

Chapter 8

Circulating Endothelial Progenitor Cells

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Abstract

Recent studies have found that bone marrow-derived cells give rise to endothelial cells during states of tissue repair and disease. We have found that one key integrin, integrin- $\alpha 4\beta 1$, promotes the homing of circulating endothelial progenitor cells (EPCs) to sites of ongoing tissue repair. This integrin facilitates the adhesion of EPCs to the vascular endothelium in inflamed tissue or within tumors. We demonstrate how to identify, isolate, purify, and characterize EPCs. We also demonstrate *in vivo* analysis of the roles of bone marrow-derived cells in tumor growth and angiogenesis by demonstrating adoptive transfer, bone marrow transplantation, tumor models, and immunohistochemistry for markers of blood and endothelial vessels. Finally, we show how to characterize cell adhesion mechanisms regulating bone marrow-derived progenitor cell trafficking.

Key words: Angiogenesis, CD34, endothelial progenitor cell, Lin⁻, lymphangiogenesis, peripheral blood mononuclear cell, Sca1, stem cell.

1. Introduction

Bone marrow-derived precursor cells have recently been shown to regulate neovascularization of tumors and ischemic tissues (1). Progenitor cells home to ischemic or hypoxic tissues (angiogenic niches), where they incorporate into sprouting blood vessels at low, but measurable, frequencies. The nature of this cell type remains unclear, however. Isner and colleagues first isolated mononuclear cells (MNCs) from human peripheral blood that were enriched for expression of the hematopoietic stem cell marker CD34 (2). On culture in endothelial growth media, these cells expressed endothelial lineage markers, such as CD31, Tie2, and vascular endothelial growth factor receptor 2 (VEGFR2),

and incorporated into blood vessels in ischemic tissues. These cells were therefore described as bone marrow-derived endothelial progenitor cells (EPCs) or “hemangioblasts.” Since then, Rafii and colleagues identified a VEGFR2 and AC133-positive subpopulation of CD34-positive circulating cells that form endothelial cell-like colonies *in vitro* (3, 4).

Recently, other studies have shown that mononuclear progenitor cells expressing monocyte markers such as CD11b or CD14 also give rise to endothelial cell-like colonies *in vitro* and possibly *in vivo* (5–10). Hildbrand and colleagues described a CD34⁺CD11b⁺ cell population from cord blood that gives rise to endothelial cell colonies *in vitro* (9). Interestingly, CD34⁺/CD14⁺ human peripheral blood monocytes have also been shown to express endothelial cell markers such as von Willebrand factor and vascular endothelial cadherin (VE cadherin) after several weeks in culture in the presence of endothelial growth media (10). Interestingly, a novel Gr⁺CD11b⁺ bone marrow-derived cell population was shown to contribute to neovascularization (11). These myeloid cells comprised 5% of the total cell population within colorectal carcinoma and Lewis lung carcinoma tumors. In fact, tumor angiogenesis was increased in the presence of Gr⁺CD11b⁺ cells, which also exhibited high levels of MMP9. Deletion of MMP9 abolished the angiogenesis-promoting activity. Tumor-derived Gr⁺CD11b⁺ cells expressed the endothelial cell markers VE cadherin and VEGFR2 and incorporated into tumor endothelium (11). These findings suggest that endothelial-like cells arise from bone marrow-derived hematopoietic progenitor cell populations (CD34⁺ AC133⁺ cells) or myeloid progenitor cell-like populations (CD14⁺ or CD11b⁺ cells). It is possible that endothelial cells and myeloid cells share a common precursor.

A number of studies therefore suggest a common link between monocytic cells and microvascular endothelial cells and further support the idea that tumor vasculogenesis occurs through the recruitment of bone marrow-derived endothelial precursor cells, which may include subsets of myeloid lineage cells. Continued characterization of the bone marrow-derived cells that contribute to neovascularization and analysis of the mechanisms by which these cells modulate neovascularization will clarify the cell types that promote vasculogenesis. Together, these studies suggest that myeloid lineage bone marrow-derived cells give rise to vascular endothelium, and that several lineage markers, including Gr, CD11c, and CD11b, identify putative EPCs.

Some studies suggest that macrophages, rather than EPCs, are the key bone marrow-derived cell type that contributes to adult neovascularization. Studies by de Palma and colleagues identified a Tie2⁺ subpopulation of CD11b⁺ tumor-associated myeloid cells that promotes angiogenesis in tumors (12). Although these

cells stimulate angiogenesis, they do not actively incorporate into blood vessels. Further studies from the same group showed that Tie2-expressing monocytic cells are specifically recruited to spontaneously arising mouse pancreatic tumors and to orthotopic xenografts of human glioma (12). From these studies, it is clear that myeloid cells play key roles in tumor angiogenesis; importantly, these cells may include EPCs, macrophages, and other myeloid lineage cells.

Our lab recently characterized the specific mechanisms by which myeloid and endothelial precursor cells adhere to tumor endothelium and extravasate into tumor tissue. Bone marrow-derived cells express a number of functional integrins ($\alpha 2\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha M\beta 2$ [CD11b], and $\alpha X\beta 2$ [CD11c]) that may play roles in these cells within neovascular microenvironments (13, 14). Our studies revealed that integrin- $\alpha 4\beta 1$, a receptor for vascular cell adhesion molecule (VCAM) and fibronectin, selectively promotes the homing of both EPCs (15) and monocytes (14) to neovascular tissue, and that this integrin is essential for the participation of these cells in angiogenesis and tumor growth. Human CD34⁺ and murine Lin⁻Sca1⁺ progenitor cells as well as bone marrow-derived myeloid cells (CD14⁺ CD11b⁺) adhered to endothelial cells *in vitro* and tumor endothelium *in vivo* via integrin- $\alpha 4\beta 1$. Treatment of mice bearing Lewis lung carcinoma tumors with antagonists of integrin- $\alpha 4\beta 1$ significantly suppressed the number of monocytes and EPCs within tumors and reduced blood vessel density. Our studies indicated that suppression of monocyte/macrophage homing to tumors by the application of an integrin- $\alpha 4\beta 1$ antagonist could be a useful supplementary approach to suppress tumor angiogenesis and growth.

In this chapter, we describe how to isolate peripheral blood or bone marrow-derived MNCs and from them, CD34⁺ human progenitor cells and Lin⁻ murine progenitor cells; how to culture endothelial cells and progenitor cells; how to characterize cell surface markers, such as integrins; how to evaluate cell adhesion mechanisms *in vitro*; and how to study cell trafficking and contributions to tumor growth *in vivo*.

2. Materials

2.1. Progenitor Cell Isolation

1. 50mL conical tubes (human cell isolation).
2. 15-mL conical tubes (rodent cell isolation).
3. Phosphate-buffered saline, Ca²⁺ and Mg²⁺ free, with 0.5 mM EDTA (ethylenediaminetetraacetic acid) (PBS-E).

4. Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) for human cell isolation.
5. Histopaque 1083 (Sigma-Aldrich) for mice, rat, or rabbit cell isolation.
6. 10-cc syringe with 18-gage bevel-tip needles.
7. Ammonium chloride red blood cell lysis solution: 155 mM NH_4Cl , 10 mM NaHCO_3 , and 0.1 mM EDTA.
8. Blood draw: 100 mL from humans (or two buffy coats from blood bank), 5–40 mL from rabbit, 1–2 mL from mice (or mouse bone marrow), 5 mL from rat.

2.2. Progenitor Cell Purification

1. Fc-receptor blocking agent.
2. Fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (clone AC136, Miltenyi Biotech, Auburn, CA).
3. PE-conjugated anti-CD133 (AC133/1, Miltenyi Biotech).
4. Anti-c-kit (clone 2B8) from eBioscience (San Diego, CA).
5. Anti-Sca-1 (clone E13-161.7) from eBioscience.
6. Antibiotin-FITC (Miltenyi Biotech).
7. Lineage cell depletion kit from Miltenyi Biotech.
8. Anti-CD34 conjugated supramagnetic colloidal beads from Miltenyi Biotech.
9. MACs buffer: PBS pH 7.2 supplemented with 0.5 % bovine serum albumin (BSA) and 2 mM EDTA.
10. Magnetic cell separator (MidiMACS, Miltenyi Biotech).
11. LS^+/VS^+ columns (Miltenyi Biotech).

2.3. FACS Analysis

1. PE-conjugated mouse antihuman $\alpha 4\beta 1$ (clone 9F10, BD Biosciences, San Jose, CA).
2. PE-conjugated anti- $\alpha 5\beta 1$ (clone IIA1, BD Biosciences).
3. PE-conjugated anti- $\beta 2$ (clone CLB-LFA 1/1, eBiosciences).
4. Mouse antihuman $\alpha \nu \beta 3$ (FITC-conjugated LM609, Chemicon International, Temecula, CA).
5. Mouse antihuman $\alpha \nu \beta 5$ (PE-conjugated P1F6) (Chemicon International).
6. PE-conjugated rat antimouse $\alpha 5\beta 1$ (5H10-27) (BD Biosciences).
7. PE-conjugated rat antimouse $\alpha \nu$ (RMV-7) (BD Biosciences).
8. PE-conjugated rat antimouse $\beta 2$ (M18/2) from (BD Biosciences).
9. PE-conjugated antimouse $\alpha 4\beta 1$ (R1-2) (BD Biosciences).
10. Anti-VCAM (clone P8B1) (Chemicon International).

11. Antihuman β 1 (clone P4C10) was from Chemicon International.
12. Rat antihuman β 7 (clone FIB504) (BD Biosciences).
13. Alexa 488-conjugated secondary antibodies from Invitrogen (Carlsbad, CA).
14. PBS containing 1% BSA (radioimmunoassay grade).
15. FcR blocking reagent.

2.4. Endothelial Progenitor Cell Culture

1. Plasma fibronectin (BD Biosciences).
2. EPC growth medium: EBM-2 containing 5% fetal calf serum (FCS), human vascular endothelial growth factor 1 (VEGF1), human fibroblast growth factor 2 (FGF2), human epidermal growth factor, insulin-like growth factor, gentamicin, and ascorbic acid but no prednisone (Cambrex, East Rutherford, NJ).
3. 6-well plastic culture plates.
4. PBS, Ca^{2+} and Mg^{2+} free, with 0.5 mM EDTA (PBS-E).
5. Glass chamber slides.
6. Gelatin (Sigma-Aldrich).
7. DiI acetylated-LDL (Biomedical Technologies, Stoughton, MA).
8. FITC-UEA-1 lectin (Vector Laboratories, Burlingame, CA) for human cells.
9. FITC-*Bandeira simplicifolia* lectin (Vector Laboratories) for rat, mice, rabbit.
10. 1% paraformaldehyde.
11. Vectashield (Vector Laboratories).

2.5. Endothelial Cell Culture

1. 7.5% sodium bicarbonate (e.g., 9330, Irvine Scientific, Irvine, CA).
2. 1M HEPES buffer (e.g., 9319, Irvine Scientific).
3. Fetal bovine serum.
4. M199 powdered medium (10 $\times$ 1 L, Gibco brand 31100-035, Invitrogen).
5. To prepare M199 basal medium: Mix the contents of one M199 powder package in 950 mL sterile, endotoxin-free water. Add 16.7 mL 7.5% sodium bicarbonate and 10 mL 1M HEPES buffer. Mix, adjust to pH 7.4 if necessary, and sterile filter using 0.45- μ m sterile filter units.
6. Endothelial cell growth supplement (ECGS) (Upstate Biotechnology 02-102) stock: Add 5 mL basal medium to one 15-mg vial of ECGS.

7. Heparin (500,000 units; Sigma-Aldrich): For 100X heparin, pour powdered heparin into a 50-mL conical tube. Rinse the heparin bottle with 5 mL basal medium and add 27.7 mL basal medium. Mix and store stock at -20°C .
8. M199 complete endothelial cell growth medium: Using a 500-mL 0.45- μm sterile filter unit, combine and filter 390 mL basal M199 medium, 100 mL fetal bovine serum, 5 mL 100X heparin, and 5 mL ECGS.
9. Gentamicin (10 mg/mL).
10. Alternatively, use endothelial growth medium (EGM-2: EBM-2 containing 2% FCS, human VEGF1, human FGF2, human epidermal growth factor, insulin-like growth factor, gentamicin, ascorbic acid, prednisone) (Cambrex).
11. Trypsin/EDTA (CC-5012) and trypsin-neutralization solution (CC-5002, Cambrex).

2.6. Adhesion Assays

1. Plasma fibronectin (BD Biosciences).
2. Vitronectin (BD Biosciences).
3. Human dermal microvascular endothelial cells (Cambrex).
4. Human umbilical vein endothelial cells (HUVECs; Cambrex).
5. Costar 48-well, nontissue culture-treated plates (Costar cat. no. 3547).
6. Adhesion buffer: Hank's balanced salt solution, 10 mM HEPES, pH 7.4, 2 mM MgCl_2 , 2 mM CaCl_2 , 0.2 mM MnCl_2 , 1% BSA.
7. 3% heat-denatured BSA: Heat a solution of 3 g/100 mL BSA on a stirring heating plate until it begins to turn cloudy; remove immediately and allow to cool at room temperature. Sterile filter through a 0.45- μm filter unit.
8. 3.7% paraformaldehyde in PBS: Heat 3.7 g paraformaldehyde in 50 mL H_2O to 60°C in fume hood. Add 1 N NaOH in drops to dissolve thoroughly; let cool, then add 50 mL 2X PBS; bring to pH 7.4.
9. Antihuman $\alpha 5\beta 1$ (JBS5, Chemicon International).
10. Antimurine $\alpha 5\beta 1$ (5H10-27, BD Bioscience).
11. Antihuman $\alpha v\beta 5$ (P1F6, Chemicon International).
12. Antimurine αv (RMV-7, BD Bioscience).
13. Antihuman $\alpha v\beta 3$ (LM609, Chemicon International).
14. Antihuman $\alpha 4\beta 1$ (HP2/1, Chemicon).
15. Antimurine $\alpha 4\beta 1$ (PS/2, BD Biosciences).
16. Antihuman $\beta 1$ integrin (P4C10, Chemicon International).
17. Antihuman $\beta 2$ integrin (BD Biosciences).

18. Antimurine $\beta 2$ (M18/2, BD Biosciences).
19. Recombinant soluble VCAM (rsVCAM, R&D Systems, Minneapolis, MN).
20. 5-and-6-4-chloromethylbenzoylamino-tetramethyl-rhodamine (CMTMR; Invitrogen). Prepare stock solution of 10 mM CMTMR in dimethyl sulfoxide (DMSO).

2.7. Bone Marrow Transplant

1. FVB/N-Tie2LacZ, FVB/N-Tie2GFP, or C57Bl/6 ActEGFP donor mice (Jackson Lab, Bar Harbor, ME); FVB/N and C57Bl6 recipient mice.
2. Sulfamethoxazole and trimethoprim antibiotics.
3. Prepare MAC buffer: PBS without CaCl_2 or MgCl_2 containing 2 mM EDTA and 0.5% BSA (ice cold).
4. Two 5-mL syringes filled with 25-gage needle for each mouse.
5. Prewarmed Histopaque 1083 and ammonium chloride red blood cell lysing solution (155 mM NH_4Cl , 10 mM NaHCO_3 , and 0.1 mM EDTA).

2.8. Tumor and Matrigel Studies

1. Lewis lung carcinoma and other tumor cell lines.
2. Growth factor-reduced Matrigel (BD Biosciences).
3. Recombinant human bFGF or VEGF.
4. Rat antimouse $\alpha 4\beta 1$ (low-endotoxin PS/2, BD Biosciences).
5. Rat antimouse $\beta 2$ integrin (low-endotoxin M1/70, BD Biosciences).
6. Rat anti-murine CD31 (MEC13.3, BD Bioscience).
7. Low-endotoxin anti- $\alpha v\beta 3$ (LM609) or antihuman $\alpha 4\beta 1$ (HP2/1).
8. Purified CD34⁺ cells.
9. EGFP⁺Lin⁻ or EGFP⁺Lin⁺ cells.
10. FITC-*Bandiera simplicifolia* lectin.
11. Low-endotoxin immunoglobulin G_{2b} (IgG2b) (BD Biosciences).
12. Low-endotoxin anti- $\alpha 4\beta 1$ (PS/2, BD Biosciences).
13. Low-endotoxin anti-VCAM (MK-1, BD Biosciences).
14. rsVCAM (R&D Systems).

2.9. Immunohistochemistry

1. Radioimmunoassay-grade BSA (Sigma-Aldrich).
2. Alexa 488 (green)-conjugated secondary antibody (Invitrogen).
3. Alexa 568 (red)-conjugated secondary antibody (Invitrogen).

4. Various antibodies, such as antifibronectin (clone TV-1) from Chemicon International; rat antimouse VCAM (M/K-2, Chemicon International); antipan species VCAM (H-276, product number sc-8304, Santa Cruz Biotechnology); anti-F4/80 (AbD Serotec, Raleigh, NC).
5. Rat antimouse CD31 (MEC 13.3, BD Biosciences).
6. Mouse antihuman Ki67 (Chemicon International, Temecula, CA).
7. Tissue Tek OCT (Electron Microscopy Sciences, Hatfield, PA).
8. Mounting medium (e.g., Vectashield, Vector Laboratories).
9. Phosphate-buffered saline.
10. Glass coverslips.
11. Glass slides.

3. Methods

3.1. Progenitor Cell Isolation

1. Put 15 mL room-temperature Histopaque 1077 into 50-mL tubes (or 5 mL Histopaque1083 in 15-mL tubes for rodent cells) for density gradient centrifugation.
2. Dilute freshly isolated blood in 2–4 volumes PBS-E (or dilute buffy coat from blood bank at same proportions in PBS-E).
3. Carefully layer 35 mL diluted blood suspension on top of Histopaque layer (*see Note 1*).
4. Centrifuge immediately at 400*g* for 25 min at room temperature without braking at centrifuge (*see Note 2*).
5. After centrifugation, carefully aspirate about half of the upper layer and discard. The opaque interface contains the MNCs.
6. Prepare clean centrifuge tubes (conical 50 or 15 mL) containing 10 mL PBS-E for the collection of the cells.
7. Carefully remove the mononuclear layer with a 10-cc syringe and 18-gage needle. Transfer cells to tubes containing PBS-E, pooling MNCs.
8. Centrifuge at 300*g* for 10 min at 20°C to pellet and to remove Histopaque.
9. Aspirate supernatant, resuspend cells in a small amount of PBS-E (5–10 mL), and pool cells from each two or three tubes into one 50-mL tube. Bring volume up to 50 mL with

PBS-E. Mix gently with pipet, and centrifuge again at 200*g* for 10 min at 20°C to remove platelets.

10. Aspirate supernatant and resuspend each pellet, pooling all cells in total volume of 5 mL PBS-E.
11. Add 15–20 mL of ammonium chloride red blood cell lysis solution and incubate on ice for 5–10 min. In case of large amounts of red blood cells, add 20 mL ammonium chloride and keep in ice for 10 min. In case of animal blood, use a ratio of 4:1 ammonium chloride to PBS-E in a 15-mL tube.
12. Add PBS-E to 50 mL (human cells) or 15 mL tubes (murine cells) and centrifuge at 1200 rpm for 5 min at 5°C to remove residual ammonium chloride.
13. Aspirate supernatant, resuspend in 5 mL PBS-E (or in 1 mL for murine cells) and determine number of cells/milliliter using a hemocytometer (*see Note 3*).

3.2. Progenitor Cell Purification

3.2.1. Human Progenitor Cell Purification

1. Add 100 μ L FcR blocking reagent per 10^8 isolated human MNCs to prevent unspecific or Fc-receptor-mediated binding of magnetic beads to nontarget cells.
2. Label cells by adding 100 μ L CD34 MicroBeads per 10^8 total cells, mix well, and incubate for 30 min in the refrigerator at 6–12°C.
3. To assess the purity of the isolated human progenitor cells, add 10 μ L FITC-conjugated anti-CD34 antibody recognizing a separate epitope from the QBEND/10 (e.g., CD34-PE, Clone: AC136) and 10 μ L PE-conjugated anti-CD133 and incubate for a further 10 min in the refrigerator at 6–12°C.
4. Wash cells carefully in wash buffer and resuspend in 1–10 mL of buffer.
5. Pass cells through 30- μ m nylon mesh or preseparation filter (Miltenyi Biotech) to remove clumps. Collect flowthrough cells (*see Note 4*).
6. Place the LS⁺/VS⁺ column on the magnetic separator; rinse with buffer. Apply cells to the LS⁺/VS⁺ column on the separator; wash the column three times with 3 mL MACs buffer. CD34⁺ cells bound to the magnetic beads will be retained on the column. The flowthrough will contain CD34⁻ cells.
7. Remove the LS⁺/VS⁺ column from the separator, place column on a tube, and pipet 5 mL buffer on top of column to elute bound CD34⁺ cells.
8. Firmly flush out retained cells with pressure using the plunger supplied with the column.
9. Repeat magnetic separation twice by applying the eluted cells to a new prefilled positive selection column, wash, and elute retained cells in 2.5 mL buffer.

10. Perform FACs analysis on a sample of the purified human progenitor cells to assess purity (*see Note 5*).

3.2.2. Murine Progenitor Cell Isolation

1. Add 100 μ L FcR blocking reagent per 10^8 isolated murine MNCs to prevent unspecific or Fc-receptor-mediated binding of magnetic beads to nontarget cells.
2. Centrifuge cells at 300*g*. Wash one time. Resuspend the pellet in 400 μ L of MACs buffer. For depletion of Lin⁺ cells, cells will be magnetically labeled with a cocktail of biotinylated antibodies against a panel of so-called lineage antigens (CD5, CD45R [B220], CD11b, Gr-1 (Ly-6G/C), 7-4, and Ter-119). Label cells by adding 100 μ L Lin antibodies per 10^8 total cells in 400 μ L, mix well, and incubate for 30 min in the refrigerator at 6–12°C.
3. Wash cells carefully and resuspend in 1–10 mL of buffer. Add antibiotin MicroBeads and incubate 30 min in the refrigerator.
4. Pass cells through 30- μ m nylon mesh or preseparation filter (Miltenyi Biotech) to remove clumps. Collect flowthrough cells (*see Note 4*).
5. Apply cells to the LS⁺/VS⁺ column mounted on the magnetic separator; wash the column three times with 3 mL buffer, collecting the flowthrough. These are the Lin⁻ progenitor cells.
6. Repeat magnetic separation twice by applying the eluted cells to a new prefilled negative selection column, collecting flowthrough and washes each time.
7. To assess the purity of the murine cells, incubate aliquots of 50,000–100,000 purified murine cells with FcR block, then add 10 μ L antibiotin-FITC (Miltenyi Biotech), anti-c-kit-FITC or anti-Sca-1-FITC antibodies. Incubate 20 min on ice, wash twice with PBS, and analyze by flow cytometry. Progenitor cells will be Lin⁻ and Sca1⁺ (*see Note 5*).

3.3. FACs Analysis

1. To evaluate the expression of integrins on EPCs, aliquot 5×10^5 cells into polypropylene snap-cap tubes, one tube per antibody to be tested. Prepare also one tube for a positive control and one tube for a negative control. Centrifuge cells at 200*g* and remove supernatant. Resuspend in PBS plus BSA.
2. Incubate cells in FcR Blocking Reagent.
3. Add 100 μ L of antibody per tube (e.g., a 1/30 dilution of a hybridoma supernatant or a 1/100 dilution of a rabbit polyclonal, prepared in tissue culture medium containing 1% BSA). Incubate 20–60 min on ice.

4. Wash cells twice by centrifugation with medium containing 1% BSA.
5. For antibodies that are not directly labeled, add 100 μ L of secondary antibody (FITC conjugated or rhodamine conjugated) per tube of cells (e.g., 0.25 mg/mL of rabbit anti-mouse, goat antirat, or goat antirabbit IgG in tissue culture medium). Incubate for 20–60 min on ice, in the dark).
6. Wash cells twice by centrifugation with cold PBS containing 1% BSA. Fix cells with 200 μ L of cold 0.5% paraformaldehyde in PBS. Store in the dark at 4°C until analysis.

3.4. Progenitor Cell Culture

1. Under sterile conditions, coat 6-well tissue culture plates with 10 μ g/mL fibronectin.
2. Plate 10×10^6 mononuclear or CD34⁺ cells per 6-well plastic plate in 2 mL culture medium. Do not remove media until d 4 (*see Note 6*).
3. Wash gently with PBS, Ca²⁺ and Mg²⁺ free.
4. Add PBS containing 1 mM EDTA (PBS-E) and incubate 10–15 min at 37°C.
5. Collect the detached cells by repeated flushing of plate with PBS-E (*see Note 7*).
6. Centrifuge cells at 200*g* for 5 min at 5°C.
7. Resuspend cells in EPC growth medium and replat on fresh fibronectin-coated plastic dishes at 1×10^6 cells/6-well dish.
8. On d 7, change media.
9. To assess the number of endothelial-like cells in these cultures, plate 2×10^6 MNCs/well of a glass chamber slide coated with fibronectin plus 0.5% gelatin or vitronectin plus 0.5% gelatin.
10. On d 4–14, add 5 μ L of an undiluted solution of DiI-Ac-LDL to one well (500 μ L medium) and incubate for 1 h at 37°C.
11. Remove media containing DiI-Ac-LDL from the culture.
12. Wash cells carefully with PBS.
13. Fix with 1% paraformaldehyde at 4°C (500 μ L each).
14. Wash once in PBS.
15. Dilute Ulex lectin to 30 μ L/300 μ L in PBS and add 300 μ L to each well for 1 h at room temperature in the dark. For rodents, use BS1-lectin at 5 μ L/500 μ L PBS.
16. Gently wash the slide with PBS two or three times.
17. Apply 1 drop of Vectashield mounting medium on the well of the slide and cover with coverslip.

18. Perform fluorescence microscopy, counting double-stained cells under a fluorescence microscope.

3.5. Endothelial Cell Culture

1. Thaw one vial of endothelial cells and place into warmed medium immediately at a density of 5×10^3 cells/cm². Use 1 mL medium/cm² until cells are more than 40% confluent, then use 2 mL/cm².
2. Change medium every other day.
3. Split cells 1:4 when they are 60–80% confluent using 0.025% trypsin, 0.01% EDTA (this is 10X lower than usual concentrations) (*see Note 8*).
4. Freeze several vials after first passage. Cells are useful for up to six passages before they begin to senesce.

3.6. Adhesion Assays

3.6.1. Adhesion of EPC to Endothelial Cells

1. To prepare HUVEC monolayers: seed 200,000 HUVECs in EGM- per well of a 48-well plate 24 h before adhesion assay.
2. To prepare EPC or BMDC population, pellet purified cells by centrifugation and resuspend in warmed adhesion medium containing 25 μ M CMTMR. Incubate at 37°C for 60 min. Wash the cells twice by centrifugation and resuspend in prewarmed adhesion medium. Rinse and resuspend cells at 800,000 cells/mL in adhesion buffer.
3. Remove supernatant from HUVEC monolayers, using only a Pipetman to aspirate (*see Note 9*).
4. Add 200,000 EPCs or other bone marrow-derived cells in 0.25 mL per well of HUVEC monolayer.
5. Incubate in 37°C incubator 30 min to 2 h.
6. Aspirate supernatant with Pipetman (*see Note 10*).
7. Wash three times with warm adhesion buffer: Add warm adhesion buffer to wells and remove gently with Pipetman.
8. Remove all medium with Pipetman (*see Note 11*).
9. Wash once with PBS.
10. Fix with 3.7% paraformaldehyde.
11. Store at 4°C until viewing on microscope.

3.6.2. Inhibition of Adhesion of EPCs to Endothelial Cells

1. Prepare HUVEC monolayers: Seed 200,000 HUVECs per well of a 48-well plate 24 h before adhesion assay.
2. To prepare EPC or BMDC population, pellet purified cells by centrifugation and resuspend in warmed adhesion medium containing 25 μ M CMTMR. Incubate at 37°C for 60 min. Wash the cells twice by centrifugation and resuspend in prewarmed adhesion medium. Rinse and resuspend cells at 800,000 cells/mL in adhesion buffer.

3. Prepare 1-mL aliquots of murine cells. Add 25 μg rsVCAM, 25 μg antimurine $\alpha 4\beta 1$, 25 μg antimurine $\alpha 5\beta 1$, 25 μg antimurine $\alpha \nu$, or 25 μg antimurine $\beta 2$ integrin antibody cells. Alternatively, prepare similar concentrations of anti-human integrin antibodies when using human CD34⁺ cells.
4. Remove supernatant from HUVEC monolayers using only Pipetman to aspirate (*see Note 11*).
5. Place 0.25 mL progenitor cells (200,000) per well (six wells per antibody). Incubate in 37°C incubator. Aspirate supernatant of three wells per antibody with Pipetman after 15 min; wash once with warm adhesion buffer. Add warm adhesion buffer to wells and replace in incubator.
6. At 30 min after start of adhesion, aspirate supernatant of remaining three wells per antibody with Pipetman; wash once with warm adhesion buffer.
7. Remove all medium with Pipetman, wash once with PBS; fix with 3.7% paraformaldehyde. Store at 4°C until viewing on microscope.

3.6.3. Adhesion of EPCs to ECM

1. Coat 48-well plates with 5 $\mu\text{g}/\text{mL}$ fibronectin, vitronectin, rsVCAM, or BSA. Incubate overnight at 4°C.
2. Discard coating solution and rinse with protein-free PBS (divalent cation free). Block nonspecific binding sites with heat-treated 3% BSA in PBS. Block wells with 200 μL per well at room temperature 1 h at 37°C incubator.
3. Wash once with PBS.
4. Resuspend EPCs in adhesion buffer, 800,000 cells per milliliter. Label cells as 3.6.2.3.
5. Prepare 5-mL aliquots of cells. Add 25 $\mu\text{g}/\text{mL}$ antimurine $\alpha 4\beta 1$, 25 $\mu\text{g}/\text{mL}$ antimurine $\alpha 5\beta 1$, 25 $\mu\text{g}/\text{mL}$ antimurine $\alpha \nu$, 25 $\mu\text{g}/\text{mL}$ antimurine $\beta 2$ integrin antibodies to aliquots of cells.
6. Aliquot triplicates per ECM plate per each antibody condition. Add three 250- μL aliquots of anti- $\alpha 4\beta 1$, anti- $\alpha 5\beta 1$, anti- $\alpha \nu$, anti- $\beta 2$, rsVCAM, and no antibody per ECM protein. Incubate 15–30 min.
7. Aspirate supernatant of remaining three wells per antibody with Pipetman; wash once with warm adhesion buffer.
8. Remove all medium with Pipetman, wash once with PBS; fix with 3.7% paraformaldehyde. Store at 4°C until viewing on microscope.

3.7. Bone Marrow Transplant

1. Treat recipient mice with a suspension of sulfamethoxazole and trimethoprim (SMZ) for 1 wk prior to irradiation and 2 wk after bone marrow transplantation.

2. Irradiate female mice of FVB/N or C57Bl6 genotype (4–6 wk) with a single dose of 1000 rad (10 Gy). Bone marrow should be transplanted within the same day.
3. Euthanize mice. Sterilize with ethanol.
4. Aseptically remove femurs, using separate sterile surgical tools for cutting soft tissues and bones. Remove muscle tissue from femurs. Cut off ends of each bone and flush with MAC buffer (2 mL/femur and 1 mL/tibia) into plastic centrifuge tubes on ice. Triturate to break up clumps and form single cell suspension.
5. Centrifuge at 400*g* for 10 min at room temperature.
6. Resuspend in 10 mL ammonium chloride buffer and incubate for 4–5 min at room temperature.
7. Transfer to 50-mL centrifuge tube and stop reaction by adding 30 mL PBS without MgCl₂ or CaCl₂.
8. Centrifuge at 400*g* for 10 min at room temperature.
9. Resuspend in 4 mL MAC buffer and transfer to 15-mL Falcon tube. Add 4 mL of RT Histopaque 1083 to the bottom of the tube. Centrifuge at 400*g* for 25 min at room temperature without brakes.
10. Aspirate surface layer, then transfer cloudy layer containing the purified bone marrow cells into a new 15-mL centrifuge tube. Wash twice with PBS by resuspending in PBS and centrifuging at 400*g* for 10 min at 4°C.
11. Add ice-cold PBS without MgCl₂ or CaCl₂.
12. Resuspend at 2.5×10^7 cells/mL in PBS (store on ice until ready for injection into recipient mice). Warm bone marrow cells to room temperature before injecting into recipient mice.
13. Inject room temperature bone marrow cells into irradiated recipient mice by tail vein injection (5×10^6 cells in 200 μ L/animal).
14. Monitor animals for 4 wk after bone marrow transplantation; animals will be fully recovered and ready for *in vivo* angiogenesis studies.

3.8. Tumor Homing Studies

1. Label human CD34⁺ cells with CMTMR: Pellet purified cells by centrifugation and resuspend in warmed culture medium containing 25 μ M CMTMR. Incubate at 37°C for 60 min. Wash the cells twice by centrifugation and resuspend in prewarmed culture medium. Rinse and resuspend cells at 800,000 cells/mL in culture medium.
2. To test the roles of various integrins in progenitor cell homing to tumor neovasculature, incubate 0.5×10^6 CMTMR-labeled

CD34⁺ human progenitor cells, CMTMR-labeled cultured EPCs, in EBM basal culture medium, medium with 50 µg/mL of low-endotoxin anti-αvβ3 (LM609), or antihuman α4β1 (either HP2/1 from Biogen or 9F10 from BD Bioscience) on ice for 30 min.

3. Inject cells by tail vein injection in a final volume of 100 µL into nude mice bearing 0.5-cm LLC or other tumors. After 1 h, sacrifice animals. Excise tumors.
4. Alternatively, inject 1×10^6 purified EGFP⁺Lin⁻ or Lin⁺ cells into C57Bl6 mice bearing 0.5-cm LLC tumors.
5. Treat animals injected with EGFP⁺Lin⁻ cells for 5 d by intravenous administration of 100 µL saline, 200 µg low-endotoxin anti-αMβ2 (Pharmingen) or 200 µg low-endotoxin anti-α4β1 (PS/2, Biogen) every other day.
6. After 5 d, sacrifice animals and excise tumors plus surrounding connective tissue. Embed in OCT to cryopreserve. Section into 10-µm thick cryosections.
7. Determine microvascular density by immunostaining to detect CD31 and CMTMR or EGFP. Count CMTMR⁺ CD31⁺ or EGFP⁺ CD31⁺ vessels per ×200 microscopic field in 10 randomly selected fields per tumor. Calculate average microvessel density in all tumors per treatment group.

3.9. Immunohistochemistry

1. Remove frozen section on glass microscope slides from -80°C freezer.
2. Dry the glass portion of the slide surrounding the sections with a Kimwipe and draw a circle around the section with the hydrophobic pen (PAP-Pen).
3. Fix slides for 30 s in cold acetone (place in slide-washing container filled with acetone for 30 s, then remove).
4. Wash twice for 5 min each in PBS. After this step, never let the section dry out.
5. Block the sections for 2 h with 8% normal goat serum or 2.5% BSA in a humidified chamber created in a Tupperware container. To create a humidified chamber, place a damp paper towel in the bottom of a Tupperware or other container and then place a pipet tip container lid on top of the paper towel. Place the slide flat on the lid and place a drop (100 µL) of block buffer on each encircled section. Replace the Tupperware lid and incubate at room temperature for 2 h.
6. Rinse the slides twice in PBS by dipping five times into container filled with PBS.
7. Dry the glass portions of the slide and remove excess liquid from the section by touching the corner of a Kimwipe to the edge of the section.

8. Apply 100 μL of the primary antibody dilution, typically at 1–10 $\mu\text{g}/\text{mL}$ in PBS-containing block buffer, to the encircled section on the left of the slide. Apply block buffer to the section on the right of the slide (negative control). Incubate for 2 h at room temperature.
9. Wash the slides by dipping in PBS-filled containers five times for 10 dips, then ending with one 5-min PBS wash. Dry the slide and wick the excess liquid from the section as described 3.9.7.
10. Incubate in secondary antibody dilution (1/250–1/1000) in 2.5% BSA in PBS by applying 100 μL to the encircled section. Incubate 1 h at room temperature. If the primary antibody is directly labeled, skip this step.
11. Wash the slide by dipping five times for 10 dips in PBS, then ending with one 5-min wash.
12. Dry the slide and wick the excess liquid from the section. Apply 20 μL of Vectashield (or other mounting medium) and place a coverslip gently on the section.
13. Place a drop of nail polish on the corners of the coverslip to fix it in place. When dry, store slide in refrigerator in a flat slide holder or in a slide box.

4. Notes

1. This is best accomplished using a 25-mL pipet for human cells.
2. Centrifugation at low temperatures may result in cell clumping and lower recovery.
3. Using this method, 3×10^8 EGFP⁺ bone marrow-derived MNCs were purified by gradient centrifugation from the femurs and tibias of six ACTB-EGFP mice.
4. Wet filter with buffer before use.
5. Using this method, 3×10^6 CD34⁺ human cells were purified from 7×10^9 peripheral blood MNCs at 98% purity; 4.8×10^6 murine Lin[−] cells were isolated from bone marrow-derived MNCs by two rounds of negative selection for a panel of lineage markers (CD5, B220, CD11b, Gr-1, 7-4, and Ter119; Miltenyi Biotech). The selected population was 90% positive for lineage markers and 80% positive for c-kit expression. Lin⁺ cells were 100% positive for lineage markers. The 0.4×10^6 Lin-Sca 1⁺ cells (70% Sca1⁺) were isolated from Lin[−] cells by three rounds of immune selection for the stem cell marker Sca1⁺ (Miltenyi Biotech).

6. “EPCs” become spindle shaped; remove floating cells. It is important to note that several investigators have found that these “EPCs” express monocyte markers in addition to endothelial markers; thus, the exact nature of these *in vitro* differentiated cells remains to be established.
7. Add trypsin at 500 μ L per well and incubate 2 min at 37°C only if necessary to detach the remaining cells. Avoid trypsin when FACs analysis will be performed.
8. Do not allow cells to become confluent as they are sensitive to contact inhibition.
9. Do not use vacuum aspiration, which may disturb the monolayer.
10. Do not use vacuum aspiration as it will disturb the monolayer.
11. Do not use vacuum aspiration.

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