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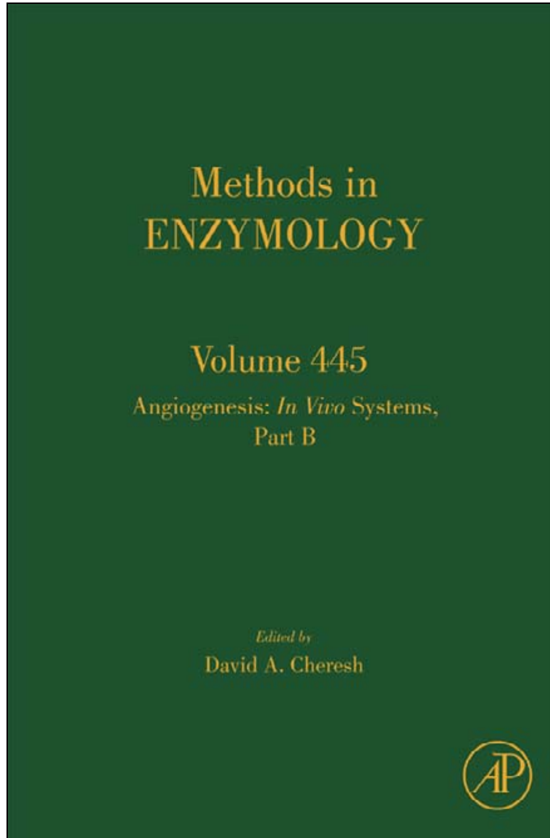

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CHAPTER THIRTEEN

An *In Vivo* Experimental Model for Postnatal Vasculogenesis

Juan M. Melero-Martin *and* Joyce Bischoff

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Abstract

Rapid and complete vascularization of ischemic tissues and thick engineered tissues is likely to require vasculogenesis. Therefore, the search for clinically relevant sources of vasculogenic cells and the subsequent development of experimental models of vasculogenesis is of utmost importance. Here, we describe a methodology adapted from the Matrigel plug assay to deliver human blood–derived endothelial progenitor cells (EPCs) and mature smooth muscle cells (SMCs) subcutaneously into immunodeficient mice. One week after

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implantation, an extensive microvascular network composed of the human EPCs and SMCs is formed within the Matrigel. The presence of human EPC-lined lumens containing host erythrocytes can be seen throughout the implants indicating not only the formation (*de novo*) of a vascular network, but also the development of functional anastomoses with the host circulatory system. This is a very versatile assay that allows (1) dialing the final microvessel density by varying either the total number of cells in the original cell suspension or the ratio between EPCs and SMCs, (2) studying the effect of substituting another type of perivascular cell for mature SMCs or another type of endothelial cell, (3) tracking each of the implanted cell types by labeling (e.g., GFP tagging) prior to implantation, and (4) studying the effect of genetically modifying the cells prior to implantation. Additionally, this assay is relatively simple to perform and it does not require an incision or surgical procedure. This murine model of human vasculogenesis is ideally suited for studies on the cellular and molecular components of microvessel development, pathologic neovascular responses, and for the development and investigation of strategies to enhance neovascularization of engineered human tissues and organs.

1. Introduction

Current strategies to generate vascular networks *in vivo* are based on our understanding of the mechanisms of blood vessel formation. During embryogenesis, blood vessels are first formed *de novo* by the patterned assembly of angioblasts in a process termed "vasculogenesis" (Cleaver and Melton, 2003; Flamme *et al.*, 1997; Risau and Flamme, 1995). Thereafter, the formation of new blood vessels occurs primarily via the sprouting of endothelial cells (ECs) from pre-existing vasculature (angiogenesis) (Carmeliet, 2003; Conway *et al.*, 2001; Risau, 1997). After birth and during adulthood, the formation of new blood vessels occurs mainly through angiogenesis in a tightly controlled process where EC proliferation and migration are regulated by secreted cytokines, surrounding cells, and extracellular matrix (Carmeliet, 2003; Risau, 1997). Postnatal vasculogenesis involves the recruitment of endothelial progenitor cells (EPCs) as well as an intricate collaboration with hematopoietic cells (De Palma *et al.*, 2005; Grunewald *et al.*, 2006; Yoder *et al.*, 2007). While EPC-derived ECs line the vessel lumen, accessory cells support vessel formation through secretion of cytokines and alteration of the extracellular matrix (Carmeliet, 2003; Hiraoka *et al.*, 1998; Stetler-Stevenson, 1999; Zentilin *et al.*, 2006). Dysregulated angiogenesis and vasculogenesis have been implicated in the pathogenesis of numerous diseases including vascular retinopathies, rheumatoid arthritis, vascular tumors such as infantile hemangioma, and cancer (Folkman, 1995).

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Our understanding of angiogenesis as a mechanism of blood vessel formation has evolved, in part, from numerous experimental assays that recapitulate the multistep angiogenic processes (Carmeliet, 2003; Carmeliet and Jain, 2000; Folkman, 1995, 2007; Risau, 1997). These include *in vitro* assays for endothelial proliferation, migration and capillary morphogenesis and *in vivo* assays such as the corneal micropocket assay (Gimbrone *et al.*, 1974), the chick chorioallantoic membrane (CAM) assay (Auerbach *et al.*, 1974; Ausprunk *et al.*, 1974, 1975; Folkman, 1974), and the Matrigel plug assay (Passaniti *et al.*, 1992), among other models (for review, see Norrby, 2006). Angiogenesis in these *in vivo* models is driven by exogenously added factors or relies on ongoing developmental processes, as is the case in the CAM assay. In some sense, the corneal micropocket and the Matrigel plug assays model regenerative processes in that angiogenesis, an essential component of tissue regeneration, is promoted by the growth of new blood vessels toward an angiogenic signal or factor (Isner and Asahara, 1999; Isner *et al.*, 1996; Lee *et al.*, 2002; Li *et al.*, 2005).

The mechanisms governing vasculogenesis are studied primarily during embryonic development in several model organisms and in murine embryonic stem-cell–derived embryoid bodies *in vitro*. Experimental models for postnatal vasculogenesis are less numerous. However, there is extensive interest in postnatal vasculogenesis because of the potential application(s) in tissue-engineering and regenerative therapies. One approach that stimulated our thinking was the work of Schechner and colleagues (2000), in which they showed the preassembly of human umbilical vein ECs (HUVECs) into nascent microvascular networks *in vitro*, and the ability of these preformed endothelial networks to remodel into functional vessels when implanted subcutaneously into immunodeficient mice. In this pioneering work, HUVECs were plated in three-dimensional (3D) collagen/fibronectin gels *in vitro* for 24 h during which time the HUVECs organized into cellular cords with nascent lumens. The endothelial networks embedded within the collagen/fibronectin gels were then implanted into immunodeficient mice and followed for up to 2 months. Blood vessels lined with human endothelial cells and filled with red blood cells were detected after 1 to 2 months, demonstrating the inherent ability of human endothelial cells to assemble into vascular networks that connect with the host (murine) blood vessels. The HUVECs provided an appropriate starting point for this model, but the cells required transduction of the antiapoptotic gene Bcl-2 to augment survival. Later studies by the same group showed that cord blood and adult blood–derived endothelial cells did not require Bcl-2 transduction to form human vessels in this model (Shepherd *et al.*, 2006). Furthermore, Yoder and colleagues (2007) used this model as a functional assay to demonstrate the crucial distinction between EPCs, called ECFC in their study, and hematopoietic/monocytic cells that express endothelial markers and have been called early EPCs, angiogenic EPCs, or CFU-ECs. (EPCs formed vessels whereas the hematopoietic/

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monocytic cells did not.) In summary, although the Schechner model system was developed to study immune cell interactions with human endothelium, it has also provided a valuable tool for investigators to test endothelial cell populations for "vasculogenic" potential—that is, the ability to form, *de novo*, networks of new vessels that can connect with the pre-existing host vasculature (Koike *et al*., 2004; Schechner *et al*., 2000).

The cellular requirements for building blood vessel networks for tissue engineering and tissue regeneration is an area of active investigation. We showed in 2004 that a combination of human EPC-derived endothelial cells and mature human smooth muscle cells (SMCs) assembled into microvessel-like structures *in vitro* when co-seeded on a biodegradable scaffold of polyglycolic acid/poly-L-lactic acid (PGA/PLLA). In the absence of SMCs, the ECs were viable and maintained endothelial characteristics, but did not undergo morphological rearrangements suggestive of lumen formation (Wu *et al*., 2004). This study indicated that although endothelial cells have an inherent ability to form vascular cords and lumens, SMCs are required in certain, perhaps less favorable, 3-D environments such as that presented by the PGA/PLLA mesh. It also suggested that a two-cell system composed of ECs and a smooth muscle support cell might prove to be more robust and efficient for building vascular networks *de novo*.

The assay we described here supports this concept. We showed that cord blood–derived EPCs combined with mature human smooth muscle as a single-cell suspension in Matrigel form functional human blood vessel networks within 7 days after implantation into nude mice (Melero-Martin *et al*., 2007). Cord blood EPCs alone or human SMCs alone do not form vessels and Matrigel without cells is inert. Vessel density can be increased or decreased, over a range from 10 to 100 vessels/mm^2, by varying cell numbers. Human cells can be identified by human specific antibodies or by GFP labeling of the cells. Other sources of human ECs, adult peripheral blood–derived EPCs, HUVECs, and human dermal microvascular endothelial cells (HDMECs) also form vessels at 7 days when combined with human SMCs, albeit at lower microvessel densities at this 80:20 ratio. Hence, this approach offers a versatile, quantifiable, and relatively simple model system to study postnatal vasculogenesis *in vivo* using human cells. The model can be used to study the vasculogenic potential of other sources of human endothelial and perivascular cells, such as human embryonic stem cells differentiated into endothelial and smooth muscle lineages (Gerecht-Nir *et al*., 2003; Kaufman *et al*., 2001; Levenberg *et al*., 2002; Levenberg *et al*., 2007; Wang *et al*., 2004). The model can be used to screen for anti- and/or proangiogenic compounds. And finally, the model can be used to study the role(s) of specific genes in the formation and function of a vascular network composed of human endothelium.

We refer to this experimental model as "postnatal vasculogenesis" because (1) vessels are formed from cells isolated from postnatal human

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blood and tissues, (2) vessels form in an adult animal, and (3) vessels do not arise from pre-existing vessels but instead from single cells suspended in Matrigel. However, angiogenesis plays an important role in this assay because connections to the murine vasculature are needed to achieve red blood cell–filled vessels, the functional read-out in this assay. Whether the ability of human vessels to form productive connections with host vessels should be considered an aspect of vasculogenesis and angiogenesis would likely generate a lively discussion.

2. Isolation of Blood-Derived Endothelial Progenitor Cells

The discovery of EPCs in peripheral blood was exciting because it suggested a promising opportunity to noninvasively obtain large quantities of autologous ECs for either therapeutic vascularization or tissue engineering, both of which require some form of postnatal vasculogenesis. However, the process for obtaining blood-derived EPCs with the ability to form blood vessels *in vivo* has not been straightforward. Most of the original studies identified circulating EPCs as cells expressing CD34, CD133, and the VEGF receptor 2 (KDR) (Asahara *et al.*, 1997; Peichev *et al.*, 2000; Reyes *et al.*, 2002; Shi *et al.*, 1998). However, it is known now that these cellular markers are shared by hematopoietic cells that can be mobilized into circulation from the bone marrow to home sites of neovascularization (Rafii and Lyden, 2003; Yoder *et al.*, 2007). Although the hematopoietic and endothelial cell types are fundamentally different, many studies have referred to blood- or bone-marrow–derived adherent cells that express progenitor and endothelial markers such as CD34, CD133 and VEGFR-2+ cells as EPCs (Prater *et al.*, 2007). The hematopoietic accessory cells have been referred to as "colony-forming units-ECs" (CFU-ECs) (Gehling *et al.*, 2000), "circulating angiogenic EPCs" (Rehman *et al.*, 2003), "early EPCs" (Gulati *et al.*, 2003; Hur *et al.*, 2004), and "colony-forming units-Hill (CFU-Hill)" (Hill *et al.*, 2003). On the other hand, the cells with direct involvement as the cellular lining of the blood vessel lumen have been referred to as "late outgrowth ECs" (Lin *et al.*, 2000), EPCs (Kaushal *et al.*, 2001), "late EPCs" (Hur *et al.*, 2004), and "endothelial colony-forming cells" (ECFCs) (Ingram *et al.*, 2004). Thus, the term EPCs has been applied to blood and bone-marrow cells with hematopoietic and endothelial features. Despite the ambiguous terminology, the functional distinction between the two groups of cells is becoming clearer. Yoder and colleagues (2007) demonstrated, in an elegant study, that most of the cells that have been long referred to as EPCs are in fact descendants of hematopoietic stem cells (HSCs); the cells express functional activities of myeloid

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cells and have no ability to differentiate into functional ECs in perfused blood vessels *in vivo*.

Cells with *bona fide* blood vessel–forming ability, which we refer to as EPCs, and Yoder and Ingram refer to as ECFCs (Ingram et al., 2004), comprise a very small population of the circulating cells. EPCs are found at a concentration of about two to five cells per milliliter in human umbilical cord blood, and at a concentration of about 0.05 to 0.2 cells/ml in adult peripheral blood (Ingram *et al.*, 2004) and in the vessel wall (Ingram *et al.*, 2005). Both the low frequency of EPCs in circulation and the lack of a unique set of distinctive cellular markers have made the isolation of EPCs by flow cytometry or other immunological techniques very challenging. As a result, the most successful methodology for isolating EPCs is based on methods similar to those originally reported for endothelial outgrowth from peripheral blood (Lin *et al.*, 2000). In this method, adult peripheral blood mononuclear cells (MNCs) or human umbilical cord blood MNCs are collected and plated onto collagen-coated plates in endothelial-specific growth media (Ingram *et al.*, 2004; Lin *et al.*, 2000; Yoder *et al.*, 2007). Nonadherent cells are discarded and EC-like colonies emerge from the adherent cell population 14 to 21 days after plating adult MNCs and 5 to 7 days after plating human umbilical cord blood MNCs. The colonies display a cobblestone appearance typical of ECs, can be plated as single cells and routinely expanded for over 70 population doublings (Ingram *et al.*, 2004, 2005; Lin *et al.*, 2000; Melero-Martin *et al.*, 2007; Yoder *et al.*, 2007). EPCs obtained by this methodology are phenotypically indistinguishable from cultured mature ECs in terms of cobblestone morphology and expression of adhesion molecules and receptors (Ingram *et al.*, 2004, 2005; Lin *et al.*, 2000; Melero-Martin *et al.*, 2007; Yoder *et al.*, 2007). However, in functional assays, EPCs exhibit enhanced migratory and proliferative activity compared to mature ECs derived from existing vasculature (Ingram *et al.*, 2004; Khan *et al.*, 2006; Melero-Martin *et al.*, 2007). More importantly, our group and two other independent groups have shown that EPCs obtained by this methodology possess *de novo* vessel-forming ability *in vivo* (Au *et al.*, 2008; Melero-Martin *et al.*, 2007; Yoder *et al.*, 2007), and therefore these EPCs constitute one of the cellular building blocks for our experimental *in vivo* model of vasculogenesis.

2.1. Materials/reagents

Heparin solution (American Pharmaceutical Partners, cat. # 504011)
19-gauge butterfly needle (Kendall, cat. # 225174)
Ficoll-Paque Plus (Amersham Pharmacia, cat. # 17-1440-02)
50-ml Accuspin tubes (Sigma-Aldrich, cat. # A2055)
Ammonium chloride solution (StemCell Technologies, cat. # 07850)
Endothelial basal medium, EBM-2 (Lonza, cat. # CC-3156)

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EGM-2 Singlequot supplements (Lonza, cat. # CC-4176)
Glutamine-penicillin-streptomycin solution, 100× GPS (Mediatech, Inc., cat. # 30-009-CI)
Fetal bovine serum, FBS (Hyclone, cat. # SV30014.03). Heat inactivated at 56 °C for 30 min
Bovine serum albumin, BSA (Sigma-Aldrich, cat. # A7906)
Gelatin (Fisher, cat. # DF0143-17-9)
CD31 Dynal beads solution (DYNAL, cat. # 111.55)
Magnetic particle concentrator (DYNAL, cat. # 120.20)
Trypsin-EDTA solution, 1× (Mediatech, Inc., cat. # 25-052-CI)
Dulbecco's phosphate buffered saline, PBS (Sigma-Aldrich, cat. # D5652)
Glucose (Sigma-Aldrich, cat. # G6152)
Sodium citrate (Sigma-Aldrich, cat. # S4641)
Citric acid (Sigma-Aldrich, cat. # 251275)
Sodium carbonate, Na_2CO_3 (Sigma-Aldrich, cat. # 223530)
Human plasma fibronectin (FN) (Chemicon International, cat. # FC-010)
Cloning rings, 150 μl (Sigma-Aldrich, cat. # C1059)
Sterile double-distilled water, dH_2O

2.2. Recipes

PBS, 1 l
- 9.6 g of PBS (Sigma-Aldrich, cat. # D5652)
- 1 l of dH_2O
- Autoclaved at 121 °C for 30 min

6% ACD-A solution, 1 l
- 22.3 g of glucose
- 22 g of sodium citrate
- 8 g of citric acid
- 1 l of dH_2O

Isolation buffer (PBS/0.6% ACD-A/0.5% BSA), 500 ml
- 50 ml of 6% ACD-A solution
- 2.5 g of BSA
- 450 ml of PBS
- Filter sterilized with a 0.2-μm–pore size vacuum filter

EBM-2/20% FBS, 500 ml (herein called EPC medium)
- 395 ml of EBM-2
- 100 ml of FBS (20% final)
- 5 ml of 100× GPS
- All the EGM-2 Singlequot supplements except for hydrocortisone (i.e., VEGF, hFGF-B, R-IGF-1, hEGF, Heparin, ascorbic acid, and GA-1000)
- Filter sterilized with a 0.2-μm–pore-size vacuum filter, divided into 45-ml aliquots and freeze down (–20 °C) until needed.

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Isolation medium, 100 ml
81.24 ml of EPC medium
3.76 ml of FBS (to maintain 20% final)
15 ml of autologous plasma (obtained as described below; 15% final)
Filter sterilize with a 0.2-μm–pore-size vacuum filter
1% gelatin solution, 500 ml
5 g of gelatin
500 ml of PBS
Autoclaved at 121 °C for 30 min. Filter sterilized with a 0.2-μm–pore-size vacuum filter
FN-coating solution (0.1 M Na_2CO_3), 500 ml
5.3 g of Na_2CO_3
500 ml of dH_2O
Adjust pH to 9.4 with HCl
Filter sterilized with a 0.2-μm–pore-size vacuum filter

2.3. Procedures

2.3.1. Isolation of cord blood–derived EPCs (cbEPCs)

1. Coat 100-mm tissue-culture plates with 1% gelatin solution (10 ml per plate) and incubate at 37 °C for 30 to 60 min. Prior to use, remove the gelatin solution and wash the plates once with PBS.
2. Set up 50-ml conical tubes with 10 ml of isolation buffer for blood collection.
3. Add 1 ml heparin solution into the syringe prior to drawing cord blood. Draw blood (typically 20 to 40 ml) from the umbilical vein using a 19-gauge butterfly needle. Collect every 25 ml of blood directly into 50-ml conical tubes with 10-ml isolation buffer. Place conical tubes with blood samples on ice.
4. Add 15 ml of Ficoll-Paque Plus to each 50-ml Accuspin tube. Spin at 1200 rpm for 1 min to sediment the Ficoll-Paque below the frit.
5. Add 30 ml of blood/isolation buffer to the top of each Accuspin tube. Spin at 2700 rpm for 15 min at room temperature (RT) with the brake of the centrifuge off.
6. Gently collect autologous plasma supernatant above the mononuclear cell layer. (Do not disturb the cell layer.) Save this autologous plasma to make up isolation medium. To minimize disturbances, do not remove all the plasma above the cell layer.
7. Using an 18-gauge needle on a 10-ml syringe, collect the mononuclear cell layer and transfer it to a 50-ml conical tube on ice. Add 5 ml of isolation buffer to every 10 ml of cells collected.
8. Spin the mononuclear cells at 2700 rpm for 5 min. Remove supernatant and resuspend the cell pellet in 10 ml of isolation buffer. Transfer to a

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15-ml conical tube and spin at 1200 rpm for 10 min. Remove supernatant.

9. Add 1 ml of isolation buffer to dislodge the cell pellet and resuspend the cells. Add 3 ml of ammonium chloride solution to lyse erythrocytes. Incubate on ice for 5 to 10 min.
10. Add 5 ml of isolation buffer and centrifuge at 1300 rpm for 5 min. Remove supernatant. If the cell pellet is not devoid of erythrocytes (i.e., pellet still has a red color), incubate again with 1 ml of isolation buffer and 3 ml of ammonium chloride solution on ice for 5 min. Add 5 ml of isolation buffer and centrifuge at 1300 rpm for 5 min. Remove supernatant.
11. Resuspend the cell pellet in 10 ml of isolation medium per 25 ml of cord blood started with. Take 10 μl to count the cells in a hemocytometer and work out the total number of mononuclear cells per ml of cord blood sampled (approximately 4 to 8 $\times$ 10^7 MNCs in 25 to 50 ml of cord blood).
12. Plate the mononuclear cell solution in 1% gelatin-coated 100-mm tissue culture plates. Use 2$\times$ 100-mm tissue culture plates per 25 ml of cord blood sampled (equivalent to 4.5 cm^2/ml cord blood sampled). Add 10 ml of cells suspended in isolation medium to each 10-cm plate, and place them in a humidified incubator at 37 °C and 5% CO_2 for 48 h.
13. Forty-eight hours after plating, aspirate out the unbound cell fraction (which includes all the unattached hematopoietic cells), and feed the bound-cell fraction with fresh EPC medium.
14. Feed the plates every 2 to 3 days with EPC medium. Screen plates for the presence of EC-like colonies. Cord blood–derived endothelial colonies that display cobblestone morphology will emerge in culture after 1 week (Fig. 13.1). The size, frequency, and time of appearance of these colonies will vary as reported by Ingram *et al*. (2004).
15. Allow colonies to expand such that the plate is covered by a confluent cellular monolayer. Detach the cells using trypsin-EDTA solution and proceed to purify them by selection of CD31-positive cells.

2.3.2. Isolation of adult blood derived–EPCs (abEPCs)

1. Coat six-well tissue culture plates with 1% gelatin solution (2 ml per well) and incubate at 37 °C for 30 to 60 min. Prior to use, remove the gelatin solution and wash the plates once with PBS.
2. Set up 50-ml conical tubes with 10 ml of isolation buffer for blood collection.
3. Add 1 ml heparin sodium into the syringe prior to drawing blood. Draw blood from the vein (typically 50 to 100 ml) using a 19-gauge butterfly needle. Collect 25 ml of blood directly into 50-ml conical tubes with 10 ml of isolation buffer. Place conical tubes with blood samples on ice.

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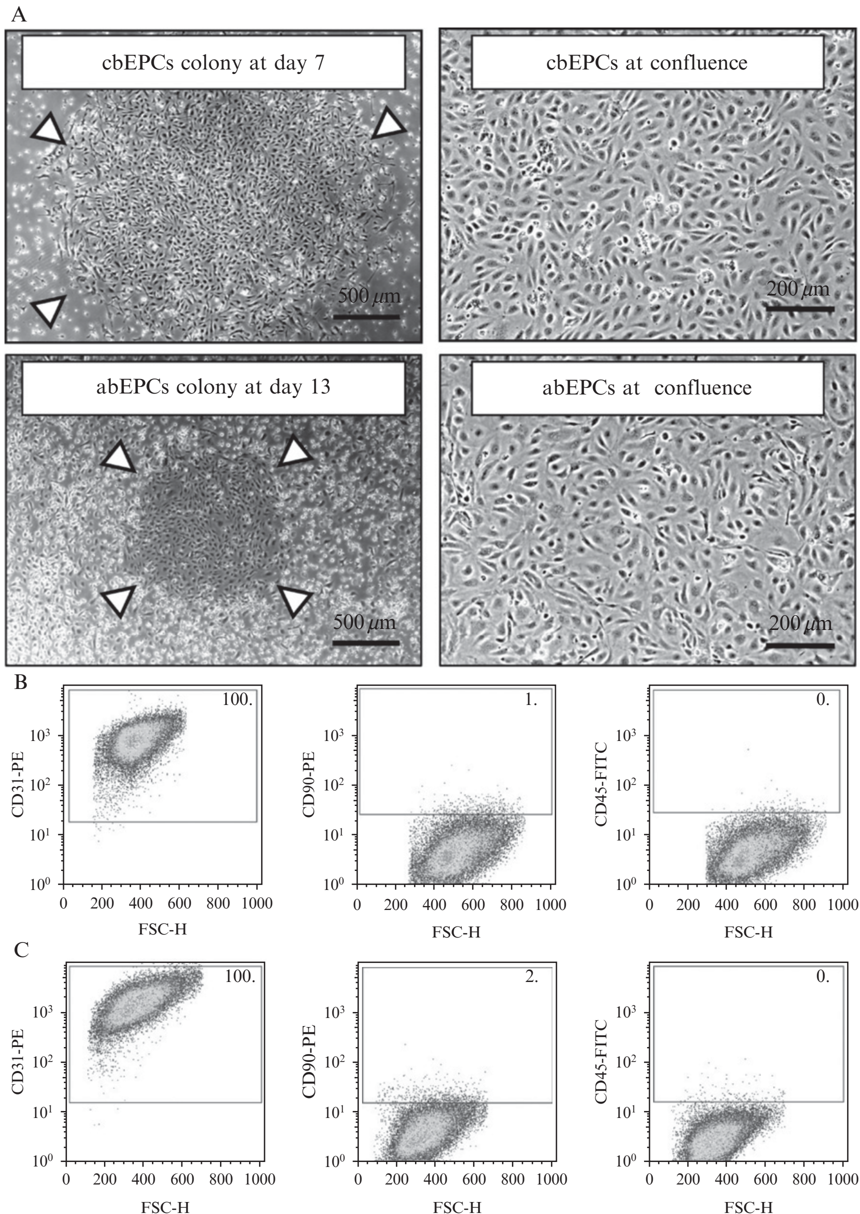

Figure 13.1 Phenotypic characterization of EPCs. (A) EPCs emerge in culture as typical EC colonies, after 5 to 7 days when isolated from cord blood, and after 14 to 21 days when isolated from adult peripheral blood (left panels). CD31-selected EPCs present typical cobblestone morphology at confluence (right panels). Flow cytometric analysis of cultured (B) cbEPCs and (C) abEPCs show uniform expression of EC marker CD31, and negative expression of mesenchymal marker CD90 and hematopoietic markers CD45. This type of analysis should be performed routinely to verify that the cell population to be used for *in vivo* vasculogenesis is not contaminated with either mesenchymal or hematopoietic cells at any stage of their expansion *in vitro*.

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4. Add 15 ml of Ficoll-Paque Plus to each 50-ml Accuspin tube. Spin at 1200 rpm for 1 min to sediment the Ficoll-Paque to below the frit.
5. Add 30 ml of blood/isolation buffer to the top of each Accuspin tube. Spin at 2700 rpm for 15 min at RT with the brake of the centrifuge off.
6. Gently collect autologous plasma above the mononuclear cell layer. (Do not disturb the cell layer.) Save this autologous plasma to make up isolation medium. To minimize disturbances, do not remove all the plasma above the cell layer.
7. Using an 18-gauge needle on a 10-ml syringe, collect the mononuclear cell layer and transfer it to a 50-ml conical tube on ice. Add 5 ml of isolation buffer to every 10 ml of cells collected.
8. Spin the mononuclear cells at 2700 rpm for 5 min. Remove supernatant and resuspend the cell pellet in 10 ml of isolation buffer. Transfer to a 15-ml conical tube and spin at 1200 rpm for 10 min. Remove supernatant.
9. Add 1 ml of isolation buffer to dislodge the cell pellet and 3 ml of ammonium chloride solution to lyse erythrocytes. Incubate on ice for 5 to 10 min.
10. Add 5 ml of isolation buffer and centrifuge at 1300 rpm for 5 min. Remove supernatant. If cell pellet is not completely free of erythrocytes (i.e., pellet still has a red color), incubate it again with 1 ml of isolation buffer and 3 ml of ammonium chloride solution on ice for 5 min. Add 5 ml of isolation buffer and centrifuge at 1300 rpm for 5 min. Remove supernatant.
11. Resuspend cells in 12 ml of isolation medium per 25 ml of blood started with. Take 10 μl to count the cells in a hemocytometer and work out the total number of mononuclear cells per ml of blood sampled (approximately 10 to 15 $\times$ 10^7 MNCs in 50 ml of blood).
12. Plate the mononuclear cell solution in 1% gelatin-coated six-well tissue culture plates (2 ml per well). Use one six-well plate for each 25 ml of peripheral blood sampled (equivalent to 2.5 cm^2/ml blood sampled). Place the plates in a humidified incubator at 37 °C and 5% CO_2.
13. Forty-eight hours after plating, add 2 ml of fresh isolation medium to each well. Do not aspirate the unbound cell fraction yet; place the plates back in the humidified incubator at 37 °C and 5% CO_2 for another 48 h.
14. Four days after plating, aspirate the unbound cell fraction and feed the bound cell fraction with fresh EPC medium (2 ml per well).
15. Feed the plates every 2 to 3 days with EPC medium. Screen plates for the presence of EC-like colonies. Peripheral blood-derived endothelial colonies (identified by typical cobblestone morphology) (Fig. 13.1) will emerge in culture after 2 to 3 weeks since isolation. The size, frequency, and time of appearance of these colonies will vary as reported by Ingram *et al.* (2004). Keep feeding the plates and leave the endothelial-like

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colonies to grow in size until they reach a diameter of approximately half the diameter of a cloning ring (3 mm).

16. Before proceeding to collect the endothelial-like colonies, coat six-well tissue culture plates with 2 ml of FN-coating solution and 10 μl of human plasma fibronectin (final fibronectin concentration of 1 μg/cm^2) per well. Incubate the plates at 37 °C for at least 60 min. Prior to use, remove the FN-coating solution and wash the plates once with PBS. Add 2 ml of EPC medium to each well where colonies will be plated. Each colony will be collected and replated in an individual well.
17. Mark the location of the colonies of interest on the bottom of the wells with a marker. Verify that the colonies are circled by observing with an inverted microscope. Aspirate the medium from wells where colonies are present and wash each well with PBS. Aspirate the PBS and using a sterile forceps, place a cloning ring over the location of a colony to be selected and press down gently. The grease at the bottom of the cloning ring will help it stick to the culture plate. Add 100 μl of trypsin-EDTA solution inside each cloning ring and incubate at 37 °C until the cells become loosely attached or detached, as observed in the inverted microscope. Transfer the 100 μl of cells in trypsin-EDTA from the cloning ring to the new FN-coated well with 2 ml of EPC medium. Add 100 μl of EPC medium to the cloning ring and gently resuspend any remaining cells inside the cloning ring; transfer this wash to the new FN-coated well with 2 ml of EPC medium. Finally, place the plates in the humidified incubator at 37 °C and 5% CO_2.
18. Feed the plates every 2 to 3 days with EPC medium. At confluence, detach the cells using trypsin-EDTA solution and replate the cells from each well into one 100-mm, FN-coated tissue culture plate. Culture the plates in the humidified incubator at 37 °C and 5% CO_2.
19. Allow cells to reach confluence. Detach the cells using trypsin-EDTA solution and proceed to purify them by selection of CD31-positive cells.

2.3.3. Magnetic bead purification of cultured CD31-positive EPCs

1. Coat 100-mm tissue-culture plates with 5 ml of FN-coating solution and 60 μl of human plasma fibronectin per plate (final fibronectin concentration of 1 μg/cm^2). Incubate the plates at 37 °C for at least 60 min. Before use, remove the FN-coating solution and wash the plates once with PBS.
2. For each confluent 100-mm cultured plate, aspirate the culture medium and wash the cells with 10 ml of PBS. Remove PBS and add 2 ml of trypsin-EDTA solution to each 100-mm plate. Gently rock the plates to evenly distribute the trypsin-EDTA solution. Incubate for 1 to 2 min. Gently tap the plate to facilitate cell detachment and verify under an inverted microscope that cells are detaching and in suspension.

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3. When cells completely detach, add 8 ml of EPC medium and collect the cell solution into a 15-ml conical tube. Take 10 μl to count the cells in a hemocytometer and work out the total number of cells harvested.
4. Centrifuge the cells at 1200 rpm for 5 min, remove the supernatant and wash the cell pellet in 5 ml of isolation buffer. Centrifuge the cells at 1200 rpm for 5 min, remove the supernatant and resuspend the cell pellet in 500 μl of isolation buffer. Transfer the cell solution into a 1.5-ml sterile centrifuge tube.
5. Add 12.5 μl of anti-CD31—coated Dynal beads and incubate for 5 min at 4 °C with intermittent mixing. CD31-positive cells will attach to the anti-CD31–coated magnetic beads.
6. Place the centrifuge tube in the magnetic cell concentrator and hold for 1 min. Anti-CD31–coated magnetic beads will move toward the magnet (accumulating on the wall of the tube) leaving the nonattached cells (CD31-negative cell fraction) free in suspension. Gently aspirate the CD31-negative cell fraction.
7. Remove the centrifuge tube from the magnetic cell concentrator. Add 0.5 ml of isolation buffer and mix gently by pipetting. Place the tube back in the magnetic cell concentrator, hold for 1 min, and discard the negative fraction. Repeat the wash a total of three times.
8. After the final wash, resuspend the CD31-positive cells in 10 ml of EPC medium and plate the resulting cell solution on a 100-mm, FN-coated tissue culture plate. These cells can be referred to as passage 1 (P1) EPCs.

2.3.4. Notes

1. During the initial steps of the isolation procedure when the MNC fraction is plated on 100-mm dishes or 6-mm multiwell dishes, cells are fed with EPC medium with 15% of autologous plasma.
2. Because the EPCs comprise a very small population of the mononucleated cells, about two to five cells per milliliter in human umbilical cord blood and 0.05 to 0.2 cells per milliliter in adult peripheral blood (Ingram *et al.*, 2004), the initial period of cell attachment is 2 days for cord blood samples and 4 days for adult peripheral blood samples. Thereafter, the unattached cells should be discarded and the cultures fed with EPC medium without autologous plasma.
3. As reported previously (Ingram *et al.*, 2004; Yoder *et al.*, 2007), EPC colonies should be evident 14 to 21 days after plating adult peripheral blood MNCs. However, since the number of expected colonies from adult peripheral blood is very low, a careful and thorough screening of the culture plates is advised, beginning in the second week after the procedure. To facilitate the screening (and the later picking of colonies), MNCs from adult blood samples can be divided among individual wells of a six-well plate instead of using 100-mm culture plates.

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4. Plates from adult peripheral blood preparations will present higher numbers of attached hematopoietic cells (e.g., monocytes and macrophages) than those from cord blood samples (Yoder *et al.*, 2007). The myeloid cells will adhere to and populate the culture plates such that colonies of abEPCs will be left with limited free space to expand. Therefore, investigators are advised to pick the abEPC colonies with cloning rings and transfer the cells to new culture wells where they can be expanded freely from the hematopoietic cells.
5. Due to the inherent heterogeneity of blood samples, one concern about this method is the potential contamination with hematopoietic cells. However, hematopoietic cells that do attach to the culture plates are then difficult to detach during the trypsinization step. If such cells do detach, they do not proliferate appreciably in subsequent passages. Moreover, the superior proliferative capacity of EPCs over hematopoietic cells in culture will rapidly outpace any hematopoietic cells present at passage 1. In addition, the presence of hematopoietic cells is only significant in preparations from adult peripheral blood, wherein the EPC colonies are selected using cloning rings, thereby reducing the chances of contaminating cells.
6. An additional concern is the potential contamination with mesenchymal cells present in the blood samples. Circulating mesenchymal cells have been reported in adult peripheral blood (Simper *et al.*, 2002) and cord blood samples (Kim *et al.*, 2004; Le Ricousse-Roussanne *et al.*, 2004; Lee *et al.*, 2004). Therefore, blood-derived mesenchymal cells constitute a real potential contaminant since they can adhere and proliferate in the culture plates with ease. However, mesenchymal cells do not express the surface cell marker CD31, and therefore they will be depleted from the culture during the magnetic bead purification of CD31-positive EPCs.

3. Expansion and Characterization of Blood-derived Endothelial Progenitor Cells

Prior to their use *in vivo*, EPCs will require a period of culture expansion *in vitro*. Although conditions for expanding EPCs can be found elsewhere in the literature, we have shown that up to 10^{13} cbEPCs (25 ml of cord blood) and 10^{8} EPCs (50 ml of adult peripheral blood) can be obtained after only 40 days in culture following the methods described here (Melero-Martin *et al.*, 2007). Similar expansion potential has been also reported by other authors for both blood-derived EPCs (Ingram *et al.*, 2004; Lin *et al.*, 2000). These remarkable numbers of human EPCs are likely to exceed (in the case of cord blood), and be sufficient (in the case of adult blood), the quantity that would be needed for most autologous regenerative therapies.

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However, it is important to consider the effect that culture conditions and the extent of expansion may impose on cellular phenotype and function.

We and others have reported that EPCs expanded *in vitro* are phenotypically indistinguishable from cultured ECs (Ingram *et al.*, 2004, 2005; Lin *et al.*, 2000; Melero-Martin *et al.*, 2007; Yoder *et al.*, 2007). For example, flow cytometric analysis of EPCs shows uniform expression of EC markers CD31, VEGF-R2, and vWF, and negative expression of mesenchymal marker CD90 and hematopoietic markers CD45 and CD14. This type of analysis should be performed routinely to verify that the cell population to be used for *in vivo* vasculogenesis is not contaminated with either mesenchymal or hematopoietic cells at any stage of their expansion *in vitro* (Melero-Martin *et al.*, 2007). Additionally, immunofluorescence staining should show that EPCs express CD31 and VE-cadherin at the cell–cell borders and vWF in a punctuate pattern in the cytoplasm, clear indications of EC properties (Melero-Martin *et al.*, 2007). Despite the consistent and uniform expression of endothelial markers, EPCs can undergo cellular and functional changes in culture. For instance, cord blood–derived EPCs have been reported to change their morphology, growth kinetics, migration, proliferative responses toward angiogenic factors, and *in vivo* vasculogenic ability as they are expanded in culture (Ingram *et al.*, 2004; Khan *et al.*, 2006; Melero-Martin *et al.*, 2007). Therefore, it is important to keep track of the number of population doublings that EPCs undergo *in vitro* prior to their use *in vivo*. In this section we provide details for (1) routine culture of EPCs, (2) evaluation of the accumulative number of population doublings during expansion, and (3) simplified phenotype confirmation by flow cytometry. In summary, we would like to stress the importance of these quality control measures to ensure that the cells to be used are homogenous and have expected endothelial features.

3.1. Additional materials/reagents

High-glucose Dulbecco's Modified Eagle Medium, 1× DMEM (Gibco, cat. # 10564)
MEM nonessential amino acid solution (NEAA), 100× (Sigma-Aldrich, cat. # M7145)
0.5 *M* EDTA solution, pH 8.0 (Gibco, cat. # 15575-038)
Mouse IgG1-PE (BD Pharmingen, cat. # 555787)
Mouse IgG1-FITC (BD Biosciences, cat. # 349041)
CD31-PE (Ancell, cat. # 180-050)
CD90-PE (BD Pharmingen, cat. # 555596)
CD45-FITC (BD Biosciences, cat. # 347463)
Sterile double-distilled water, dH_2O

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3.2. Additional recipes

DMEM/10% FBS medium, 500 ml
 440 ml of DMEM
 50 ml of FBS (10% final)
 5 ml of 100× NEAA
 5 ml of 100× GPS
 Filter sterilized with a 0.2-μm–pore-size vacuum filter
FACS buffer (PBS/0.5% BSA/2m*M* EDTA), 100 ml
 9.6 ml of PBS
 0.5 g of BSA
 0.4 ml of 0.5 *M* EDTA solution

3.3. Procedures

3.3.1. Expansion of blood-derived EPCs

Feed P1 blood-derived EPCs every 2 to 3 days using EPC medium (10 ml of medium per each 100-mm tissue-culture plate). At confluence, subculture the cells as follows:

1. Aspirate out the culture medium and wash the cells with 10 ml of PBS.
2. Remove PBS and add 2 ml of trypsin-EDTA solution to each 100-mm plate. Gently rock the plates to evenly distribute the trypsin-EDTA solution. Incubate for 1 to 2 min. Gently tap the plate to see the detached cells in suspension under an inverted microscope.
3. When cells completely detach, add 8 ml of EPC medium and collect the cell solution into a 15-ml conical tube. Take 10 μl to count the cells in a hemocytometer and work out the total number of cells harvested. Calculate the number of population doublings (PD) as follows:

$$PD = \frac{Ln\left(\frac{XF}{X0}\right)}{Ln(2)}$$

where *X*0 (cells) refers to the initial cell number seeded in this culture, *XF* (cells) refers to the final cell number observed at the time of harvesting, and *PD* refers to the number of doublings that the cell population underwent during this passage.

4. Plate the cells in FN-coated (1 μg/cm^2) tissue-culture plates at a seeding density of 5000 cell/cm^2 using EPC medium. Place the plates in a humidified incubator at 37 °C and 5% CO_2 and feed them every 2 to 3 days with EPC medium. These cells can be referred to as passage 2 (P2) EPCs.

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Repeat this procedure for subsequent passages. Keep track of the accumulative value of PD as the cell population is expanded.

3.3.2. Cell characterization by flow cytometry (CD31, CD90, and CD45)

1. Aspirate out the culture medium from one confluent 100-mm culture plate and wash the cells with 10 ml of PBS.
2. Remove PBS and add 2 ml of trypsin-EDTA solution to each 100-mm plate. Gently rock the plates to evenly distribute the trypsin-EDTA solution. Incubate for 1 to 2 min. Gently tap the plate to see the detached cells in suspension under an inverted microscope.
3. When cells completely detach, add 8 ml of DMEM/10% FBS medium and collect the cell solution into a 15-ml conical tube. Take 10 μl to count the cells in a hemocytometer and work out the total number of cells harvested.
4. Centrifuge the cells at 1200 rpm (<300 g) for 5 min. Remove supernatant and resuspend the cell pellet in 10 ml of FACS buffer and centrifuge again at 1200 rpm (<300 g) for 5 min. Remove supernatant.
5. Resuspend the cell pellet in 500 μl of FACS buffer (100 μl per label). Split the cell sample by transferring 100 μl of the cell suspension into five individual 1.5-ml centrifuge tubes. Add 1 μl of conjugated antibody (IgG-FITC, IgG-PE, CD31-PE, CD90-PE, and CD45-FITC) and incubate for 20 min at 4 °C with frequent mixing (tapping).
6. Following incubation, add 1 ml of FACS buffer to the tubes and centrifuge at 3000 rpm (800 g) for 3 min.
7. Wash the pellet in 1 ml of FACS buffer and centrifuge again. Repeat the wash once again.
8. Resuspend the cell pellets in 1% paraformaldehyde (made in PBS) and transfer the cells to FACS tubes. Keep the samples at 4 °C in the dark up to 1 week before analysis. Flow cytometric analyses can be performed using standard instruments (e.g., a Becton Dickinson FACScan flow cytometer) and the collected data analyzed by specialized software (e.g., FlowJo software from Tree Star Inc.).

3.3.3. Notes

1. When expanding cells in culture, it is common among investigators to report the passage number of the cultured cells. However, it is more accurate to keep track of the number of population doublings (PD) to reflect the degree of expansion exerted on them. The functional changes that cultured cells experience as a result of their expansion *in vitro* are likely to correlate with the number of PD, but not necessarily with the number of passages. Two different investigators can easily reach the same number of PD after two different passage numbers.

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2. Anti-VE-cadherin (CD144) can be used in place of anti-CD31 to verify endothelial phenotype. PE-conjugated, anti-human VE-cadherin is available from R&D Systems.
3. Expanded EPCs can be cryo-preserved using standard methods (e.g., using 90% FBS and 10% DMSO as freezing medium and liquid nitrogen for storage). We have tested EPCs cryo-preserved at different passages (up to passage 15), and their phenotype as well as their *in vitro* and *in vivo* functions were properly maintained (Melero-Martin *et al.*, 2007).

4. Growth of Human Smooth Muscle Cells

This assay requires SMCs as a source of perivascular cells. Human SMCs can be isolated from discarded vascular tissues (e.g., human saphenous vein-derived SMCs). Additionally, investigators can purchase commercially available human SMCs (e.g., Cell Application, Inc. ScienCell Res Lab; Cascade Biologics; Lonza Inc.).

4.1. Procedure

Plate SMCs in noncoated tissue-culture plates at a seeding density of 10,000 cells/cm^2. Place the plates in a humidified incubator at 37 °C and 5% CO_2 and feed them every 2 to 3 days with DMEM/10% FBS medium (10 ml of medium per 100-mm tissue-culture plate). At 80% confluence, subculture the cells as follows:

1. Aspirate out the culture medium and wash the cells with 10 ml of PBS.
2. Remove PBS and add 2 ml of trypsin-EDTA solution to each 100-mm plate. Gently rock the plates to evenly distribute the trypsin-EDTA solution. Incubate for 1 to 2 min. Gently tap the plate to see the detached cells in suspension under an inverted microscope.
3. When cells completely detach, add 8 ml of DMEM/10% FBS medium and collect the cell solution into a 15-ml conical tube. Take 10 μl to count the cells in a hemocytometer and work out the total number of cells harvested. Calculate the number of PDs with the previous equation.
4. Plate the cells in noncoated tissue-culture plates at a seeding density of 10,000 cell/cm^2 using DMEM/10% FBS medium. Place the plates in a humidified incubator at 37 °C and 5% CO_2 and feed them every 2 to 3 days with DMEM/10% FBS medium.

Repeat this procedure for subsequent passages. Keep track of the cumulative value of PD as the cell population is expanded.

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5. *In vivo* Vasculogenic Assay

The Matrigel plug assay was introduced by Passaniti and co-workers in 1992 (Passaniti *et al.*, 1992), and is regarded as a useful assay for *in vivo* screening of potential pro- and anti-angiogenic compounds (Auerbach *et al.*, 2003). Matrigel, which is an extract of the murine Engleberth-Holm-Swarm tumor, is composed of basement membrane proteins. Although it takes the form of a liquid at 4 °C, Matrigel reconstitutes into a gel or plug at body temperature when injected subcutaneously into mice, where it is progressively surrounded by granulation tissue. This assay was originally conceived as a mechanism to study the growth of new vessels into the Matrigel in response to an angiogenic factor. For instance, Matrigel plugs elicit an intense vascular response when supplemented with acidic FGF (Passaniti *et al.*, 1992), basic FGF, or VEGF (Kano *et al.*, 2005). One aspect of this assay that has been often criticized is the fact that Matrigel has not been fully defined chemically. It contains collagen IV, laminin, nidogen/entacin, heparin sulfate proteoglycan, and growth factors such as epidermal growth factor, transforming growth factor beta, platelet-derived growth factor, insulin-like growth factor-1, nerve growth factor, and bFGF (Baatout, 1997; Vukicevic *et al.*, 1992). This suggests that caution should be exercised in the interpretation of experiments on cellular activities related to Matrigel (Vukicevic *et al.*, 1992). However, subcutaneous implantation of Matrigel alone (without any additional angiogenic factor) does not initiate an angiogenic response from the host; when plugs of Matrigel alone are implanted for 1 to 2 weeks into immunodeficient mice, they remain largely inert with no vascular structures inside the implants and only a few cells invading the plugs (Melero-Martin *et al.*, 2007). On the contrary, implantation of Matrigel containing EPCs and SMCs supports the progressive formation of vascular networks, reproducing aspects of postnatal vasculogenesis such as cellular assembly, lumen formation and network remodeling. Additionally, this assay is relatively simple to perform; it does not require an incision or surgical procedure, which reduces the potential influence of wound healing. Finally, the assay can be carried out in either athymic nu/nu or NOD/SCID mice.

5.1. Additional materials/reagents

Phenol-red–free BD Matrigel Matrix (BD Bioscience, cat. # 356237)
Six-week-old male athymic nu/nu mouse (Massachusetts General Hospital, Boston)
Histological Tissue-Tek unicassette (Sakura Finetek, cat. # 4117-02)
Isoflurane liquid for inhalation (Buxter Healthcare Corporation, cat. # NDC 10019-360-40)
10% neutral buffered formalin (Sigma-Aldrich, cat. # HT501128)

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5.2. Additional recipes

Matrigel aliquots, 1ml

Matrigel should be divided into aliquots as recommended by the manufacturer. Briefly, thaw 10 ml of Matrigel on ice overnight and transfer the liquid Matrigel into ten 1.5-ml centrifuge tubes (1-ml aliquots). Freeze the Matrigel aliquots at −20 °C until needed. The day of the experiment, thaw the required aliquots on ice 1 to 2 h prior to their use.

5.3. Procedures

Before the experiment, make sure that sufficient EPCs and SMCs are available in culture; 1,500,000 EPCs and 375,000 SMCs will be required for each implant and mouse.

1. Aspirate the medium of each culture plate and wash the cells with 10 ml of PBS. Remove PBS and add 2 ml of trypsin-EDTA solution to each 100-mm plate. Gently rock the plates to evenly distribute the trypsin-EDTA solution. Incubate for 1 to 2 min. Gently tap the plate to see the detached cells in suspension under an inverted microscope.
2. When cells completely detach, add 8 ml of DMEM/10% FBS and collect the cell solution into a 15-ml conical tube. Take 10 μl to count the cells in a hemocytometer and work out the total number of EPCs and SMCs harvested.
3. Transfer 7,500,000 EPCs (5 × 1,500,000 cells) and 1,875,000 SMCs (5 × 375,000 cells) together into a single 50-ml conical tube. This is the total amount of cells required for five individual mice. Centrifuge at 1200 rpm and remove the supernatant. The total number of cells/implant can be varied from one-third to three times this amount to achieve lower or higher microvessel density (Melero-Martin *et al.*, 2007). Furthermore, the ratio of EPCs to SMCs can also be varied to achieve different degrees of vessel formation.
4. Resuspend the cell pellet on 1 ml of ice-cold Matrigel. Mix the cells very gently to avoid bubbles within the Matrigel. Load the cell–Matrigel mixture into a 1-ml sterile syringe, and place a 26-gauge needle with its cap on the tip of the syringe. Keep the loaded syringe deep on ice until injection.
5. Prior to the injection, anesthetize the immunodeficient mice by placing them in a gas chamber delivering isoflurane. Allow the mice to inhale the isoflurane for approximately 2 min until they are asleep (monitor their heart beats by inspection). For each mouse, inject 200 μl of the cell–Matrigel mixture subcutaneously into the upper dorsal region using a 26-gauge needle. Matrigel forms a gel at 37 °C so that the implant should form a small bump just under the skin (Fig. 13.2). After the injection,

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place the mice on a layer of gauze for comfort and warmth and observe them until they become ambulatory. Then, observe the mice daily for the first 3 days.

6. One week after the injections, euthanize the mice by placing them in a gas chamber delivering compressed CO_2 gas. Once euthanized, cut open the skin near the area of the injection and surgically removed the Matrigel plug (Fig. 13.2). Digital photographs of the retrieved Matrigel plugs with a scale are advised.
7. Place the harvested Matrigel plugs into histological cassettes and deep them into 10% neutral buffered formalin overnight at RT. After fixation, wash the 10% neutral buffered formalin away with dH_2O and place the histological cassettes at 4 °C in PBS until histological evaluation.
8. For histological evaluation, the implants are embedded in paraffin and sectioned (7-μm–thick sections) using standard histological procedures. Standard protocols for hematoxilin and eosin (H&E) can be found elsewhere.
9. Quantify microvessel density by evaluation of 10 randomly selected fields (0.1 mm^2 each) of H&E stained sections taken from the middle part of the implants. Microvessels can be identified as lumenal structures containing red blood cells and counted (Fig. 13.3). Report microvessel density as the average number of red blood cell-filled microvessels from the fields analyzed and expressed as vessels per square millimeter; 93 ± 18 vessels/mm^2 correspond to the average values plus/minus standard deviation obtained from four individual mice using cbEPCs at passage 3 plus SMCs (Melero-Martin *et al.*, 2007).

5.3.1. Notes

1. The time between resuspension of the cells in Matrigel and injection into the mice should be kept to a minimum (30 to 60 min).

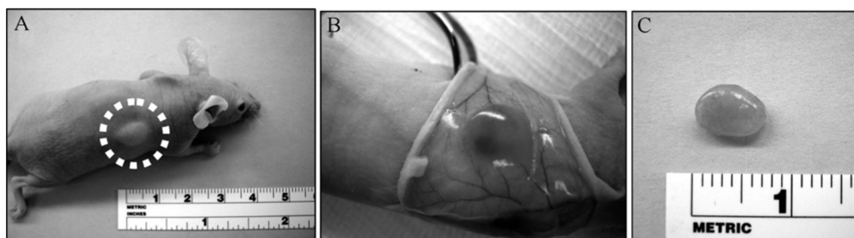

Figure 13.2 Appearance of cell/Matrigel plugs. Human cord blood EPCs and smooth muscle cells were suspended in Matrigel as described in the text. Right panel shows location and appearance of a cell/Matrigel suspension implanted subcutaneously into a nude mouse. Middle panel shows appearance of the cell/Matrigel plug 7 days after implantation. The left panel shows the appearance of the cell/Matrigel plug removed from the mouse after 7 days and prior to processing for histology.

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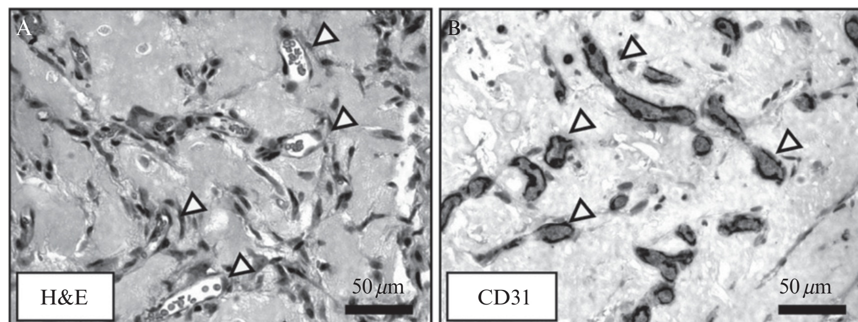

Figure 13.3 *In vivo* vasculogenic potential of EPCs. Matrigel implants containing human EPCs and SMCs were evaluated after 1 week. (A) H&E staining of implants revealed the presence of an extensive network of microvessels containing red blood cells. (B) Immunohistochemical staining at 1 week with anti-human CD31 antibody revealed that the lumenal structures were formed by the implanted EPCs.

2. Note that in this assay it is difficult to generate identical 3D plugs, even though the total Matrigel volume is kept constant (Auerbach *et al.*, 2000). Therefore, injections should be performed gently and the cell–Matrigel mixtures slowly released. In this regard, further improvements can be achieved by using subcutaneous chambers that allow for constant 3D form and volume of the Matrigel plug; these chambers have been reported to make the original Matrigel plug assay more reproducible in mice and rats (Kragh *et al.*, 2003; Ley *et al.*, 2004).
3. To further characterize the microvascular structures detected, sections of the retrieved Matrigel plug should be immunohistochemically stained with a human-specific CD31 antibody using standard staining protocols. Lumenal structures will stain positive for human CD31 (Fig. 13.3), confirming that those lumens are formed by the implanted human EPCs and not by the host cells. This evaluation is important because it demonstrates that the formation of microvascular vessels within the implant is the result of a process of *in vivo* vasculogenesis carried out by the implanted cells, and is not due to blood vessel invasion and sprouting (i.e., an angiogenic response from nearby host vasculature). We recommend using the monoclonal mouse anti-human CD31 antibody from DakoCytomation (Clone JC70A, cat. # M0823) at a 1:20 dilution. The human specificity of this antibody was confirmed by the negative reaction obtained with a diversity of mouse tissue sections that were stained in parallel (Melero-Martin *et al.*, 2007). SMCs can be localized by immunostaining with anti-alpha smooth muscle actin (clone 1A4, Sigma). However, this antibody reacts with both murine and human alpha–smooth muscle actin.

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6. Conclusion

Rapid and complete vascularization of ischemic tissues and thick engineered tissues are likely to require vasculogenesis. Therefore, the search for clinically relevant sources of vasculogenic cells and the subsequent development of experimental models of vasculogenesis are of utmost importance. Here, we describe a methodology adapted from the Matrigel plug assay to deliver human blood–derived EPCs and mature SMCs subcutaneously into immunodeficient mice. One week after implantation, an extensive microvascular bed that forms anastomoses with the host vasculature will be created by the implanted cells inside the plugs. The presence of human EPC–lined lumens containing host erythrocytes can be seen throughout the implants indicating not only the formation (*de novo*) of a vascular network, but also the development of functional anastomoses with the host circulatory system. Varying the number of cells in the original cell suspension can be used to manipulate the microvessel density achieved at 7 days. Altering the ratio between EPCs and SMCs, substituting another type of perivascular cell for mature SMCs, and GFP tagging the EPCs or perivascular cells are potential modifications (Melero-Martin *et al.*, 2008). This murine model of human vasculogenesis is ideally suited for studies aimed at cellular and molecular components of microvessel development and pathologic neovascular responses, and for the development and investigation of strategies to enhance neovascularization of engineered human tissues and organs.

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