the samples for 30 min at RT.

10. Remove secondary antibodies followed by DPBS washing for three times and DAPI solution (1:5000) is added to for 10 min at RT for nuclear staining.

11. Rinse samples with DPBS and remove gaskets carefully from glass slides using a razor blade.

12. Mounting medium and a cover-slip are added to the glass slide followed by sealing samples with nail varnish. The sample slides can be viewed immediately after the varnish is dry, or be stored in the dark at 4°C for up to a month.

13. Slides are viewed under fluorescent microscopy. Excitation at 543 nm includes the Cy3 fluorescence (red emission), excitation at 490 nm/488 nm includes the FITC/Cy2 fluorescence (green emission), respectively, and excitation at 350 nm includes the DAPI fluorescence (blue emission). Software (Adobe Photoshop® CS3, Adobe Systems) can be used to adjust the fluorescent signal intensity in each image to detect immunoreactivity of endothelial markers. An example result is shown in Fig. 1b.

3.3.3. Colony/Tube Formation Assay

1. GFrMatrigel® is prepared on ice or in refrigerator (4°C) for 3 h before assay.

2. Coat 24-well culture dish with 200 μl of GFrMatrigel®/each well on ice by cold pipette tip.

3. Incubate the 24-well culture dish at 37°C for 30 min.

4. Seed mouse cultured EPCs obtained from BM mononuclear cells according to the above protocol (Subheading 3.2) at a density of 5×10^4/well with 500 μl of EGM-2 medium onto growth factor-reduced GFrMatrigel®-coated dish.

5. Samples are cultured in CO₂ incubator (5% CO₂ at 37°C) for 48 h (see Note 9), and the cells in colony and tube formation are viewed under phase contrast microscope.

6. After capturing images by a computer-assisted software with microscope, the images are analyzed using a Image J® software for measuring total tube length per colony and averaged. Tube length accounts for vasculogenic capacity of the cells that have endothelial-like cell phenotype. An example result is shown in Fig. 2.

3.4. Human Peripheral Blood Mononuclear Cell Isolation

1. Six 50 mL conical tubes with each 15 mL of 5 mM EDTA/DPBS solution are prepared for blood collection.

2. After wearing a tourniquet on either right or left upper arm, take blood using a drip infusion kit with 18G i.v. catheter, and add 35 mL of blood in each 50 mL conical tube (total 210 mL).
3. Prepare eight 50 mL conical tubes with each 12.5 mL Histopaque 1077TR at RT.

4. Divide the blood diluted with 5 mM EDTA/DPBS solution (step 3) into eight 50 mL conical tubes with 12.5 mL of Histopaque1077TR (step 4) gently overlaying cell solution on Histopaque1077 (see Note 5).

5. Centrifuge the samples at 1,100 × g speed for 30 min at RT without brake.

6. Collect cloudy mononuclear cell layer with 18G needle-10 mL disposable syringe (see Note 6) and transfer to four new 15 mL conical tubes, and fill the tubes with 5 mM EDTA/DPBS solution.

7. Four 50 mL conical tubes with 10 mL of 5 mM EDTA/DPBS solution are prepared, and each mononuclear cell sample (step 7) is added to the four 50 mL conical tubes.

8. Centrifuge samples at 1,150 × g speed for 5 min at 4°C with low brake.

9. Discard supernatant and add 10 mL of 5 mM EDTA/DPBS solution suspending cell pellet by pipetting.

10. Add 20 mL of 5 mM EDTA/DPBS solution and centrifuge at 240 × g speed for 5 min at 4°C with low brake.

11. Discard supernatant and add 2 mL of 5 mM EDTA/DPBS solution suspending cell pellet by pipetting.

12. Add 8 mL of NH₄Cl mixing well and incubate at 4°C (on ice) for 10 min.

Fig. 2. Colony and Tube formation assay with Day7 mouse BM-derived cultured EPCs. Cultured EPCs are obtained from BM mononuclear cells after 7 days in culture in EPC differentiation medium (EGM-2) and further cultured on GFMatrigelTR for 48 h (Day 2) and 7 days (Day 7). After forming colonies with spicula, remarkable elongated tube-like structure originated from colonies is observed, indicating vascular forming capacity. On Day 2, after culture with 10%FBS/DMEM medium, attached cells form colonies, but not tube-like structure. (data not shown).
13. Add 40 mL of 5 mM EDTA/DPBS solution and centrifuge at 350 × g speed for 15 min at 4°C with low brake.
14. Discard supernatant and add 2 mL of EGM-2 medium in each tube.
15. Combine four samples into one 15 mL conical tube (total 8 mL), and count cell number.

**3.5. Human CD34+ Cell Isolation**

1. Resuspend 2 × 10⁸ human PB mononuclear cells obtained according to the above protocol (Subheading 3.4) with buffer in a final volume of 600 µL.
2. Add 200 µL of FcR Blocking Reagent to the cell suspension to inhibit unspecific or Fc-receptor binding of CD34 MultiSort MicroBeads to nontarget cells.
3. Label cells by adding 200 µL of CD34 MultiSort MicroBeads, mix well, and incubate for 30 min at 4°C (see Note 10).
4. Wash cells by adding 1 mL of buffer, centrifuge at 300 × g for 10 min, remove supernatant completely, and then resuspend in 500 µL of buffer.
5. Place an MS Column (combined with the appropriate Column Adapter) in the magnetic field of the MACS Separator.
6. Prepare the column by washing with 500 µL of buffer.
7. Apply cell suspension on top of the column and let the unlabeled cells pass through followed by rinsing with 500 µL of buffer three times.
8. Remove column from separator and place column on a 15 mL conical tube.
9. Add 1 mL of buffer onto the column and flush out magnetically labeled fraction using the plunger supplied with the column (see Note 11).
10. To achieve a higher purity, apply magnetically labeled fraction onto a new freshly prepared column, and let the unlabeled cells pass through followed by rinsing with 500 µL of buffer three times.
11. The magnetically labeled cell fraction are eluted and collected in the conical tube.
12. Incubate magnetically labeled cells with 20 µL of MACS<sup>TR</sup> MultiSort Release Reagent/mL of cell suspension for 10 min at 4°C.
13. Separate cells over a new MS Column to remove any remaining magnetically labeled cells, and prepare the column by washing with 500 µL of buffer.
14. Apply cell suspension on top of the column and let the unlabeled cells pass through followed by rinsing with 500 µL of buffer three times.
15. Wash cells from released fraction removing supernatant completely, and resuspend the cell pellet in buffer in a final volume of 40 µL.

16. Add 60 µL of MACS MultiSort Stop Reagent/40 µL of cell suspension and mix well.

17. The cells in 100 µL of buffer are magnetically sorted CD34+ cells.

3.6. Human CD34+ Cell Characterization by FACS Analysis

1. Freshly isolated CD34+ cells (Subheading 3.4, step 17) are centrifuged at 300 × g for 10 min. After removing supernatant, suspend the cell pellet with buffer (3% FBS/HBSS) at a concentration of 8 × 10^6 cells/mL, and make ten samples in 1.5 mL micro tubes with 2 × 10^5 cells/25 µL of cell solution each.

2. Add 2 µL of FcR Blocking Reagent in each cell sample and mix well, and then incubate for 30 min at 4°C.

3. Add antibodies in each cell solution as indicated in Table 2 and incubate for 30 min at 4°C followed by addition of 1 mL buffer and mixing (see Note 12).

4. After centrifugation at 2,000 × g speed for 2 min at 4°C, wash the cells with 1 mL buffer + by pipetting and repeat this step twice.

5. Remove supernatant and suspend the cells with 300 µL of PI buffer each.

6. The samples are ready for FACS analysis with FACS Calibur 4A.

7. For calibration, after turning on the power and starting FACS Comp software, uptake FACS calibration beads (Calibrite 3 beads) in the machine by pressing [RUN] button.

8. After the automatic calibration, wash the circuit with FACS Flow solution in the same manner as step 7 (see Note 13).

9. After starting CellQuestProTR software (BD Biosciences), uptake the sample #1 (Table 2) in the machine by pressing [RUN] button and the sample is analyzed automatically.

10. For compensation, adjust voltage levels for the next PE, APC, and FITC measurement and also for PI on the dot plot screen in the result of sample tube #1 (Table 2) (negative controls).

11. Uptake samples #2, #3, and #4 (Table 2) in the machine by pressing [RUN] button and the samples are analyzed automatically.

12. In the results of sample tubes #2 (FITC control), #3 (PE control), and #4 (APC control), adjust compensation values at FL3-FL1, FL3-FL2, and FL3-FL4 channels to avoid interference of FITC, PE, and APC signals to PI signal, respectively (see Note 14).
For sample analysis, input the cell number (10,000) to be counted at “Acquisition & Storage” area on the screen.

Uptake samples #5, #6, and #7 (Table 2) in the machine by pressing [RUN] button and save the automatically analyzed data.

Next, uptake the sample #8 (Table 2) in the machine by pressing [RUN] button and the sample is analyzed automatically.

Adjust voltage levels for the next APC and FITC measurement and also for PI on the dot plot screen in the result of sample tube #8 (Table 2) (negative controls).

For sample analysis, input the cell number (10,000) to be counted at “Acquisition & Storage” area on the screen.

Uptake samples #9 (Table 2) in the machine by pressing [RUN] button and save the automatically analyzed data.

Adjust voltage levels for the next APC and FITC measurement and also PI on the dot plot screen in the result of sample tube #10 (Table 2) (negative controls).

### Table 2
**Combination of antibodies with human CD34+ Cells for FACS analysis**

<table>
<thead>
<tr>
<th>Tube #</th>
<th>IgG or antibodies (antibody 1 + antibody 2)</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FITC-mouse IgG(<em>{1k}) (isotype control for CD31,CD34) + PE-mouse IgG(</em>{1k}) (isotype control for CD34, KDR) + APC-mouse IgG(_{1k}) (isotype control for CD133, CD117, Tie2)</td>
<td>2 µL + 2 µL + 2 µL</td>
</tr>
<tr>
<td>2</td>
<td>FITC anti-human CD45 (for FITC compensation)</td>
<td>2 µL</td>
</tr>
<tr>
<td>3</td>
<td>PE-anti-human CD45 (for PE compensation)</td>
<td>2 µL</td>
</tr>
<tr>
<td>4</td>
<td>APC-anti-human CD45 (for APC compensation)</td>
<td>2 µL</td>
</tr>
<tr>
<td>5</td>
<td>PE-anti-human CD34 + APC-anti-human CD133</td>
<td>2 µL + 2 µL</td>
</tr>
<tr>
<td>6</td>
<td>PE-anti-human CD34 + APC-anti-human CD117/c-kit</td>
<td>2 µL + 2 µL</td>
</tr>
<tr>
<td>7</td>
<td>FITC-anti-human CD31 + APC-mouse anti-human Tie2</td>
<td>2 µL + 2 µL</td>
</tr>
<tr>
<td>8</td>
<td>APC-Streptavidin + Biotin-mouse IgG(<em>{2k}) (isotype control for VE-cadherin) + FITC-mouse IgG(</em>{1k})</td>
<td>2 µL + 2 µL + 2 µL</td>
</tr>
<tr>
<td>9</td>
<td>FITC-anti-human CD31 + APC-Streptavidin + Biotin-mouse anti-human VE-cadherin</td>
<td>2 µL + 2 µL + 2 µL</td>
</tr>
<tr>
<td>10</td>
<td>APC-Streptavidin + Biotin-antimouse IgG(<em>{1k}) + Purified mouse IgG(</em>{1k}) (for isotype control for KDR) + PE-mouse IgG(_{1k})</td>
<td>2 µL + 2 µL + 2 µL + 2 µL</td>
</tr>
<tr>
<td>11</td>
<td>PE-anti-human CD34 + APC-Streptavidin + Biotin-antimouse IgG(_{1k}) + Purified mouse anti-human VEGF-R2/KDR</td>
<td>2 µL + 2 µL + 2 µL + 2 µL</td>
</tr>
</tbody>
</table>
20. Uptake samples #11 (Table 2) in the machine by pressing [RUN] button and save the automatically analyzed data.

21. After all measurements, circuit in the machine should be cleaned with FACS Clean solution and rinsed with FACS Rinse solution and be shut down.

22. For data analysis, select “Analysis” for dot plot on the CellQuest Pro software screen.

23. After retrieving the saved data, set up a gate in the targeted cell fraction and develop the gated part in another dot plot graph.

24. In the dot plot graph of negative control samples, set quadrant location on PE/APC or FITC/APC axis until the percent of PE/APC or FITC/APC negative control shows 100% negative for PE, FITC, and APC signals.

25. Apply the same values of quadrant location as negative control to the dot plot graph of the samples (#1, #2, #3, #4, #8 and #10), and then the percent of targeted cell fractions in total CD34+ cells are automatically calculated. An example result is shown in Fig. 3.

Fig. 3. Characterization of human CD34+ cells by FACS analysis. CD34+ cells are isolated by MACS system and the phenotype is assessed by FACS with stem/progenitor and endothelial markers. Although little cell population was positive for Flk1, VE-cadherin, and Tie2 that are generally expressed in well-differentiated ECs, high expressions of CD31, c-kit, and CD133 were observed in CD34+ cells, suggesting that most of the freshly isolated CD34+ cells were immature endothelial progenitors.
4. Notes

1. RepCell\textsuperscript{TR} (or UpCell\textsuperscript{TR}) (11) allow us to detach any strong adherent cells from culture dish easily without trypsinization. Since trypsinization more or less affects (reduces) cell surface marker expressions and the viability, this temperature-sensitive cell culture dish (RepCell\textsuperscript{TR} or UpCell\textsuperscript{TR}) is suitable for harvesting cells specifically for FACS analysis.

2. Since FBS strongly affects EPC differentiation to endothelial lineage depending on the lot and concentration in culture medium, the same lot of FBS should be used in all subsequent EPC-related assays in vitro.

3. To avoid BM cell death by grinding minced bone pieces, the procedure of gentle frequent tapping and rotation by stone stick is important for preserving cell viability. If white pellet can be observed with red blood cell pellet after the first centrifugation (step 9), dead mononuclear cells are in the white pellet, and a reduced number of mononuclear cells with less viability will be collected. Pellet color should be red at step 9.

4. Twenty milliliters of final cell suspension makes two samples of 15 mL tubes and is convenient for centrifugation (step 8).

5. For successful isolation of mononuclear cells, the temperature of Histopaque 1083\textsuperscript{TR} should be similar to RT, so Histopaque 1083\textsuperscript{TR} should be prewarmed at RT before step 8.

6. Collect all cell pellets on the wall of conical tube as well as mononuclear cells. The pellet also contains a number of mononuclear cells.

7. If cell detachment is not enough after 10–15 min incubation at RT, RepCell dish can be incubated in refrigerator for 5–10 min alternatively.

8. Triton-X100 treatment is only needed for staining of cytoplasmic molecules (eNOS and vWF). For cell surface molecule (CD31, Tie2, Flk-1, VE-cadherin, CD-105) staining, this treatment is not necessary.

9. Colony and tube formation can be observed 24 and 48 h after incubation, respectively. These findings can also be observed up to 14 days after incubation. Appropriate time points for observation should be determined by investigators.

10. Work fast, keep cells cold, and use precooled solutions. This procedure will prevent capping of antibodies on the cell surface and nonspecific cell labeling.

11. For optimal performance, it is important to obtain a single-cell suspension. Pass cells through 30 µm nylon mesh to remove cell clumps which may clog the column.
12. For staining in biotin-avidine system, samples should be incubated with each antibody separately, i.e., incubate the sample with biotin-antimouse IgG followed by washing step (step 4) and then do with APC-streptoavidine.

13. This washing step for circuit should be performed in each sample measurement.

14. Channel for each fluorescent is set up as: FL1 = FITC, FL2 = PE, FL3 = PI, FL4 = APC. To detect dead cells (PI positive cells) and avoid overlap of PI and other fluorescent, i.e., FITC, PE, or APC for measurement, adjustment of compensation value for PI signal should be performed at this step again.

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**References**


