Table 8.1 Functional characteristics of hEPC colonies in hEPC differentiation

	Primitive EPC CFU (PEPC-CFU)	Definitive EPC-CFU (DEPC-CFU)
Colony-forming cell features	Weakly adhesive round cells (10–20 μm)	Strongly adhesive spindle-like cells with monolayer structure (50–200 μm)
Cell density per colony	High	Low
2nd colony-forming potential	High	Low~ Absence
Gene expression:		
CD31	Low	High
VEGFR-2,VE-Cad, eNOS, Tie2, vWF	Low	High
CD45, CD14 immunocytochemistry:		
VEGFR-2, VE-Cad, eNOS	Yes	Yes
acLDL-DiI uptake and	Yes	Yes
UEA1 lectin-FITC binding		
Angiogenic/ vasculogenic activity:		
Proliferation	Dominant	
Adhesion		Dominant
Tube formation promotion		Dominant
Sprouting activity		Dominant
In vivo vascular regeneration		Dominant

Low, High, and Dominant indicates the comparison between PEPC-CFU and DEPC-CFU. Yes indicates the positive feature in each item of immunocytochemistry.

single cells or bulk cells from hEPC-enriched, arbitrary fractions, their cultured cells or nonselected populations; and (2) cell fate analysis of single cells or bulk cells following suspension conditions. Our newly introduced analytical system clarifies the cell fate of each sample cell, reviews the differentiation hierarchy of hEPCs, and identifies the determinants of hEPC proliferation, commitment, and differentiation *in vitro* and *in vivo* (Figure 8.2). In this chapter, we present a step by step protocol for the hEPC-CFA of isolated cell populations from UCB, PB, or BM.

Materials

Laboratory reagents

1. Cells: The experimental protocol of fresh PB, UCB, or BM should be performed under the approval of the institutional ethical committees of the Cord Blood and/or Clinical Investigation Committee.

Alternatively, isolated MNCs, $CD34^+$, or $CD133^+$cells in UCB, PB, or BM can be purchased (e.g. ALLCELLS™: http://www.allcells.com).

All blood samples should be handled under the biological guideline for human samples. Investigators should work with a class 2 safety cabinet, and wear gloves and other personal protective clothing, which should be disinfected with bleach before being discarded.

2. MethoMethoCult™ GF^+H4435 (Stem Cell Tec, cat. no. 04435)
3. Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, cat. no. 31980-030)
4. BSA (Sigma-Aldrich, cat. no. A4503)
5. PBS without calcium and magnesium (Sigma-Aldrich, cat. no.P4417-100TB)
6. 0.5 M EDTA solution (Nacalai Tesque, cat. no.14362-24)
7. 0.05% (wt/vol) Trypsin/ 0.2% (wt/vol) EDTA ·4Na solution (Sigma-Aldrich, cat. no. T3924)
8. Ammonium chloride, NH_4Cl (Wako Pure Chemical Industries Ltd., cat. no. 017-02995)
9. Potassium bicarbonate, $KHCO_3$ (Wako Pure Chemical Industries Ltd., cat. no.166-03275)
10. EDTA-2Na (Wako Pure Chemical Industries, Ltd., cat. no. 345-01865)
11. Histopaque-1077 (Sigma-Adrich, cat. no. 10771)

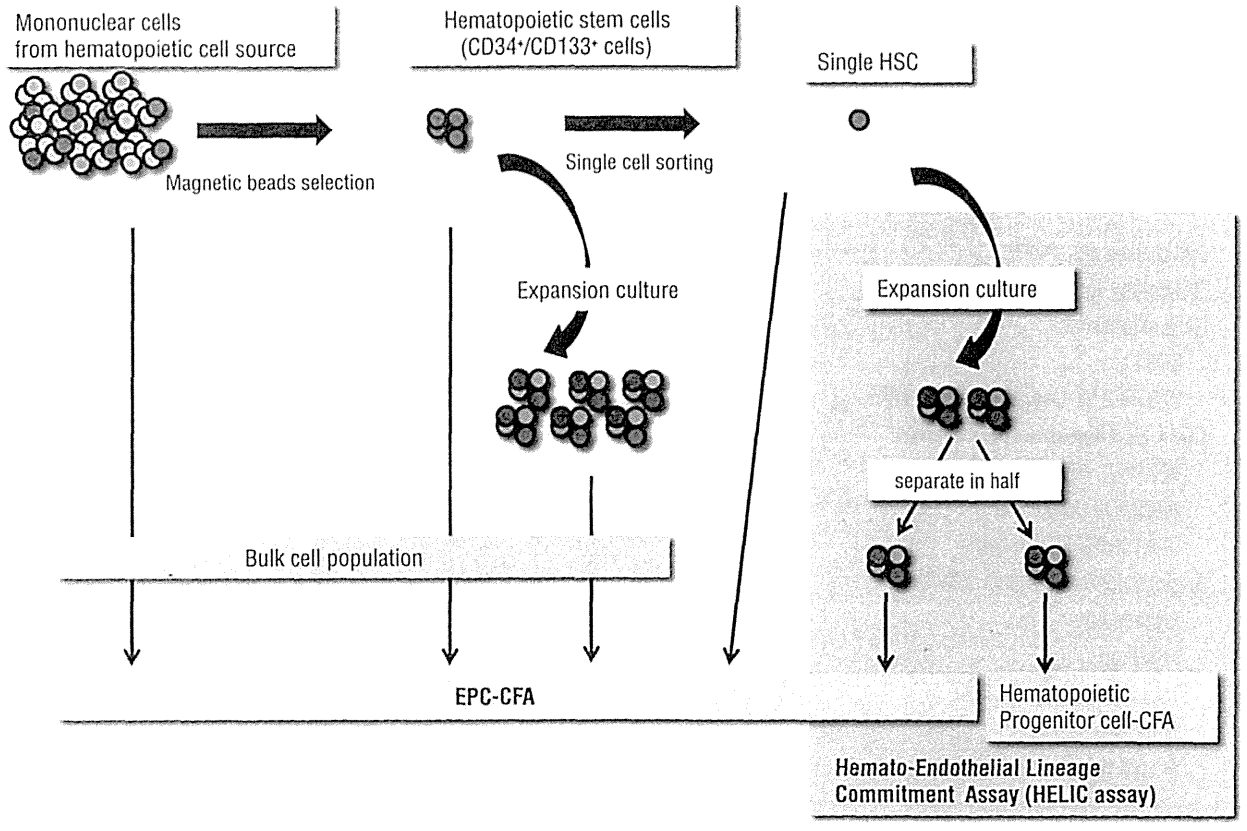

Figure 8.2 Application of EPC-CFA for primary or expansion cultured cell populations.
EPC-CFA is applicable to primary or expansion cultured cells, not only for bulk non-selected MNCs or selected $CD34^+/CD133^+$ cells, but also for single selected $CD34^+/CD133^+$ cells. In particular, the hematoendothelial lineage commitment assay (HELIC assay), which is applicable to EPC-CFA, uses single hematopoietic stem cells to analyze their commitment into hematopoietic and/or endothelial lineages. In brief, EPC-CFA and a conventional hematopoietic progenitor cell-CFA can be performed by using half of the expanded cells from a single hematopoietic stem cell. Subsequently, the cell fate of the single stem cells into endothelial and/or hematopoietic cell lineages can be quantitatively evaluated by analyzing the frequency of the stem cells generating EPC-CFUs in EPC-CFA and/or hematopoietic lineage-CFUs (e.g. erythrocyte-CFU, granulocyte/ macrophage-CFU, monocyte/ macrophage-CFU) in hematopoietic progenitor cell-CFA. (Source: Masuda H. 2013 [2]. Reproduced with permission from Elsevier).

12. CD34 MicroBead Kit (Miltenyi Biotec., cat. no. 130-046-702)
13. CD133 MicroBead Kit (Miltenyi Biotec., cat. no. 130-050-801)
14. Cellbanker (Nippon Zenyaku Kogyo Co., Ltd., cat. no. BLC-1)
15. Reagents of semisolid culture medium for EPC-CFA (Table 8.2)
16. Reagents for expansion culture (EX-culture), and hematoendothelial lineage commitment (HELIC) assay (Table 8.3)
17. $CaCl_2 \cdot 2H_2O$ (Wako Pure Chemical Industries, Ltd, cat. no. 031-00435)

Laboratory equipment

1. Culture dishes or plates (Table 8.4)
2. Polypropylene conical tubes, 15 mL (TPP, cat. no. 91015)
3. Polypropylene conical tubes, 50 mL (TPP, cat. no. 91050)
4. Screw-cap sampling tubes, 1.5 mL (Assist, cat. no. A.1500)
5. 14-mL polypropylene round-bottom tubes (BD falcon, 352059)
6. CryoTube™ vials (Nunc, cat. no. 366656)
7. Bio freezing vessels, Bicell (Nihon Freezer Co., Ltd.)

Table 8.2 Reagents for semisolid master mixture for EPC-CFA

Reagents	Company, Cat. no.	Final concentration
MethoCult™ SF^{BIT} H4236	Stem Cell Tec, 04236	
rhSCF	Peprotec, 300-07	100 ng/mL
rhVEGF	Peprotec, 100-20	50 ng/mL
rhbFGF	Peprotec, 100-18B	50 ng/mL
rhEGF	Peprotec, 100-15	50 ng/mL
rhIGF-1	Peprotec, 100-11	50 ng/mL
rhIL-3	Peprotec, 200-03	20 ng/mL
Heparin	Ajinomoto Pharma, 699053417	2 U/mL
FBS	SAFC Biosciences, 12303	30% (vol/vol)
Penicillin/ streptomycin	Invitrogen, 5140-122	1/100 (vol/vol)

rhbFGF, recombinant human basic fibroblast growth factor; rhEGF, recombinant human epidermal growth factor; rhIGF-1, recombinant human insulin like growth factor-1; rhIL-3, recombinant human interleukin-3; rhSCF, recombinant human stem cell factor; rhVEGF, recombinant human vascular endothelial growth factor.

Table 8.3 Reagents of EX-culture medium

Reagents	Company, Cat no.	Final concentration
Stem Span™ SFEM	Stem Cell Tec, 09650	
rhSCF	Peprotec, 300-07	100 ng/mL
rhFlt-3 ligand	Peprotec, 300-19	100 ng/mL
rhTPO	Peprotec, 300-18	20 ng/mL
rhVEGF	Peprotec, 100-20	50 ng/mL
rhIL-6	Peprotec, 200-06	20 ng/mL
Penicillin/ streptomycin	Invitrogen, 5140-122	1/100 (vol/vol)

rhFlt-3 ligand, recombinant human FMS-like tyrosine kinase 3 ligand; rhIL-6, recombinant human interleukin-6; rhSCF, recombinant human stem cell factor; rhTPO, recombinant human thrombopoietin; rhVEGF, recombinant human vascular endothelial growth factor; SFEM, serum-free expansion medium.

Table 8.4 Materials for EX-culture, EPC-CFA, and HELIC assay

Culture vessels	Company, Cat. no.	Application
35-mm Primaria™ tissue culture dish	BD Falcon, 353801	EPC-CFA of bulk cells
96-well Primaria™ tissue culture plate	BD Falcon, 353872	EPC-CFA of single cells, EX-culture of HELIC assay
24-well Primaria™ tissue culture plate	BD Falcon, 353847	EX-culture of bulk cells, EPC-CFA of HELIC assay
24-well suspension culture plate	Greiner, 662102	HPC-CFA of HELIC assay
18-gauge blunt end needle	NIPRO, 02-161	Seeding cells for EPC-CFA or HPC-CFA
Gridded scoring dish	Stem Cell Tec., 27500	Counting EPC-CFUs or SAU
14-mL Polypropylene round-bottom tube	BD Falcon, 352059	Storage of semisolid medium for EPC-CFA or HPC-CFA

8. Polystyrene microcentrifuge tube with plug cap, 1 mL for fluorescence-activated cell sorting (FACS) (Fisher Scientific, cat. no. 04-978-145)
9. 20-μL pipette tip (MbP, cat. no. 2069)
10. 200-μL pipette tip (MbP, cat. no. 2069)
11. 1000-μL pipette tip (MbP, cat. no. 2079E)
12. 20-μL pipette (Gilson, cat. no. F123600)
13. 200-μL pipette (Gilson, cat. no. F123601)
14. 1000-μL pipette (Gilson, cat. no. F123602)
15. Tuberculin syringe, 1 mL (Terumo, cat. no. SS-01T)
16. Syringe, 5 mL (Terumo, cat. no. SS-05LZ)
17. Syringe, 20 mL (Terumo, cat. no. SS–20ESZ)
18. Syringe, 50 mL (Terumo, cat. no. SS–50ESZ)
19. 2-gauge winged infusion set (Terumo, cat. no. SV-21DLK)
20. 23-gauge winged infusion set (Terumo, cat. no. SV-23DLK)
21. 18-gauge needle (Terumo, cat. no. NN-1838R)
22. Bottle top vacuum filtration system (Iwaki, cat. no. 11-067-008)
23. Disposable Pasteur pipette, 9 inch (Iwaki, cat. no. IK-PAS-9P)
24. Centrifuge (Hitachi, cat. no. Himac CF 8DL) for 15-mL or 50-mL conical tube
25. Centrifuge (Tomy, cat. no. MX-160) for 1.5-mL screw-cap tube
26. Vacuum pump for negative-pressure filtration or aspiration (ULVAC, cat. no. DAP-15)
27. Parafilm (Hitech Inc., cat. no. F-20001)
28. Cell filtration column of 30-μm nylon mesh (Consul, cat. no. 130-33S)
29. Polystyrene tubes (BD Falcon, cat. no. 352008)
30. Cell strainer capped polystyrene tube (BD Falcon, cat. no. 352235)
31. Vortex-Genie 2 (M&S Instruments Inc.)
32. Phase-contrast light microscope (Eclipse TE300, Nikon)
33. Fluorescent microscopy (IX70, Olympus)
34. autoMACS™ Separator (Miltenyi Biotec.)
35. FACSAria™ cell sorter(BD)
36. LSRFortessa™ cell analyzer (BD)

Methods

MNC isolation from fresh PB or UCB

Preparation of working solution

1. Ammonium chloride solution (0.15 M NH_4Cl) for hemolysis:
   a. Measure 8.26 g NH_4Cl, 1.0 g $KHCO_3$, and 0.037 g EDTA-2Na.
   b. Dissolve together in 1000 mL of sterilized MilliQ water.
   c. Adjust the solution to pH 7.3 and store at 4°C after autoclave and cooling down.
2. PBS:
   a. Dissolve one PBS tablet in 1000 mL MilliQ water.
   b. Adjust to pH 7.4, and store at 4°C after autoclaving and cooling down.
3. PBS/ 2 mM EDTA: Add 0.5 M EDTA solution to autoclaved PBS (pH 7.4) at the ratio of 1:250 (vol/vol).
4. 10% (w/vol) BSA/PBS:
   a. Measure 50 g of BSA and dissolve in 500 mL PBS.
   b. Sterilize by filtration through bottle top vacuum filtration system with a vacuum pump.
   c. Aliquot and store at –20°C until use.
   For preparation of 0.1% BSA/PBS, dilute 10% BSA/PBS solution with PBS at the ratio of 1:100 (w/vol) prior to use.
5. MACS™ buffer: Adjust 2 mM EDTA/ 0.5% BSA/ PBS solution, sterilize by filtration through bottle top vacuum filtration system with a vacuum pump and store at 4°C until use.

Procedure

Guideline. Depending on an experimental design, the required PB volume will vary, e.g. 10 mL for EPC-CFA alone or more than 50 mL for isolation of $CD34^+/CD133^+$ cells. The protocol described here is for the isolation of $CD34^+$ or $CD133^+$ cells as a large-scale experiment, requiring 15 mL of Histopaque-1077 per 50-mL conical tube. The small-scale protocol for EPC-CFA alone requires 5 mL of Histopaque-1077 per 15-mL conical tube. In the case of UCB, use the large-scale protocol.

1. Prepare at room temperature (RT) 15 mL of Histopaque-1077 per 50-mL conical tube.
2. Gently layer 30 mL of PB or UCB on 15 mL Histopaque-1077 in a 50-mL conical tube.
3. Centrifuge at 400 *g* at RT for 30 minutes without acceleration and with low deceleration.
4. Confirm a clear white monolayer.

Tips. Blood samples and Histopaque-1077 should be preincubated at RT (15°C to 25°C) to form a clear

monolayer of MNCs and acquire sufficient MNC numbers. Note that a blurred white monolayer will yield insufficient MNCs.

5. Aspirate upper a plasma layer with a 9-inch sterilized Pasteur pipette attached to a vacuum pump.

Tip. Leave an upper plasma layer to 1 cm above the visible white monolayer containing MNCs to avoid the aspiration of MNCs.

6. Carefully transfer the entire layer of MNCs to 50-mL conical tube with a 1000-μL pipette.

Tip. Thoroughly harvest the part of the monolayer attached to the inside wall of the conical tube to isolate as many MNCs as possible.

7. Completely resuspend the harvested MNC suspension to 15 mL with 2 mM EDTA/PBS solution.
8. Centrifuge at 850*g* at 4°C for 20 minutes.
9. Aspirate the supernatant with a sterilized Pasteur pipette attached to a vacuum pump.
10. Warm NH_4Cl solution at 37°C in advance.
11. Add 5 mL of 0.15 M NH_4Cl solution to lyse erythrocytes, resuspend the cell pellet completely and incubate at 37°C for 7 minutes.

Tip. If a large number of erythrocytes seem to be contaminated in advance, add NH_4Cl solution up to half of the maximal volume in tube and incubate at 37°C for 7 minutes.

12. Fill up to maximal volume with 2 mM EDTA/PBS solution.
13. Centrifuge at 320*g*, 4°C for 10 minutes.
14. Carefully aspirate the supernatant until the cell pellet alone remains; then completely resuspend the cell pellets with 1 mL of 2 mM EDTA/PBS solution.
15. Fill up to maximal volume with 2 mM EDTA/PBS solution.
16. Centrifuge at 200*g*, 4°C for 10 minutes.
17. Carefully aspirate the supernatant until the cell pellet alone remains, and resuspend MNCs completely with 2 mL of either working medium for EPC-CFA or MACS™ buffer for isolation of $CD34^+$ or $CD133^+$ cells by AutoMACS™.
18. Count the suspended MNCs for downstream application.
19. When performing EPC-CFA of PBMNCs, suspend the cell pellet with 30% FBS/ IMDM at 2×10^5 cells /100 μL per dish for three dishes.
20. Isolate $CD34^+$ or $CD133^+$ cells by using commercially available antibody-coated beads (CD34 MicroBead Kit, CD133 MicroBead Kit from Miltenyi Biotec).
21. Evaluate the purities of the positive cells by flow cytometry (e.g. LSRFortessa™ cell analyzer (BD)). The antibodies for evaluation should be different clones from the ones for cell isolation. The example antibody clones are listed in Table 8.5.

Tips. Freshly isolated $CD34^+$ or $CD133^+$ cells may be cryopreserved until use. We usually cryopreserve

Table 8.5 Antibodies for confirmation of selected $CD34^+$/$CD133^+$ cells

Antibodies etc.	Clone	Isotype	Company, Cat. no.
CD34-FITC	581	Mouse IgG1	BD Biosciences, 555821
CD34-FITC	AC136	Mouse IgG2a	Miltenyi Biotec, 130-081-001
CD133/1-PE	AC133	Mouse IgG1	Miltenyi Biotec, 130-080-801
CD133/1-APC	AC133	Mouse IgG1	Miltenyi Biotec, 130-090-826
CD133/2-PE	293C3	Mouse IgG2b	Miltenyi Biotec, 130-090-853
CD133/2-APC	293C3	Mouse IgG2b	Miltenyi Biotec, 130-090-854
Mouse IgG1-FITC	A851	Mouse IgG1	BD Biosciences, 555748
Mouse IgG2a-FITC	S43.10	Mouse IgG2a	Miltenyi Biotec, 130-091-837
Mouse IgG1-PE	IS5-21F5	Mouse IgG1	Miltenyi Biotec, 130-092-212
Mouse IgG1-APC	IS5-21F5	Mouse IgG1	Miltenyi Biotec, 130-092-214
Mouse IgG2b-PE	ISE6-11E5.11	Mouse IgG2b	Miltenyi Biotec, 130-092-215
Mouse IgG2b-APC	ISE6-11E5.11	Mouse IgG2b	Miltenyi Biotec, 130-092-217

fractionated cells to detach the antibody coated MicroBeads before use. When using cryopreserved $CD34^+$ or $CD133^+$ cells after isolation, the same isotype antibody as coated on MicroBeads (QBEND/10 or AC133) are also available for the subsequent applications (e.g. cell sorting or EX-culture).

EPC-CFA of bulk cell populations in PB, GmPB, BM, or UCB

Preparation of working medium

1. EPC-CFA working semisolid medium
   a. Reconstitute and aliquot each growth factor/ cytokine solution (Table 8.2) according to the manufacturer's data sheets, then store at –20°C until use. Aliquot and store FBS at –20°C until use.
   b. One day prior to adjustment of EPC-CFA working medium, defrost one bottle of MethoCult™ SF^{BIT} H4236 (80 mL).
   c. Add each aliquot of growth factor/ cytokine, FBS, and penicillin/ streptomycin to the bottle; thoroughly mix the whole solution by vigorously shaking the master mix (Table 8.2).
   d. Place the bottle for 20 minutes at RT to eliminate small air bubbles appearing in the mixed solution.
   e. Aliquot 2 mL semisolid solution into each 14-mL Polypropylene round-bottom tubes with a 5-mL syringe attached to a blunt-end needle.
   f. Add 1 mL of 30% (vol /vol) FBS/IMDM with a 1000-µL pipette, and seal the lid with Parafilm to avoid volume loss by evaporation, then store at –20°C until use. The final volume of 3 mL EPC-CFA working medium should be prepared for three dishes of 35-mm Primaria™ Tissue culture dishes (BD Falcon, Cat. no. 353801) per group.
2. EX-culture medium
   a. Dissolve 500 mL of Stem Span™ serum-free expansion medium (SFEM) in one bottle at 4°C over night.
   b. Aliquot 20 mL each of Stem Span SFEM per 50-mL conical tube, and store at –20°C until further use.
   c. After melting in a water bath at 37°C, adjust EX-culture medium by mixing each aliquot of growth factor /cytokine (Table 8.3).
3. Hematopoietic progenitor cell CFA (HPC-CFA) working semisolid medium for HPC-CFA or HELIC assay (optional)
   a. Dissolve one bottle of MethoMethoCult™ GF^+H4435.
   b. Aliquot 3 mL into each 14-mL polypropylene round-bottom tube and store at –20°C until use.

Preparation of cell suspension of bulk cell populations

1. Primary cell populations in PB, GmPB, BM, or UCB: Suspend the freshly isolated or cryopreserved bulk cells (MNCs, $CD34^+$, or $CD133^+$ cells) with 30% FBS/IMDM at the relevant cell number/100 µL per dish, for three dishes, in a 1.5-mL screw-cap sampling tube.

Tip. Note that EPC-CFA is irrelevant for the vasculogenic evaluation of BMMNCs, due to the interference of EPC-colony formation by the growth of contaminated BM stromal cells (Figure 8.3E).

2. EX-cultured $CD34^+$ or $CD133^+$ cells
   a. Adjust the suspension of $CD34^+$ or $CD133^+$ cells with EX-culture medium (1×10^4 cells/500 µL) for EX-culture (Table 8.3).
   b. Seed 500 µL per well of a 24-well Primaria™ tissue culture plate (BD Falcon, Cat. no. 353847), and EX-culture for 7 days.
   c. Harvest EX-cultured cells and adjust the cell suspension with 30% FBS/IMDM at the relevant cell number /100 µL per dish, for three dishes, in a 1.5-mL screw-cap sampling tube.

Tips. The last medium or buffer suspending the cells should be completely replaced with 30% FBS/ IMDM for EPC-CFA, and not with the other solution, e.g. PBS or the different % FBS/IMDM. Replacement with a different medium or buffer interferes with the optimal growth of EPC-CFUs. Prepare more than the 300 µL of the whole cell suspension volume required for the three dishes per sample or group; we usually prepare 350 µL or more for safety.

Semisolid culture for EPC colony formation in EPC-CFA

1. Add 300 µL of the adjusted cell suspension (as in section Preparation of cell suspension of bulk cell

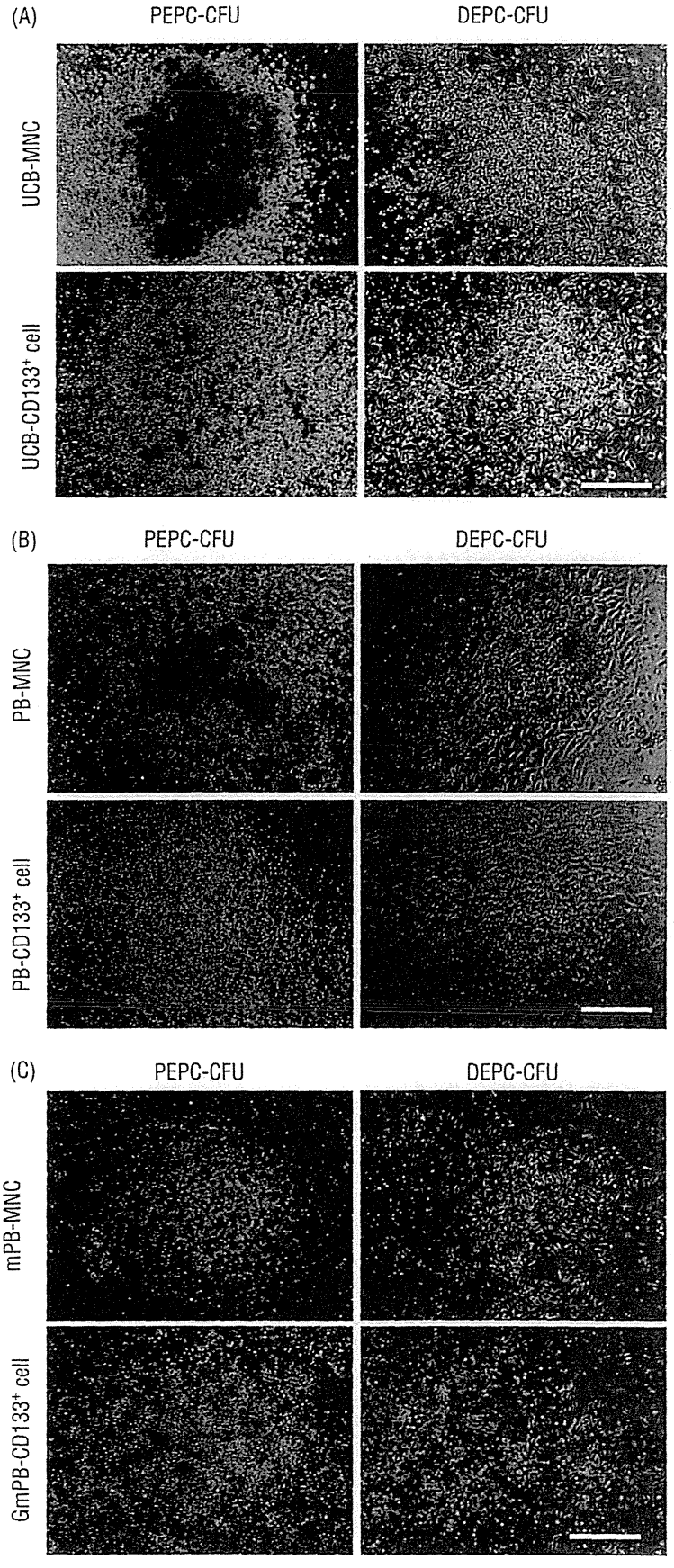

Figure 8.3 The features of EPC-CFUs generated from primary hematopoietic cell populations: (A) UCB, (B) PB, (C) GmPB, (D) BM, (E) stromal cells in EPC-CFA of BM-MNC. Scale bar = 500 μm. (Source: Masuda H 2011 [19]. Reproduced with permission from Wolters Kluwer Health).

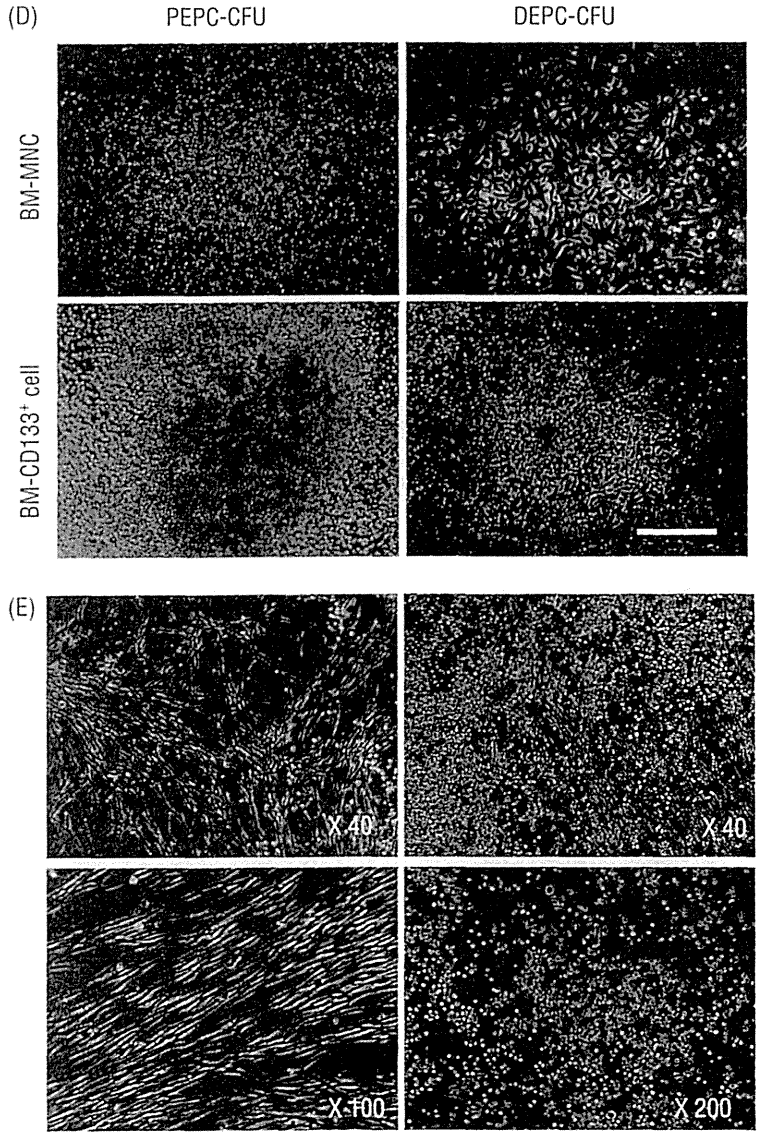

Figure 8.3 *(Continued)*

populations) into 3 mL of EPC-CFA working medium in a polypropylene round-bottom tube.

2. Vortex the tubes for 10 seconds and leave at RT for 10 minutes to eliminate small air bubbles in the medium.

3. Seed 1 mL of the semisolid cell suspension per dish into three 35-mm Primaria™ tissue culture dishes.

Tip. When seeding, pipette up and down the semisolid cell suspension three times in the tube with a 1-mL tuberculin syringe attached to a 18-gauge blunt-end needle, carefully avoiding the generation of small air bubbles.

4. Set a maximum of six semisolid culture dishes into a 15-cm nontreated culture dish as a container and put in the center a 6-cm tissue culture dish with 5 mL of sterilized MilliQ water for humidification, and close the lid of the 15-mL tissue culture dish.

5. Culture at 37°C in CO_2 incubator until the EPC-CFU measurement. Assay plates should be kept in the CO_2 incubator until immediately before observation under microscope.

Tips. (1) When observing the growth of EPC-CFU through the culture period, handle the dishes gently to avoid scattering the colony cells. They are still nonadhesive or lightly adhesive at early time points. Otherwise, it may produce irregular-shaped EPC-CFUs with nondistinct foci, e.g. EPC-CFUs fused with nearby ones or elongated, which makes it difficult to count the exact number of EPC-CFU. (2)

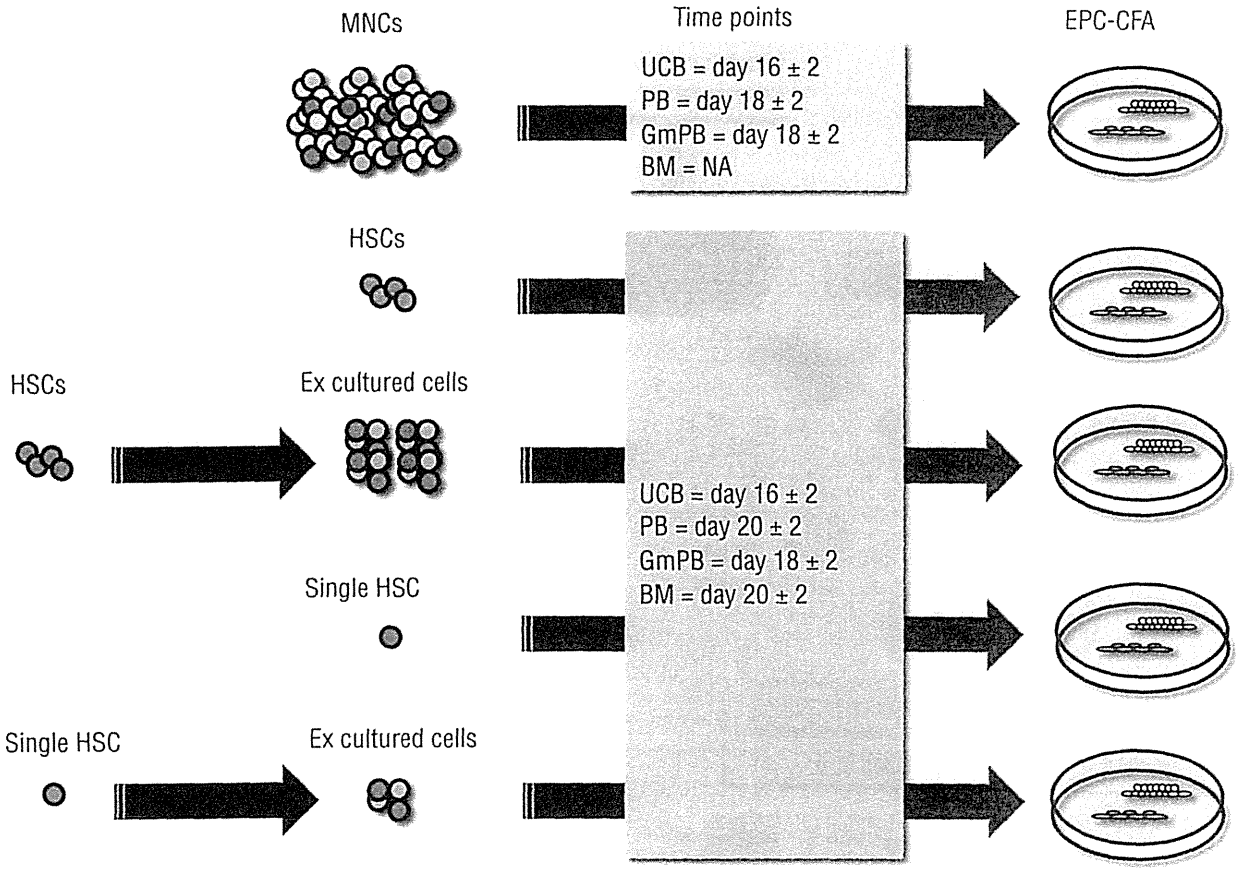

Figure 8.4 Time points of EPC-CFA in each cell population. The time points for EPC-CFA should be optimized by researchers in individual laboratories. The optimized time points necessary for EPC-CFA in our laboratory are shown in this figure. NA, not available.

Culture periods until counting EPC-CFUs are different among the cell populations (Figure 8.4).

Step by step counting procedure of EPC-CFU under phase-contrast microscopy

1. Check the color of the semisolid medium megascopically to assess the growth of EPC-CFU. In case of reddish medium, the growth of EPC-CFUs may be poor. On the other hand, when the medium is yellowish, the growth of EPC-CFUs, especially PEPC-CFUs, may be excellent.

Tips. (1) Note that when seeding MNCs, the growth of noncolony-forming EPCs with spindle-like shape or other hematopoietic colonies may also make the medium yellowish. (2) PEPC-CFUs with highly proliferative cells may be frequently observed as white spot colonies even by the naked eye.

2. Under phase-contrast light microscopy, identify adhesive cell colonies spreading two-dimensionally on the bottom of dish. This discriminates the EPC-CFUs units from the other hematological colonies, which do not adhere to the bottom of the dish.
3. Detect individual EPC-CFUs with a cell density of more than 100 cells per colony.
4. Identify the focus of each EPC-CFU, which is the origin of EPC-CFU. When counting the colonies whose growth is partly limited by the edge of dish, detect the border between colonies to their neighbors to distinguish them from each other and count each as an individual colony.
5. Define the identified colonies as either PEPC-CFU or DEPC-CFU, based on the morphological characteristics depicted in Figure 8.3 and Table 8.1.
6. Count the number of PEPC-CFU and DEPC-CFU per dish.
7. Evaluate the quantification and differentiation of EPC-CFC, as described in Figure 8.5.

Tips. (1) See Figure 8.4 to set the time points for counting the number of EPC-CFUs. (2) In the

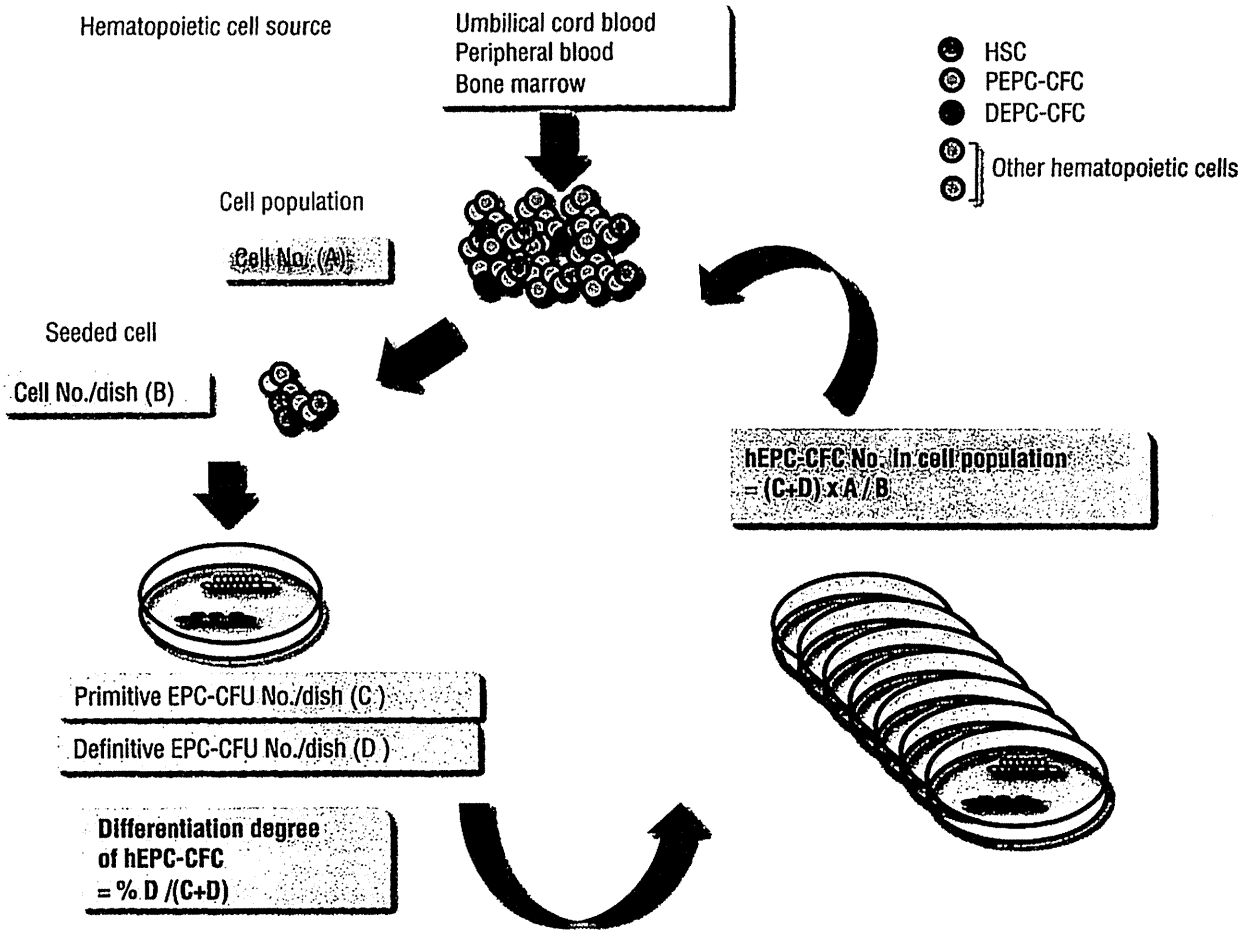

Figure 8.5 EPC-CFA to evaluate the degree of differentiation and number of hEPC-CFCs in a hematopoietic cell population. EPC-CFA allows us to evaluate the differentiation degree and number of hEPC-CFCs in a cell population from hematopoietic cell sources. When performing EPC-CFA, the cell number (A) and seeded cell number per dish (B) of the cell population are first determined. Using the counted numbers of primitive and definitive EPC-CFUs/dish (C and D), the differentiation degree is calculated as the percentage ratio of D to total EPC-CFUs (C + D). Further, total hEPC-CFCs in the whole cell population are simply estimated by the equation (C + D) × A/B. (Source: Masuda H 2013 [20]. Reproduced with permission from Elsevier).

preliminary trials, researchers should optimize the EPC-CFU number generated from each target population by dose-escalation studies in their own laboratories. The results of dose-escalation studies in our laboratory are shown in Figure 8.6.

Optional-1: EPC-CFA of single CD34$^+$ or CD133$^+$ cells in PB, GmPB, BM, or UCB

1. Prepare CD34$^+$ or CD133$^+$ cell suspension in 30% FBS/IMDM (e.g. 1×10^5 cells/mL) and leave on ice until sorting.
2. Apply 100 μL of EPC-CFA working semisolid medium per well of a 96-well Primaria™ tissue culture plate (BD Falcon, Cat. no. 353872).
3. Sort a single CD34$^+$ or CD133$^+$ cell into each well by cell sorter (e.g. FACSAria™ cell sorter(BD)), after staining the cell population with their surface antibodies listed in Table 8.5.
4. Perform EPC-CFA according to the procedure in section Semisolid culture for EPC colony formation in EPC-CFA and section Step by step counting procedure of EPC-CFU under phase-contrast microscopy.

Optional-2: HELIC assay of single CD34$^+$ or CD133$^+$ cells as cell fate assay of HSC

1. Prepare CD34$^+$ or CD133$^+$ cell suspension in EX-culture medium (e.g. 1×10^5 cells/mL) and leave on ice until sorting.

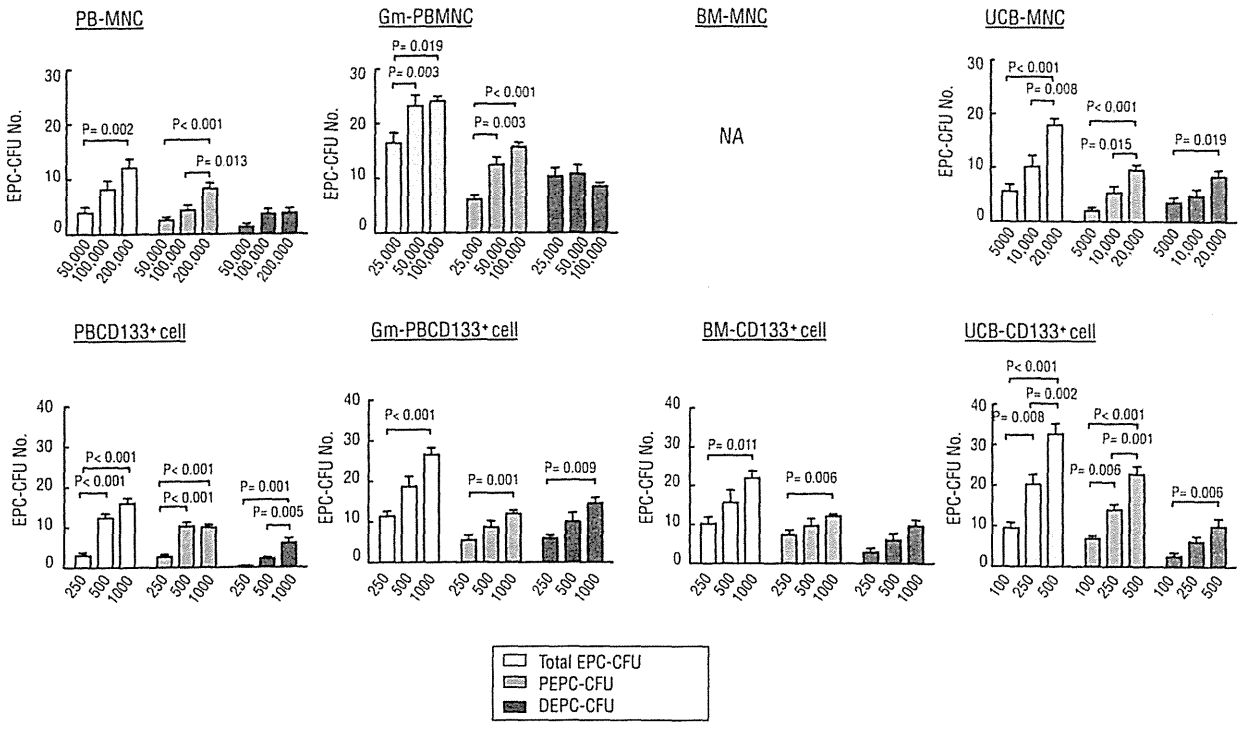

Figure 8.6 Methodological evaluation of EPC-CFA in primary cell populations of hematopoietic samples (PB, mPB, BM, UCB). The bar graphs represent values for each EPC-CFU number in MNC and $CD133^+$ cell with the corresponding seeded primary cell number indicated on the x-axis. Data are means ± SEM. n = 9 per cell population. NA, not available. (Source: Masuda H 2011 [19]. Reproduced with permission from Wolters Kluwer Health).

2. Apply 100 µL of EX-culture medium per well of a 96-well Primaria™ tissue culture plate.
3. Sort a single $CD34^+$ or $CD133^+$ cell into each well by cell sorter (e.g. FACSAria™ cell sorter (BD)), after staining the cell population with their surface antibodies listed in Table 8.5.
4. EX-culture to expand sorted single cells at 37°C for 7 days in a CO_2 incubator.
5. Confirm that only one cell is sorted into each well under phase-contrast light microscopy 12–16 hours after sorting.
6. Count EX-cultured cell number per well at day 7 (optional).

Tip. To save time when counting at day 7, confirm the wells with EX-cultured cells in advance at day 6.

7. Prepare tissue culture plates for HELIC assay. Apply 300 µL of EPC-CFA working medium per well of Primaria™ 24-well plates for EPC-CFA. Simultaneously, apply 300 µL of MethoCult™ H4435 into wells of 24-well suspension culture plates (Greiner, Cat. no. 662102) for HPC-CFA.
8. Gently mix EX-cultured cell suspensions in each well of 96-well Primaria™ tissue culture plates with a 200 µl pipette three times.
9. Reseed half of the suspension (50 µL) into 300 µL of EPC-CFA working medium per well and the another half (50 µL) into 300 µL of MethoCult™ H4435 per well.

Tip. When mixing EX-cultured cell suspension, avoid making air bubbles to disturb the exact pipetting.

10. Culture the separated EX-cultured cells for EPC-CFA and HPC-CFA at 37°C in a CO_2 incubator.

Tips. To maintain appropriate humidity level, apply 1 mL of sterilized MilliQ water into each of the four wells located at the corner of both plates. Also, the spaces among wells should be filled with sterilized MilliQ water in both kinds of plates for EPC-CFA and HPC-CFA. Thus, 20 wells per plate are available for HELIC assay.

11. Count the colony numbers of colony-forming unit–erythroid (CFU-E), burst forming unit–erythroid (BFU-E), CFU–granulocyte/ macrophage

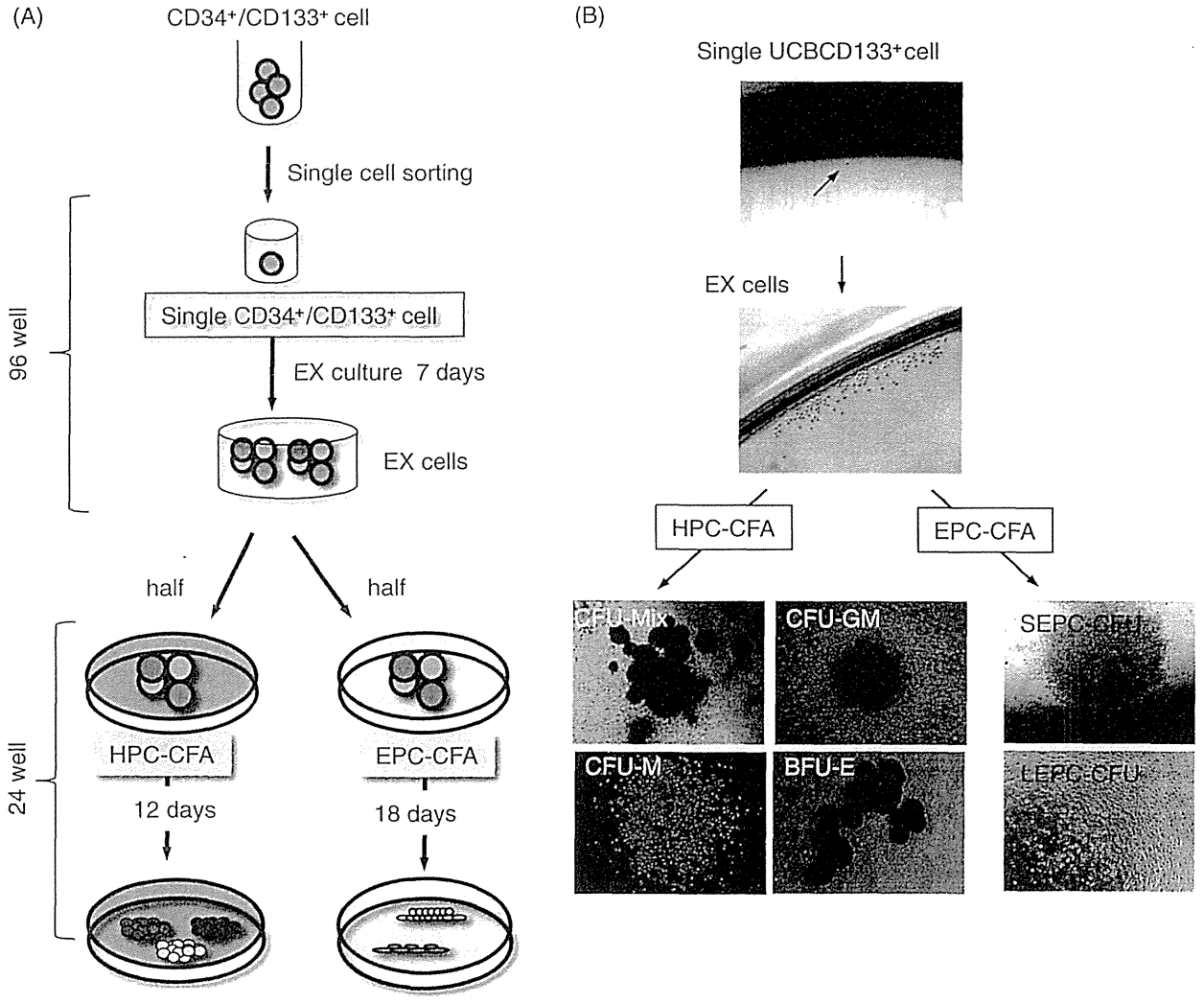

Figure 8.7 HELIC assay for single cell fate determination of the hematoendothelial lineage. (A) The schematic protocol of the HELIC assay, combining EPC-CFA with HPC-CFA for each half of the EX-cultured cells from single HSC ($CD34^+/CD133^+$ cell). (B) Representative cells and colonies at each step of the HELIC assay (×10 high power field (HPF)). BFU-E, burst forming unit erythroid; CFU-GM, CFU-granulocyte/ macrophage; CFU-M, CFU-macrophage; CFU-Mix, CFU-granulocyte/ erythrocyte/ monocyte/ macrophage. (Source: Masuda H 2011 [19]. Reproduced with permission from Wolters Kluwer Health).

(CFU-GM), CFU– macrophage (CFU-M), and/or CFU–granulocyte/ erythrocyte/ monocyte/ macrophage (mixed) per well at 12–14 days for HPC-CFA.

12. Count the colony numbers of PEPC-CFUs and/ or DEPC-CFUs per well at 14–18 days for EPC-CFA.

The whole procedure and example data of a HELIC assay are shown in Figures 8.7 and 8.8.

Application

EPC-CFA provides the methodology for accurate research into basic scientific or clinical EPC biology. The applications include the following.

1. Basic research:
   a. evaluation of the effect of target factors on EPC expansion and/or differentiation
   growth factors, cytokines, hormones, cell signaling regulators, etc.;
   b. clarification of the EPC differentiation cascade;
   c. cell fate analysis of HSC in hematopoiesis and vasculogenesis.
2. Clinical research:
   a. evaluation of pathophysiology in cardiovascular diseases etc. in terms of EPC biology;
   b. evaluation of vascular regenerative potential of cell sources for cell-based therapy.

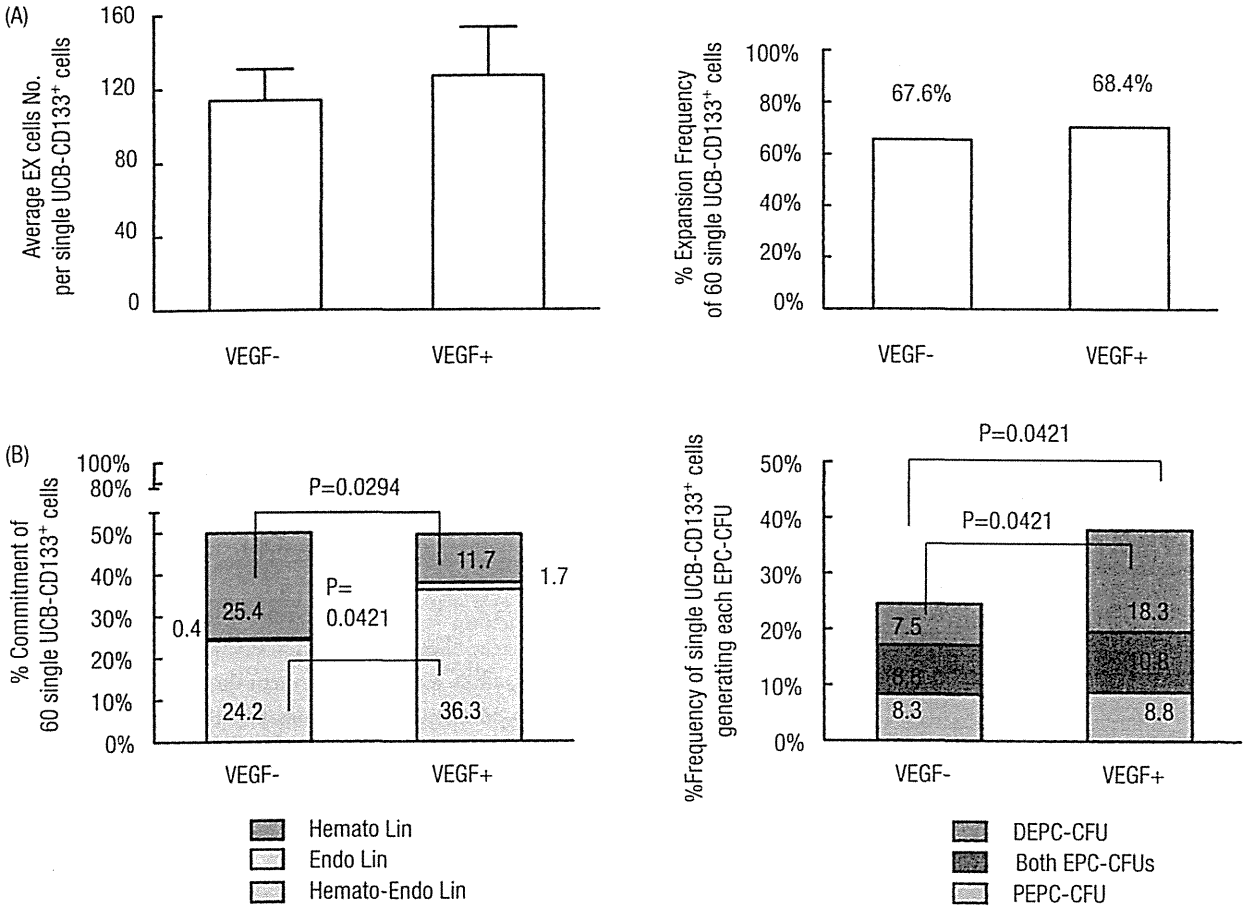

Figure 8.8 Example of an HELIC assay using UCB-CD133+ cells. (A) Left graph: Representative cell numbers per EX-cultured single UCB-CD133+ cell. Right graph: Percentage frequencies of EX-cultured cells from single UCB-CD133+ cells. (B) Left graph: Percentage commitment ratios of single UCB-CD133+ cells into hematopoietic (Hemato Lin), endothelial (Endo Lin), and both lineages (Hemato-Endo Lin). Percentage commitment frequency into Hemato Lin was estimated by counting single UCB-133 cells producing any colonies of BFU-E, CFU-GM, CFU-M and/or CFU-Mix. Right graph: Percentage frequencies of PEPC-CFUs and/or DEPC-CFUs in single UCB-CD133+ cells. Data are mean ± SEM (A left) or mean alone (A right, B); n = 4–6. (Source: Masuda H 2011 [19]. Reproduced with permission from Walters Kluwer Health).

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Competing interests statement

The authors declare that they have no competing financial interests.

References

1 Asahara T, Kawamoto A, Masuda H. Concise review: Circulating endothelial progenitor cells for vascular medicine. *Stem Cells* 2011; **29**: 1650–1655.

2 Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, *et al.* Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; **275**: 964–967.

3 Asahara T, Kalka C, Isner JM. Stem cell therapy and gene transfer for regeneration. *Gene Ther* 2000; **7**: 451–457.

4 Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, *et al.* Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *J Clin Invest* 2000; **105**: 1527–1536.

5 Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, *et al.* In vitro differentiation of endothelial cells from ac133-positive progenitor cells. *Blood* 2000; **95**: 3106–3112.

6 Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, *et al.* Expression of vegfr-2 and ac133 by circulating human cd34(+) cells identifies a population of functional endothelial precursors. *Blood* 2000; **95**: 952–958.

7 Quirici N, Soligo D, Caneva L, Servida F, Bossolasco P, Deliliers GL, *et al.* Differentiation and expansion of endothelial cells from human bone marrow cd133(+) cells. *Br J Haematol* 2001; **115**: 186–194.

8 Eggermann J, Kliche S, Jarmy G, Hoffmann K, Mayr-Beyrle U, Debatin KM, *et al.* Endothelial progenitor cell culture and differentiation in vitro: A methodological comparison using human umbilical cord blood. *Cardiovas Res* 2003; **58**: 478–486.

9 Handgretinger R, Gordon PR, Leimig T, Chen X, Buhring HJ, Niethammer D, *et al.* Biology and plasticity of cd133+ hematopoietic stem cells. *Ann N Y Acad Sci* 2003; **996**: 141–151.

10 Alessandri G, Emanueli C, Madeddu P. Genetically engineered stem cell therapy for tissue regeneration. *Ann N Y Acad Sci* 2004; **1015**: 271–284.

11 Friedrich EB, Walenta K, Scharlau J, Nickenig G, Werner N. Cd34-/cd133+/vegfr-2+ endothelial progenitor cell subpopulation with potent vasoregenerative capacities. *Circ Res* 2006; **98**: e20–25.

12 Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, *et al.* Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003; **348**: 593–600.

13 Rohde E, Bartmann C, Schallmoser K, Reinisch A, Lanzer G, Linkesch W, *et al.* Immune cells mimic the morphology of endothelial progenitor colonies in vitro. *Stem Cells* 2007; **25**: 1746–1752.

14 Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, *et al.* Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* 2004; **104**: 2752–2760.

15 Ingram DA, Caplice NM, Yoder MC. Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. *Blood* 2005; **106**: 1525–1531.

16 Eichmann A, Corbel C, Nataf V, Vaigot P, Breant C, Le Douarin NM, *et al.* Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc Natl Acad Sci U S A* 1997; **94**: 5141–5146.

17 Benndorf RA, Gehling UM, Appel D, Maas R, Schwedhelm E, Schlagner K, Silberhorn E, *et al.* Mobilization of putative high-proliferative-potential endothelial colony-forming cells during antihypertensive treatment in patients with essential hypertension. *Stem Cells Dev* 2007; **16**: 329–338.

18 Rustemeyer P, Wittkowski W, Greve B, Stehling M. Flow-cytometric identification, enumeration, purification, and expansion of cd133+ and vegf-r2+ endothelial progenitor cells from peripheral blood. *J Immunoassay Immunochem* 2007; **28**: 13–23.

19 Masuda H, Alev C, Akimaru H, Ito R, Shizuno T, Kobori M, *et al.* Methodological development of a clonogenic assay to determine endothelial progenitor cell potential. *Circ Res* 2011; **109**: 20–37.

20 Masuda H, Asahara T. Clonogenic assay of endothelial progenitor cells. *Trends Cardiovasc Med* 2013; **23**: 99–103.

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Vasculogenic Conditioning of Peripheral Blood Mononuclear Cells Promotes Endothelial Progenitor Cell Expansion and Phenotype Transition of Anti-Inflammatory Macrophage and T Lymphocyte to Cells With Regenerative Potential

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ORIGINAL RESEARCH

American Heart Association | American Stroke Association.

Vasculogenic Conditioning of Peripheral Blood Mononuclear Cells Promotes Endothelial Progenitor Cell Expansion and Phenotype Transition of Anti-Inflammatory Macrophage and T Lymphocyte to Cells With Regenerative Potential

Haruchika Masuda, MD, PhD; Rica Tanaka, MD, PhD; Satoshi Fujimura, PhD; Masakazu Ishikawa, MD, PhD; Hiroshi Akimaru, PhD; Tomoko Shizuno, BS; Atsuko Sato, BS; Yoshinori Okada, PhD; Yumi Iida, BS; Jobu Itoh, PhD; Yoshiko Itoh, PhD; Hiroshi Kamiguchi, BS; Atsuhiko Kawamoto, MD, PhD; Takayuki Asahara, MD, PhD

Background—Cell-based therapies involving mononuclear cells (MNCs) have been developed for vascular regeneration to treat ischemic diseases; however, quality control of therapeutic MNCs has not been evaluated. We investigated the therapeutic potential of peripheral blood (PB) MNCs, operated by recently developed quality and quantity (QQ) culture of endothelial progenitor cells (EPCs).

Methods and Results—PBs were collected from healthy volunteers; peripheral blood mononuclear cells (PBMNCs) isolated from these PBs were subjected to QQ culture for 7 days with medium containing stem cell factor, thrombopoietin, Flt-3 ligand, vascular endothelial growth factor, and interleukin-6. The resulting cells (QQMNCs) in EPC colony-forming assay generated significantly more definitive EPC colonies than PBMNCs. In flow cytometry, macrophages and helper T lymphocytes of QQMNCs became phenotypically polarized into angiogenic, anti-inflammatory, and regenerative subsets: classical M1 to alternative M2; T helper (Th) 1 to Th2; angiogenic or regulatory T-cell expansion. Quantitative real-time polymerase chain reaction (qRT-PCR) assay revealed the predominant proangiogenic gene expressions in QQMNCs versus PBMNCs. Using murine ischemic hindlimb models, the efficacy of QQMNC intramuscular transplantation (Tx) was compared to that of PBMNCTx, cultured "early EPC" Tx (eEPCTx), and granulocyte colony-stimulating factor mobilized $CD34^+$ cell Tx (GmCD34Tx). Laser Doppler imaging revealed the blood perfusion recovery in ischemic hindlimbs after QQMNCTx superior to after PBMNCTx and eEPCTx, but also earlier than after GmCD34Tx. Histological evaluations and qRT-PCR assays in ischemic hindlimbs demonstrated that QQMNCTx, similarly to GmCD34Tx, enhanced angiovasculogenesis and myogenesis, whereas it preponderantly inhibited inflammation and fibrosis versus PBMNCTx and eEPCTx.

Conclusions—QQ culture potentiates the ability of PBMNCs to promote regeneration of injured tissue; considering the feasible cell preparation, QQ culture-treated PBMNCs may provide a promising therapeutic option for ischemic diseases.

Clinical Trial Registration—URL: irb.med.u-tokai.ac.jp/d/2/monthly/2010.html; IRB No.: 10R-020.URL: irb.med.u-tokai.ac.jp/d/2/monthly/201312.html; IRB No.: 13R228. **(*J Am Heart Assoc*.2014;3:e000743 doi: 10.1161/JAHA.113.000743)**

Key Words: anti-inflammation • cell-based therapy • peripheral blood mononuclear cells • serum-free culture • vascular regeneration

Based on the isolation of bone marrow (BM)-derived endothelial progenitor cells (EPCs),[1–3] autologous total mononuclear cells (MNCs) freshly isolated from BM or peripheral blood (PB) have been applied to clinical vascular regenerative therapy in patients with severe ischemic heart or limb diseases.

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An accompanying Video S1 is available at http://jaha.ahajournals.org/content/3/3/e000743/suppl/DC1

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These initial clinical experiences indicate that cell-based therapy for vascular regenerations is safe, feasible, and effective.[4–7] However, the translational and clinical trials have also demonstrated insufficient or contradictory effectiveness on recovery from ischemic diseases.[8–11]

Peripheral blood and bone marrow MNCs (PBMNCs and BMMNCs) constitute hematopoietic lineage cells; most are lymphoid cells or myeloid monocytes, and very few are stem/progenitor cell types, such as hematopoietic stem/progenitor cells, EPCs, or other mesenchymal stem cells. The scarcity of EPCs in MNC populations is a main reason for failure of constant and potent contributions in clinical cases.

Enriched EPCs, such as $CD34^+$ or $CD133^+$ cells, constitute <0.01% of PBMNCs and 0.1% of BMMNCs, and the frequency of colony-forming EPCs is 0.005% in PBMNCs.[12]

Taking into account the majority in MNCs, we need to issue the critical functions of monocyte/macrophages. Monocytes are precursors of macrophages; monocytes play key roles in both proinflammatory and regenerative processes by phenotype alterations after tissue infiltrations.[13] Macrophages are a heterogeneous cell population that adapts and responds to a large variety of microenvironmental signals. For example, the cytokines and growth factors in some microenvironments induce macrophages to adopt regenerative phenotypes, whereas the inflammatory deterioration and fibrosis in uncontrolled inflammatory environment in tissues induce proinflammatory macrophage transitions and suppress regenerative processes.

Also, the inflammatory environment is interacted by major lymphoid phenotypes simultaneously stimulated by proinflammatory signals and controlled macrophage phenotype transition for proinflammatory drive.[13,14]

To suppress unproductive inflammatory process and enhance vasculogenic regeneration, purified EPCs have been transplanted into patients with severe ischemic heart or limb diseases, and these initial clinical experiences indicate that this cell-based therapy is safe and effective.[15–19] Nevertheless, the problems caused by EPC scarcity must be overcome for EPC-mediated therapy to become reproducible and effective. To this end, the following 3 step-wise strategies have been used: (1) leukapheresis; (2) daily administration of granulocyte colony-stimulating factor (G-CSF); and (3) isolation of machinery $CD34^+$ or $CD133^+$ cells. Such an isolation process of autologous EPC sources burdens the patients to be treated with "EPC therapy," in terms of medical invasiveness and costs.

More important, the number and functionality of EPCs decline as patients age[20] and in patients with cardiovascular (CV) risk factors.[21,22]

Here, we describe a method for MNC culture that enhances the vasculogenic potential of EPCs and facilitates the preparation of monocytes for regenerative phenotype activation.

Our method for quality and quantity-control culture (QQ culture) of MNCs (QQMNCs) is based on an established culture method that increases the quality and quantity of EPCs derived from enriched EPC populations, such as $CD34^+$ and $CD133^+$ cells. Notably, the therapeutic potential of QQ culture is demonstrably greater than that of naïve EPCs for CV regeneration after infarcted myocardia in rats.[23] Interestingly, we found that the vasculogenic signaling condition of MNCs in QQ culture potentiates the vascular and tissue regeneration ability of naïve PBMNCs. The regenerative function of QQMNCs turned out to be operated through activation of anti-inflammatory and angiogenic monocytes/helper T lymphocytes as well as vasculogenic EPC expansion.

Moreover, the present experimental study demonstrated that therapeutic efficacy of QQMNC transplantation (QQMNCTx) is equal to and greater than that of G-CSF mobilized $CD34^+$ cell Tx (GmCD34Tx).

Therefore, in cell-based therapy for ischemic diseases, QQMNC provides a practical option of cell sources, including PBMNC and GmCD34.

Methods

Collection of PBMNCs Cells or $CD34^+$ Cells From Healthy Volunteers

Experiments using human samples were performed with institutional approval and guidelines from the Clinical Investigation Committee at Tokai University School of Medicine (institutional review board [IRB] No.: 10R-020; irb.med.u-tokai.ac.jp/d/2/monthly/2010.html and IRB No.: 13R228; irb.med.u-tokai.ac.jp/d/2/monthly/201312.html). The whole healthy human volunteers between the ages of 20 and 55 years gave informed consent.

PB (20 to 100 mL per subject) was drawn by heparinized venous puncture at the forearm. PBMNCs were isolated by density gradient centrifugation using Lymphocyte Separation Solution (d=1.077; Nakalai Tesque, Kyoto, Japan), as previously reported.[24] $CD34^+$ cells were purified by an autoMACS separator (Miltenyi Biotec, Lund, Sweden), using magnetic beads-coated mouse anti human CD34 antibody (Ab) and a CD34 Cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions.

QQ Culture and Early EPC Culture of PBMNCs

QQ culture condition for PBMNCs

QQ culture medium of Stem Line II (Sigma-Aldrich, St. Louis, MO) contained the 5 human recombinant proteins: stem cell factor (SCF); thrombopoietin (TPO); Flt-3 ligand; vascular endothelial growth factor (VEGF); and interleukin (IL)-6.

Then, isolated PBMNCs were cultured for 7 days at the cell density of 2×10^6 cells/2 mL QQ culture medium per well of 6-well Primaria tissue culture plate (BD Falcon; BD Biosiences, San Jose, CA). Cell density in QQ culture was corresponded to the approximate density of 1×10^6 MNCs in 1 mL of PB. Culture well plates and the contents of QQ culture medium are listed in Tables 1 and 2.

Early EPC culture of PBMNCs

As previously reported,[24,25] early EPCs (eEPCs) were acquired after 7 days of culture of isolated PBMNCs using the EGM-2-MV SingleQuots kit (Lonza Walkersville, Inc., Walkersville, MD). In brief, EGM-2-MV complete medium was adjusted by adding 5% FBS (SAFC Biosciences Inc., Lenexa, KS) and supplemented growth factors, except hydrocortisone, to EBM-2 basal medium. PBMNCs were adjusted to the similar cell density (1×10^6 cells/mL) with 5% FBS/EGM-2-MV complete medium to that in QQ culture of PBMNCs. Cells were then plated on a human fibronectin-coated 6-well Primaria tissue culture plate (2×10^6 cells/2 mL per well) and cultured. The medium was changed 4 days after seeding, then cultured for 3 more days. Seven days later, nonattaching cells were removed; adherent cells were harvested with 2 mmol/L of EDTA/PBS after washing with PBS. Harvested eEPCs were suspended in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma Aldrich), adjusted the cell density (1×10^4 cells/40 μL or 2×10^5 cells/40 μL), cells were then transplanted into skeletal muscle of murine ischemic hindlimb, as described below.

EPC Colony-Forming Assay

To investigate the vasculogenic potential of PBMNCs or QQMNCs, we used semisolid culture medium and 35-mm Primaria dishes (BD Falcon; BD Biosciences) to grow and then counted the adhesive EPC colonies by EPC colony-forming assay (EPC-CFA) (MethoCult SF^{BIT}; STEMCELL Technologies Inc., Vancouver, BC, Canada) with proangiogenic growth factors/cytokines, as previously reported (Table 3).[12] Aliquots of those cells were seeded at 2×10^5 cells/dish (3 dishes per volunteer) for EPC-CFA. Sixteen to 18 days after initiation of the culture, the number of adherent colonies per dish was measured using a gridded scoring dish (STEMCELL Technologies) under phase-contrast light microscopy (Eclipse TE300; Nikon, Tokyo, Japan). Primitive EPC colony-forming units (pEPC-CFUs) and definitive EPC-CFUs (dEPC-CFUs) were separately counted.

Table 1. Materials for QQ Culture and EPC-CFA

	Company, Catalaog No.	Application
6-well Primaria tissue culture plate	BD Biosciences, #353846	QQ culture
35-mm Primaria tissue culture dish	BD Biosciences, #353801	EPC-CFA
Blunt-end needle	STEMCELL Technologies, #28110	Applying semisolid medium
Gridded scoring dish	STEMCELL Technologies, #27500	Guide when counting EPC-CFU

EPC-CFA indicates endothelial progenitor cell colony-forming assay; EPC-CFU, endothelial progenitor cell colony-forming units; QQ, quality and qunatity.

Table 2. Contents of QQ Culture Medium

	Company, Catalog No.	Final Concentration
Stemline II™ Hematopoietic Stem Cell Expansion Medium	Sigma-Aldrich, #S0192	
rh SCF	Peprotec, #300-07	100 ng/mL
rh Flt-3 ligand	Peprotec, #300-19	100 ng/mL
rh TPO	Peprotec, #300-18	20 ng/mL
rh VEGF	Peprotec, #100-20	50 ng/mL
rh IL-6	Peprotec, #200-06	20 ng/mL

IL indicates interleukin; rh, recombinant human; SCF, stem cell factor; TPO, thrombopoietin; VEGF, vascular endothelial growth factor.

Flow Cytometry

Performance of flow cytometry for lineage cell populations

Freshly isolated PBMNCs and the QQMNCs were subjected to flow cytometry (FCM) to detect surface antigen positivities

Table 3. Contents in Semisolid Culture for EPC-CFA

	Company, Catalog No.	Final Concentration
MethoCult™ SF^{BIT} H4236	STEMCELL Technologies, #04236	
rh SCF	Peprotec, #300-07	66.7 ng/mL
rh VEGF	Peprotec, #100-20	33.3 ng/mL
rh basic FGF	Peprotec, #100-18B	33.3 ng/mL
rh EGF	Peprotec, #100-15	33.3 ng/mL
rh IGF-1	Peprotec, #100-11	33.3 ng/mL
rh IL-3	Peprotec, #200-03	13.3 ng/mL
Heparin	Shimizu Pharmaceutical Co	1.33 IU/mL
FBS	SAFC Biosciences, #12303	30% (vol/vol)

EGF indicates epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; IL, interleukin; rh, recombinant human; VEGF, vascular endothelial growth factor.

of hematopoietic stem or lineage committed cells as well as endothelial lineage cells. The Abs were listed in Tables 4 through 6. Cells suspended in 2 mmol/L of EDTA/0.2% BSA/PBS buffer (5×10^5 cells/200 μL) were incubated after the addition of 10 μL of FcR blocking reagent at 4°C for 30 minutes and then equally dispensed into reaction tubes for subsequent staining (100 μL/tube). Each aliquot was incubated with 2 μL of each first Ab at 4°C for 20 minutes and then washed twice with 1 mL of 2 mmol/L of EDTA/0.2% BSA/PBS buffer. Cells were suspended in 2 mmol/L of EDTA/0.2% BSA/PBS buffer (2×10^5 cells/200 μL). FCM analysis was performed using the LSRFortessa cell analyzer (BD Biosciences) and FlowJo software (Tomy Digital Biology Co., Ltd., Tokyo, Japan). When staining for von Willebrand factor (vWF), after incubation with each first Ab, cells were incubated with biotin conjugated rat anti-mouse immunoglobulin G (IgG)1 and then conjugated to streptavidin/phycoerythrin/cyanine 7 (PE/Cy7).

Table 4. Antibodies Recognizing Hematopoietic Cell Populations for Flow Cytometry

Antibody	Clone	Isotype	Company, Catalog No.
CD34-FITC	581	Mouse IgG1 κ	BD Pharmingen, #555821
CD133-APC	AC133	Mouse IgG1	Miltenyi Biotec, #130-090-826
VEGFR-2-PE	89106	Mouse IgG1	R&D Systems, #FAB357P
CD31-FITC	WM59	Mouse IgG1 κ	BD Pharmingen, #555445
vWF	4F9	Mouse IgG1 κ	Abcam, #ab20435
CD105-APC	SN6	Mouse IgG1 κ	eBioscience, #17-1057-42
CD146-PE	P1H12	Mouse IgG1 κ	BD Pharmingen, #550315
CD3-Alexa700	HIT3a	Mouse IgG2a κ	BioLegend, #300324
CD4-APC/Cy7	RPA-T4	Mouse IgG1 κ	BioLegend, #300518
CD8-Pacific Blue	SK1	Mouse IgG1 κ	BioLegend, #344718
CD11c-PE	S-HCL-3	Mouse IgG2b κ	BD Biosciences, #347637
CD14-Pacific Blue	M5E2	Mouse IgG2a κ	BioLegend, #301828
CD16-APC/Cy7	3G8	Mouse IgG1 κ	BioLegend, #302018
CD19-PE/Cy7	HIB19	Mouse IgG1 κ	BioLegend, #302215
CD56-APC	HCD56	Mouse IgG1 κ	BioLegend, #318309
CD192(CCR2)-PerCP/Cy5.5	TG5/CCR2	Mouse IgG2b κ	BioLegend, #335303
CD206-APC/Cy7	15-2	Mouse IgG1 κ	BioLegend, #321119
CD235a-FITC	GA-R2 (HIR2)	Mouse IgG2b κ	BD Pharmingen, #559943
CD184(CXCR4)-PE/Cy7	12G5	Mouse IgG2a κ	BioLegend, #306514

APO indicates allophycocyanin; CCR2, CC chemokine receptor 2; CXCR4, C-X-C chemokine receptor type 4; PE, phycoerythrin; VEGFR, vascular endothelial growth factor receptor; vWF, von Willebrand factor.

Table 5. Antibodies Recognizing Helper T-Cell Subsets for Flow Cytometry

Antibody	Clone	Isotype	Company, Cat No.
CD4-PerCP/Cy5.5	OKT4	Mouse IgG2b κ	BioLegend, #317428
CD25-PE	BC96	Mouse IgG1 κ	BioLegend, #302606
INF-γ-Pacific Blue	4S.B3	Mouse IgG1 κ	BioLegend, #502522
IL-4-APC	8D4-8	Mouse IgG1 κ	BioLegend, #500713
Foxp3-FITC	206D	Mouse IgG1 κ	BioLegend, #320105

Foxp3 indicates forkhead box P3; IL, interleukin; INF, interferon.

Table 6. Isotype Antibodies and Reagents for Flow Cytometry

Antibody	Clone	Company, Catalog No.
Mouse IgG1 κ-FITC	MOPC-21	BD Pharmingen, #555748
Mouse IgG1 κ-APC	679.1Mc7	Beckman Coulter, #IM2475
Mouse IgG1 κ-PE	MOPC-21	BD Pharmingen, #555749
Mouse IgG1 κ	MOPC-21	BD Pharmingen, #555746
Mouse IgG1 κ-Pacific Blue	MOPC-21	BioLegend, #400131
Mouse IgG1 κ-PE/Cy7	MOPC-21	BioLegend, #400125
Mouse IgG1 κ-APC/Cy7	MOPC-21	BioLegend, #400127
Mouse IgG2a κ-Pacific Blue	MOPC-173	BioLegend, #400235
Mouse IgG2a κ-Alexa700	MOPC-173	BioLegend, #400247
Mouse IgG2b κ-FITC	27-35	BD Pharmingen, #555742
Mouse IgG2b κ-PE	27-35	BD Pharmingen, #555743
Mouse IgG2 κ-PerCP/Cy5.5	MPC-11	BioLegend, #400337
Mouse IgG2a κ-PE/Cy7	MOP-173	BioLegend, #400232
Biotin-rat anti-mouse IgG1	A85-1	BD Pharmingen, #553441
Streptavidin-PE/Cy7		BioLegend, #405206
Fc blocking reagent, human		Miltenyi Biotec, #130-059-901

Performance of FCM for activated helper T-lymphocyte subsets

PBMNCs or QQMNCs in 10% FBS/RPMI 1640 medium (1×10^6 cells/mL) were treated with 25 ng/mL of phorbol-12-myristate-13-acetate (PMA; Promega, Madison, WI) and 1 μg/mL of ionomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 12 hours at 37°C. Subsequently, for the last 3 hours, cells were incubated with 2 μmol/L of monensin (BioLegend, San Diego, CA). Thereafter, cells were washed and suspended with 2 mmol/L of EDTA/PBS buffer and stained for cell surface markers with CD4-PerCP/Cy5.5 and CD25-PE before fixation. Stained cells were washed, resuspended with 2 mmol/L of EDTA/PBS buffer, and distributed into aliquots for each staining. After treatment with fixation buffer (BioLegend) and permeabilization buffer (BioLegend), cells underwent intracellular staining with interferon-gamma (INF-γ)-Pacific Blue and IL-4/allophycocyanin (APC). Alternatively, after treatment with the FOXP3 Fix/Perm Buffer Set (BioLegend), cells underwent intranuclear staining with forkhead box protein 3/fluorescein isothiocyanate (Foxp3-FITC). Intracellular or intranuclear staining was performed, according to the supplemental protocol for each buffer. The cellular frequency of $CD4^+/INF\text{-}\gamma^+/IL\text{-}4^-$, $CD4^+/INF\text{-}\gamma^-/IL\text{-}4^+$, or $CD4^+/CD25^+/Foxp3^+$ in $CD4^+$ helper lymphocytes was evaluated as that of T helper (Th)1, Th2, or regulatory T cells.

FCM analysis

The scatter diagram of each PBMNC, or the QQ cultured cell (QQMNC) population in an individual, was gated into 3 cell-sized populations of lymphocytes, monocytes, and the larger cells. The percent (%) positivity of a hematopoietic cell population per each gate in PBMNCs or QQMNCs was evaluated and then calculated to that in the whole cells of the 3 gates. The ratio of the % positivity in the whole cells of QQMNCs to that in PBMNCs was further calculated for each cell population. Similarly, the % positivity of each helper T subset (Th1, Th2, or regulatory T cell) was calculated in $CD4^+$ T cells of the 3 gates of PBMNCs or QQMNCs; the ratio of the % positivity in $CD4^+$ T cells of QQMNCs to that in PBMNCs was calculated.

Quantitative Real-Time Polymerase Chain Reaction In Vitro

Using Trizol (Invitrogen, Carlsbad, CA), total RNA was isolated from PBMNCs or QQMNCs. Contaminated genomic DNA was digested by DNase I treatment (Invitrogen) at 37°C for 15 minutes. DNase I-treated total RNA was purified by phenol extraction and ethanol precipitation. One hundred nanograms of purified total RNA was used for cDNA synthesis with the SuperScript VILO cDNA synthesis kit (Invitrogen). cDNA mixture was diluted by 10-fold after first-strand cDNA synthesis. Using ABI Prism 7700 (Applied Biosystems, Foster City, CA), quantitative real-time polymerase chain reaction (qRT-PCR) for diluted cDNA was performed with EagleTaq Master Mix (Roche Diagnostics, Tokyo, Japan), 0.3 μmol/L of forward and reverse primers used for cDNA amplification, and 0.25 μmol/L of probe (Sigma-Aldrich), according to the manufacturer's protocol. The relative mRNA expression was calculated by ΔΔCt method with normalization against human GAPDH (hGAPDH). All primers and probes used are listed in Table 7.

In Vitro Angiogenesis Assay Using Matrigel

As previously reported,[12] PBMNCs and QQMNCs were respectively incubated in 500 μL of 2% FBS/EBM-2 with 20 μg/mL of acetylated low-density lipoprotein, labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (acLDL-Dil; Biomedical Technologies Inc., Stoughton, MA) (4×10^4 cells/500 μL) in a 1.5-mL tube for 30 minutes at 37°C in a CO_2 incubator. After centrifugation at 400*g* for 10 minutes at 4°C, and aspirating the supernatant, the cell pellets were washed by 1 mL of PBS and suspended with EBM-2/2% FBS (1.0×10^3 cells/50 μL). Labeled cells were resuspended together with human umbilical vain endothelial cells (HUVECs; EPCs: HUVECs=1×10^3:1.5×10^4 in 100 μL of 2% FBS/EBM-2). The mixed cell suspension was incubated at 37°C in a water bath and applied at 100 μL each onto preincubated Matrigel (BD Falcon) (50 μL/well) in each 96-well plate (BD Falcon; BD Biosciences). After incubation for 12 hours, the numbers of closed areas formed by HUVECs were counted using Photoshop software in the pictures taken at ×2 high power field (HPF) by a phase-contrast light microscope (Eclipse TE300; Nikon). Furthermore, acLDL-Dil-labeled PBMNCs or QQMNCs incorporated into a tube were also counted using ImageJ software in the pictures taken at ×4 HPF by a fluorescence microscope (IX70; Olympus, Tokyo, Japan). The tube and cellular numbers were counted independently by 2 blinded investigators.

In Vivo Assessment of Blood Flow Recovery and Tissue Regeneration by Cell Tx Using Murine Ischemic Hindlimb Model

Guideline for animal experiment

All animal studies conformed to national and institutional guidelines. The protocols were approved by the guidelines of the Institutional Animal Care and Use Committee of the Isehara Campus, Tokai University School of Medicine (Isehara, Japan), based on Guide for the Care and Use of Laboratory Animals (National Research Council). The experimental animal protocols for making ischemic models and laser Doppler