Human Endothelial Progenitor Cells

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Human endothelial progenitor cells (EPCs) have been generally defined as circulating cells that express a variety of cell surface markers similar to those expressed by vascular endothelial cells, adhere to endothelium at sites of hypoxia/ischemia, and participate in new vessel formation. Although no specific marker for an EPC has been identified, a panel of markers has been consistently used as a surrogate marker for cells displaying the vascular regenerative properties of the putative EPC. However, it is now clear that a host of hematopoietic and vascular endothelial subsets display the same panel of antigens and can only be discriminated by an extensive gene expression analysis or use of a variety of functional assays that are not often applied. This article reviews our current understanding of the many cell subsets that constitute the term EPC and provides a concluding perspective as to the various roles played by these circulating or resident cells in vessel repair and regeneration in human subjects.

The importance of the systemic vasculature in mediating optimal delivery, exchange, and removal of gases, nutrients, and regulatory cells and molecules to the tissues and organs of a mature subject has long been appreciated (Aird 2007). More recently, interest in the role of the vasculature in promoting organogenesis during development, stem cell homeostasis, rescue of injured tissues following an ischemic/hypoxic challenge, and the growth and spread of cancer cells within the body has grown exponentially as investigators have probed new approaches for cellular therapies in all areas of human health and disease. Concomitant with these interests in translational research, investigators have become enthralled with the discovery of novel adult stem/progenitor cell populations that may be involved in the development, repair, or

regeneration of the systemic vasculature. The first reported existence of a bone marrow–derived circulating progenitor for the endothelial lineage called the endothelial progenitor cell (EPC) in 1997 (Asahara et al. 1997) initiated a robust area of investigation in experimental animals and human subjects with nearly 9500 papers cited in the PubMed database as of January 1, 2011 using the search term “endothelial progenitor cell.”

In contrast to the vast number of papers elucidating roles of putative bone marrow–derived EPC in cancer (Lyden et al. 2001; Mancuso et al. 2001, 2006, 2009; Bertolini et al. 2006; Shaked et al. 2006; Nolan et al. 2007; Gao et al. 2008; Seandel et al. 2008; Gao and Mittal 2009), cardiovascular disorders (Eizawa et al. 2004; Schmidt-Lucke et al. 2005; Werner et al. 2005;

Editors: Michael Klagsbrun and Patricia D’Amore

Additional Perspectives on Angiogenesis available at www.perspectivesinmedicine.org

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Cite this article as *Cold Spring Harb Perspect Med* 2012;2:a006692

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Fadini et al. 2006a,b; Kunz et al. 2006; Hughes et al. 2007), and diabetes (Tepper et al. 2002; Eizawa et al. 2004; Loomans et al. 2004; Fadini et al. 2006b, 2007), little focus has been placed on fully understanding how these cells may differ in their roles, behavior, or function compared to the rare circulating endothelial cells that may also be involved in many of these same disorders. This overview will attempt to summarize our current understanding of the various cell subsets that circulate in the bloodstream and are all referred to using the same EPC terminology. Given that no specific cell surface marker or unique gene expression pattern has been identified to unambiguously mark an EPC in mouse or man, we will identify those tools currently used to identify the putative EPC phenotype and will stress the differences in cell function displayed by the various “EPC” subsets.

BLOOD VESSEL FORMATION, REPAIR, AND REMODELING ARE REGULATED BY DIFFERING MECHANISMS

Blood vessel formation in the embryo has been examined in numerous vertebrate model systems. In the mouse, angioblast precursors derived from posterior primitive streak-derived mesoderm cells emerge on embryonic day (E)7.5 to initiate the process of vasculogenesis (Risau and Flamme 1995; Sabin 2002). The angioblasts migrate into the extraembryonic yolk sac to form a primitive capillary plexus. In time other angioblasts from later primitive streak-derived mesoderm populations migrate into and colonize the embryo proper and complete the first systemic vascular capillary bed by E8.25. The first blood cells to emerge in the developing mouse are the primitive erythroid progenitor (EryP) cells that independently migrate into the yolk sac (Fig. 1) and segregate into a circumferential extravascular band of erythroid cells (Ferkowicz et al. 2003). Near the time of onset of cardiac contractions that promulgate the first evidence of systemic blood circulation, the extraembryonic blood band is circumscribed by adjacent endothelial cells and the first blood-filled capillary structures

called blood islands are formed. Over the next 36 h, the blood islands are remodeled via intussusceptive angiogenesis and arteriogenesis into the various arterial, venous, and capillary beds of the mature yolk sac. Some of these endothelial cells display the capacity to form definitive hematopoietic progenitor cells that display multipotential hematopoietic lineage potential. Of interest, the systemic vascular bed is not completely filled with circulating blood cells until nearly E10.5. Thus, while arising nearly simultaneously from mesoderm, the EryP, definitive progenitor cells, and angioblasts do not appear to be derived from a common precursor, the hemangioblast, as often cited (reviewed in Ferkowicz and Yoder 2005; Ueno and Weissman 2010). Recent studies suggesting that the hemangioblast gives rise to hemogenic endothelial cells (which subsequently form the definitive hematopoietic lineages) only partially explains the origins of the EryP and fails to explain the origin of the earliest primitive capillary plexus (in which no blood cells emerge). Thus, in the earliest stages of blood vessel formation, hematopoiesis and vasculogenesis are independently executed developmental events that arise from mesoderm precursors. The fact that these lineages arise at nearly the same time and place in the developing embryo no longer infers that they arise from a common precursor.

Once the capillary plexus is formed, further expansion of blood vessel growth occurs via angiogenesis, as well as vasculogenesis (Risau and Flamme 1995). Angiogenesis is the formation of new vessels from endothelial cells representing already existing vascular structures in the embryo. In some cases, new vessels form from sprouting capillary endothelium within the primitive capillary plexus. In other cases, the sprouting endothelial cells arise from vascular structures remodeled into arterial or venous appearing structures. It is apparent that the metabolic demands of the tissue participate in the remodeling of the primary capillary plexus to define the final vascular bed density. As reviewed elsewhere (Davis and Senger 2008; Iruela-Arispe and Davis 2009; Eilken and Adams 2010; Pitulescu and Adams 2010),

Figure 1. A late neural plate stage embryo. (A) Late neural plate stage mouse embryo with maturing Flk-1⁺ vascular plexus (green) and distinct band of CD41⁺ primitive erythroid progenitor cells in the proximal yolk sac (red). Note the paucity of angioblasts (green) in the blood band region. (B) Higher-magnification cross section of the blood band. The Flk-1⁺ angioblasts (green cells identified by asterisks) are located between the primitive erythroid progenitor cells and the yolk sac visceral endoderm (blue cells indicated by endo) cells. (C) Blood island image depicting an angioblastic cord (a) from the same stage embryo as in panels A and B. Note the similarity of the cross section of the tissue in panel B to the panel C. The primary difference is the evidence that the cells highlighted in red in panel B are now known to be primitive erythroid cells and not mesoderm (angioblastic) cords as once thought (C). (Figure adapted from Ferkowicz 2005; reprinted, with permission, from Elsevier © 2005.)

genetic programmatic features and hemodynamic stressors play important roles in the capillary plexus remodeling into arteries and veins. Some evidence supports the role of hemodynamic stress on endothelial cells as an important component that promotes hematopoietic progenitor cell emergence from hemogenic endothelium (Adamo et al. 2009). A host of molecular mechanisms are now well understood in the regulation of the endothelial sprouting that represents a key event for new vessel formation. Subsequent steps of matrix degradation, endothelial sprouting, endothelial cell migration, cell-to-cell interactions with adjacent endothelial cells, cytoplasmic vacuolation and lumenization, basement membrane synthesis, and inosculature with preexisting vessels to access systemic blood flow are subsequent steps in vessel formation (Iruela-Arispe and Davis 2009).

In certain circumstances, a denudation injury to the vascular endothelium can occur that may not perturb the underlying endothelial basement membrane but requires rapid cellular recruitment to cover the otherwise thrombogenic exposed basement membrane (Schwartz et al. 1980). For example, traumatic injury may compromise the health of the vascular intima without harming the rest of the vessel components. Localized areas of inflammation may compromise vascular endothelial health and survival, leading to enhanced endothelial turnover (Schwartz et al. 1981). Apparently, the first events that occur to repair denudation of the endothelium (in experimental models) include deposition of platelets to the exposed basement membrane, increased migratory behavior of the endothelium adjacent to the injury, and endothelial cell spreading into the injury site (Schwartz et al. 1975; Malczak and

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Buck 1977; Manderson and Campbell 1986). For small injuries, endothelial migration and spreading can result in closure of the previously denuded area within a day. Subsequently, endothelial cell proliferation (first within more distal cells but later even in those cells that had migrated over the injury site) ensues, often leading to an eventual increased cellular density over the primary injury site that is several times that of the original cell density. Thus, the major cell type involved in the resolution of a denudation injury and return of the endothelial monolayer to the original topography and function of that vessel is the endogenous adjacent endothelium resident within the vessel (Schwartz and Benditt 1976). Whether similar events occur to replace senescent endothelial cells, without ever exposing the underlying basement membrane for circulating blood cell attachment, is unclear. Prior studies in rodent and pig models of normal vascular growth or induced vascular injury suggest that proliferating clusters of endothelial cells are apparent in vessels without signs of vascular denudation, providing some support for the presence of endogenous endothelial replacement (Wright 1968, 1971, 1972; Florentin et al. 1969; Caplan and Schwartz 1973; Schwartz and Benditt 1976, 1977; Schwartz et al. 1980, 1981; Prescott and Muller 1983; Taylor and Lewis 1986).

If large areas of endothelium are removed and/or there is damage to the underlying basement membrane or an artificial vascular grafting is interposed within vessels, greater influx of circulating cells ensues as a first response to injury. In this instance, a host of circulating hematopoietic cells along with platelets readily attach to these areas of damage or to the grafted artificial material (Rafi et al. 1995). In some instances, these deposited blood cells from the circulating blood are soon replaced by migrating and spreading endogenous endothelium. In other instances, colonies of replicating endothelial cells grow on the exposed area but fail to completely repopulate the monolayer and with time these areas of exposed basement membrane develop a fibrous and nonthrombogenic covering (Berger et al. 1972; Herring et al. 1984; Clowes et al. 1985, 1986; Zilla et al. 1994,

2007). Thus, the extent and type of vascular intima repair or regeneration is perhaps dependent on the extent and type of injury or implant and perhaps the age of the host. It is also well known that there are species-specific factors that may differentially regulate reendothelialization of denuded vessels (Berger et al. 1972; Herring et al. 1984; Clowes et al. 1985).

Finally, tumor cell growth, expansion, and metastasis depends on the ability of the tumor cells to secrete a variety of molecules that coopt circulating and resident proangiogenic cells in the tumor microenvironment to promote angiogenesis (Mancuso and Bertolini 2010). Tumor angiogenesis is perhaps the most complicated context within which to understand all of the cellular elements contributing to the tumor vascular growth. Not only do tumor cells recruit local and resident cells that include tumor-associated macrophages, tissue macrophages, mast cells, monocytes, neutrophils, and platelets that promote neoangiogenesis with sprouting of nearby vascular endothelium into the tumor, but in some cases, tumor cells may become vessel-mimicking or actual vessel-forming cells (Hirschi et al. 2008; Ricci-Vitiani et al. 2010; Wang et al. 2010). It is within this complex heterogeneous tumor microenvironment that some investigators have observed recruitment of circulating putative EPCs that play a role in enhancing tumor growth and metastasis. Defining those cells that represent EPCs and truly become long-term endothelial cells that comprise the tumor vessels versus those that enhance the vessel-forming properties of recruited endothelial cells but do not directly become endothelial cells is an important issue in developing the most powerful and effective drugs to inhibit all of the cells involved in the most effective tumor therapy (Bautch 2010).

METHODS TO DEFINE HUMAN EPCs

In the human system, putative EPCs have been identified using three general approaches. One of the first and perhaps the simplest method involves collecting low density mononuclear cells (MNCs) from human peripheral blood

or cord blood and plating the MNCs in dishes coated with fibronectin in a commercial cell culture medium containing endothelial growth factors and fetal calf serum (Ito et al. 1999; Hill et al. 2003). After 4–5 days in culture, the nonattached cells are removed and the adherent cells examined for the ability to bind acetylated low-density lipoprotein (AcLDL) and *Ulex europaeus* agglutinin 1 (a plant lectin). Thus, EPCs, as defined in this assay, are characterized by their morphologic appearance, adhesion to fibronectin, cell surface protein expression, AcLDL uptake, and lectin binding. Although this is a simple method for isolating an adherent cell population, it may be flawed by the lack of specificity of the cells obtained. For example, blood platelets are known to contaminate most MNC preparations and the presence of platelets in this culture milieu can result in non-discriminate transfer of platelet plasma membrane proteins to any adherent cells also attached to the culture matrix (including certain proteins thought to be endothelial specific) (Prokopi et al. 2009). Numerous blood cells (stem, progenitor, or committed mature lineages) express the integrin receptors for fibronectin and attach to plates coated with this molecule. In fact, monocytes are known to be highly enriched from peripheral blood MNCs when plated on fibronectin-coated dishes, and adherent monocytes cultured in media containing endothelial growth factors are known to express a variety of proteins typically thought to be reserved for endothelial cells (von Willebrand factor, endothelial nitric oxide synthase, CD31, CD144, and vascular endothelial growth factor 2 receptor [KDR]) (Hassan et al. 1986; Schmeisser et al. 2001, 2003). Recent proteomic and mRNA profiling analyses have indicated that human peripheral blood MNCs cultured as adherent cells on fibronectin-coated dishes in culture medium with added endothelial growth factors display a gene expression pattern that highly resembles hematopoietic (particularly cultured myeloid and T lymphoid cells) but not human endothelial cells (Medina et al. 2010). Thus, this very straightforward method of adherent MNC growth in vitro does not promote the unique emergence of an

EPC. Thus, given the ambiguity of this isolation method, one cannot recommend using this approach for EPC isolation or enumeration. As we will discuss later, this does not mean that the hematopoietic cells isolated via this technique and cultured under these conditions do not display proangiogenic activity; in fact, one can readily obtain proangiogenic hematopoietic cells using this approach that participate in ischemic hindlimb blood flow restoration in mice and even demonstrated some ability to improve cardiac outcomes in human subjects with acute myocardial infarction (Tongers et al. 2010). We simply point out that some of the lack of clarity in the field may relate to the use of the term EPC for cells isolated using this procedure when, in fact, in nearly all cases, proangiogenic hematopoietic cells (of various lineages) are selected and these cells though promoting vessel repair but do not become long-lived endothelial lined blood vessels.

A second method of human EPC identification has relied on identification of a particular pattern of cell surface antigen expression on the cells. As noted above, there are no unique or specific protein markers that can be used to prospectively isolate an EPC (reviewed in Hirschi et al. 2008). The first description of a human EPC by Asahara et al. (1997) was dependent on the selection of circulating cells with certain cell surface markers that might be expressed by both hematopoietic and endothelial cells so as to search for a putative circulating angioblast precursor. Although this rationale was based on the long-held recognition for the close temporal and spatial emergence of blood and endothelial cells during embryogenesis, the most current understanding of blood cell and angioblast development indicates that these lineages may not be derived from a common precursor. Indeed, evidence supports the origin of hematopoietic stem and progenitor cells from hemogenic endothelium but not endothelial cells from blood cells (Yoder et al. 2007; Zovein et al. 2008; Chen et al. 2009). Asahara et al. (1997) isolated human peripheral blood CD34-expressing cells (15.7% enriched for CD34⁺-expression) and reported that cell adhesion to fibronectin-coated dishes was

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significantly greater than to type 1 collagen coated dishes and that the CD34 enriched cells displayed a spindle-shaped morphology in this culture system. Of interest, the putative CD34⁺ EPCs when cocultured with CD34⁻ MNCs on the fibronectin-coated dishes formed clusters of round cells centrally and sprouts of spindle-shaped cells at the periphery. The authors pointed out that these clustered structures were reminiscent of the blood island-like clusters normally found in the developing embryonic yolk sac. The adherent putative EPCs expressed a variety of cell surface proteins typically expressed by human umbilical vein endothelial cells, and expression of these markers increased over time in vitro. Further studies provided evidence that vascular endothelial growth factor receptor 2 (Flk-1⁺) expressing putative EPCs (enriched to 20% Flk-1⁺) homed to areas of neovascularization when injected into nude mice with induced hindlimb ischemia with some evidence for improved microvascular density after infusion. Thus, in one article, Asahara et al. (1997) brought forth concepts of circulating EPCs, in vitro observations of EPC behavior, in vivo migration of putative EPCs to sites of vascular injury, and the paradigm of postnatal vasculogenesis whereby circulating EPCs could be infused into an animal with subsequent contribution to tissue microvasculature. These were and are important observations that have stimulated many investigators to understand the biology of these cells, attempt to characterize their concentration in health and disease, and isolate and infuse these cells into patients with acute or chronic ischemic diseases. However, potential limitations of this paper included the lack of sufficient cellular enrichment to constitute a purified cell population, lack of clonal analytical studies, failure to provide any functional exclusionary evidence as to whether any of the CD34⁺ or Flk-1⁺ EPCs possessed hematopoietic potential (given the fact that many of the cultured CD34⁺ cells displayed CD45), and lack of high cellular resolution evidence that the infused cells directly formed the new blood vessels in the tissues of the mice with induced vascular injury. Nonetheless, the use of CD34 and

Flk-1 (KDR in human subjects) as markers for the putative EPCs were instituted with this publication and have continued to be used as surrogate markers for the presence of a circulating cell with vascular reparative properties or in the isolation of this putative precursor.

The choice of CD34 as a potential marker of the circulating angioblast was not surprising as it is known to be expressed on endothelial cells and is a marker used to isolate human hematopoietic stem and progenitor cells for clinical stem cell transplantation. But, CD34 is a widely expressed molecule on some mesenchymal, epithelial, and even cancer stem cell populations (Hirschi et al. 2008). Thus, use of CD34 as an individual EPC marker is inadequate and would certainly require the search for additional potentially unique markers to discriminate all these different cellular lineages from the putative EPCs. KDR (human) or Flk-1 (mouse), a receptor for vascular endothelial growth factor, is also widely expressed on blood, endothelial, and cardiac cells and thus fails to be a helpful discriminator among those cells expressing CD34. Rationalizing that EPCs may share some cell surface antigen expression patterns with hematopoietic stem or progenitor cells, Peichev et al. (2000) chose to separate peripheral blood cells by expression patterns for CD34, KDR, and CD133. CD133 (AC133, prominin-1) is a 5-transmembrane domain cell surface glycoprotein that localizes to membrane protrusions on numerous epithelial, hematopoietic, and various cancer stem cells. CD34 and CD133 were known to be highly expressed on hematopoietic stem cells and are down-regulated during hematopoietic cell differentiation. Given this expression pattern in hematopoiesis, Peichev et al. (2000) rationalized that any endothelial cells coexpressing these molecules may represent a more immature progenitor population than cells expressing either antigen alone. Furthermore, because KDR was known to be expressed by embryonic angioblasts, Peichev et al. (2000) hypothesized that this antigen may be coexpressed on subsets of CD133⁺ cells with angioblastic (EPC) activity. The authors reported that although 2% of mobilized human peripheral blood CD34⁺ cells

coexpressed KDR and CD133, mature CD34⁺KDR⁺ human umbilical vein endothelial cells (chosen as representative of a mature vascular endothelial population) failed to express CD133, and thus the circulating CD34⁺KDR⁺CD133⁺ cells were thought to represent a progenitor cell phenotype. To confirm the presence of CD133 and KDR expressing cells in vivo, Peichev et al. examined the luminal surfaces of implanted left ventricular assist devices in human subjects with heart failure and identified some surface adhering cells expressing CD133 and KDR. Based on this evidence, the investigators concluded that a human EPC could be defined as a circulating CD34⁺ cell that coexpresses CD133 and KDR. Subsequently, this pattern of cell surface antigen expression has been one of the most popular to use when attempting to identify circulating EPC in human subjects. Nonetheless, no direct evidence was presented in the original manuscript that isolated and purified human circulating CD34⁺KDR⁺CD133⁺ cells directly participate in generating the endothelial lining of the implanted left ventricular device or whether the cells attached to the device were hematopoietic or endothelial in origin. Because these antigens are all known to be expressed by hematopoietic stem and early progenitor cells, one would have liked to see the comparative enrichments of the putative EPCs compared to the potentially contaminating hematopoietic subsets that may have been present.

Although CD34, CD133, and/or KDR expression have been used to identify human circulating EPCs in thousands of papers since 2000, and many of the papers have reported statistically significant correlations between the blood concentration of the selected putative EPC subset and a disease state, few have attempted to formally compare the functional properties of isolated human circulating CD34⁺KDR⁺CD133⁺ cells in hematopoietic and endothelial assays (reviewed in Alaiti et al. 2010). In one instance, Case et al. (2007) reported that purified CD34⁺KDR⁺CD133⁺ cells were highly enriched in hematopoietic progenitor activity but did not give rise to any endothelial colonies in vitro. Perhaps not

surprising given all the rationale in the above studies, more than 99% of the hematopoietic progenitor CD34⁺KDR⁺CD133⁺ cells coexpressed CD45, the common leukocyte antigen (this antigen is not expressed in endothelial cells even at the mRNA level). Timmermans et al. (2007) have also reported that colonies of endothelial cells that display high proliferative potential are derived only from a human cord blood or bone marrow CD34⁺CD45⁻ population of cells and not from CD34⁺CD45⁺ cells (which were enriched for hematopoietic colony forming cells). Thus, several labs have independently determined that the putative EPCs expressing CD34, CD133, and KDR that express the CD45 antigen are hematopoietic cells with colony forming activity and fail to give rise to endothelial cells during in vitro culture or directly form blood vessels in vitro or in vivo (Fig. 2).

The question of whether a cell expressing CD45 from adult human peripheral blood could represent an EPC has been controversial for some time and some of this controversy can be attributable to the underlying assumptions of the flow cytometric approaches used. Significant changes have occurred in both the hardware and software used in flow cytometry rate event analysis over the past decade (Herzenberg et al. 2006; Parks et al. 2006; Duda et al. 2007a). Newer digital machines with advanced resolution and data storage capabilities permit the identification of up to 20 distinct cellular parameters (Perfetto et al. 2006). Inherent in the ability to discriminate such a plethora of biologic data are improvements in the analytical software that are required to reduce spectral overlap by applying software-generated postacquisition compensation (based on compensation bead controls) and the use of bi-exponential scaling to properly visualize events below and above the zero axis (De Rosa and Roederer 2001; Parks et al. 2006). These advances, termed polychromatic flow cytometry (PFC), have permitted discovery of novel rare cell subsets, further characterized the functionality of established cell populations, and even discovered gross errors in phenotyping cells when compared to conventional flow cytometry practices (reviewed in Abdul-Salam et al. 2005;

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Figure 2. Formation of capillary-like structures in Matrigel coated plates. Photomicrographs ($20\times$ magnification) of freshly sorted cord blood (CB)- or mobilized peripheral blood (mPB)-derived $CD34^{+}AC133^{+}VEGFR2^{+}$ cells and early passage endothelial colony-forming cells (ECFCs) plated over Matrigel. The triple positive CB- and mPB-derived cells failed to form capillary-like structures, whereas the ECFC formed numerous lumenized structures. (Figure adapted from Case 2007; reprinted, with permission, from Elsevier © 2007.)

Herzenberg et al. 2006; Parks et al. 2006; Tung et al. 2007). Although numerous publications have proposed methods for resolution of circulating EPCs from circulating endothelial cells or other blood cell elements (Asahara et al. 1997; Gehling et al. 2000; Mancuso et al. 2001, 2009; Bertolini et al. 2003, 2006; Willett et al. 2004, 2005; Abdul-Salam et al. 2005; Khan et al. 2005; Goon et al. 2006; Duda et al. 2007b; Van Craenenbroeck et al. 2008; Widemann et al. 2008), several recent publications have applied PFC to the identification of putative EPCs and have resolved the subsets into proangiogenic hematopoietic cells (Fig. 3) and circulating endothelial colony-forming cells (Estes et al. 2010a,b; Schmidt-Lucke et al. 2010). Despite the use of advanced PFC techniques, to demonstrate that nearly all $CD133^{+}CD34^{+}KDR^{+}$ cells are $CD45^{+}$ and thus by definition hematopoietic, the field will only become clarified on identification of novel cell surface molecules that unambiguously identify the EPCs (see below for proposed definition of an EPC).

The final method to identify a human circulating EPC is based on colony forming ability of the plated MNC in vitro. The original description of cluster-forming cells appearing within 5 days of plating $CD34^{+}$ cells as a putative EPC characteristic (Asahara et al. 1997) was expanded on by Ito and colleagues who also isolated and plated blood cells on fibronectin-

coated dishes (Ito et al. 1999). One day later the nonadherent cells were removed and replated onto fibronectin-coated dishes, and the number of clusters that emerged at 7 days of replating was used to indicate the number of putative EPCs. The rationale for preplating the MNC for 24 h was to remove any monocytes, macrophages, or circulating mature endothelial cells in the MNC fraction that could contaminate the putative EPC assay system. Although laudable in intent, the failure to show that all hematopoietic or endothelial elements were depleted by the preplating step diminished the impact of the improved methodology. Hill et al. (2003) further modified the EPC cluster assay, by preplating blood cells for 48 h, then replating the nonadherent cells to quantify the emergence of the EPC-derived colonies. This assay has been commercialized and the putative EPC (that produce the progeny that form the colony) have been referred to as colony forming unit-Hill (CFU-Hill; Fig. 1). The CFU-Hill assay has been used to demonstrate a significant inverse correlation between the circulating CFU-Hill concentration and Framingham cardiovascular risk score in human subjects (Hill et al. 2003). Subsequent transcriptome, proteomic, and functional analyses have determined that CFU-Hill are more closely related to human hematopoietic cells than to primary endothelial cells.

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Figure 3. Frequency analysis of $CD31^{+}CD34^{bright}CD45^{dim}AC133^{+}$ cells using two distinct methods of analysis. In the first strategy (A–D), manually compensated data collected on a digital flow cytometer were visualized on plots with logarithmic scaling. Mononuclear cells (MNCs) (red gate in A) were identified on a forward and side scatter (FSC/SSC) plot and subgated onto a bivariate antigen plot to identify $CD34^{bright}AC133^{+}$ cells (dark blue gate in B). $CD34^{bright}AC133^{+}$ cells were further gated to identify the $CD45^{dim}$ subpopulation (light blue gate in C). CD31 expression on the resulting $CD34^{bright}AC133^{+}CD45^{dim}$ cells was confirmed on a CD31 histogram (D). In this first strategy (A–D), gate boundaries were set using Boolean gating and negative isotype controls. In the second strategy (E–I), uncompensated data was collected on a digital flow cytometer, compensated after acquisition by using software, and visualized in plots with biexponential scaling (linear and logarithmic). MNCs (red gate in A) were identified on a FSC/SSC plot and then $CD14^{-}$ cells (orange gate in E) were identified. All $CD14^{-}$ events were then assessed for viability (ViViD) and glycophorin A (GlyA) (F). The $CD14^{-}GlyA^{-}ViViD^{-}$ (pink gate in F) were subgated onto a bivariate antigen plot to identify $CD14^{-}GlyA^{-}ViViD^{-}CD34^{bright}AC133^{+}$ cells (dark blue gate in G). $CD14^{-}GlyA^{-}ViViD^{-}CD34^{bright}AC133^{+}$ cells were further subgated to identify the $CD45^{dim}$ subpopulation (light blue gate in H). CD31 expression was confirmed on a CD31 histogram. In the second approach (E–I), fluorescence minus one gating controls were used to set gate boundaries. (Figure adapted from Estes et al. 2010; reprinted, with permission, from John Wiley & Sons © 2010.)

Another assay system identifies outgrowth endothelial cells (OECs) possessing clonal endothelial colony-forming cell (ECFC) ability within 1–3 wk of culture, when blood cells are plated on matrix coated dishes with added growth factors (Gulati et al. 2003, 2004; Bompaïs et al. 2004; Hur et al. 2004; Ingram et al.

2004, 2005; Yoon et al. 2005; Guven et al. 2006; Shepherd et al. 2006; Melero-Martin et al. 2007; Nagano et al. 2007; Timmermans et al. 2007; Au et al. 2008). A hierarchy of clonal proliferative potential is displayed by the ECFC with some colonies growing to more than 10,000 progeny from a single cell plated 14

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days earlier (Ingram et al. 2004, 2005). The cord blood ECFC display high telomerase activity and vigorous in vivo human vessel formation when suspended in a matrix and implanted into immunodeficient mice (Ingram et al. 2004). The ability of these ECFCs to display spontaneous vasculogenic properties, to integrate long-term into the systemic vasculature of the host animal, and to remodel into arteries and veins in vivo distinguishes this EPC from all other types of cells that have been given this term (Bompais et al. 2004; Gulati et al. 2004; Hur et al. 2004; Ingram et al. 2004, 2005; Yoon et al. 2005; Guven et al. 2006; Shepherd et al. 2006; Melero-Martin et al. 2007; Nagano et al. 2007; Timmermans et al. 2007; Au et al. 2008).

Although the general consensus purports that endothelial cell turnover in systemic blood vessels is low in adult subjects, ample evidence has also been presented to suggest that the endothelium in some blood vessels is easily detectable (Wright 1968, 1971, 1972; Caplan and Schwartz 1973; Schwartz and Benditt 1976; Kunz et al. 1978; Taylor and Lewis 1986). In fact, in young experimental animals, endothelial replication rates have been reported as high as 60% in certain focal areas within the aorta. Experimental injury to the aortic endothelium (direct denudation) or disorders such as hypertension, hyperlipidemia, and endotoxemia all lead to an increase in endothelial replication in rodent models (Wright 1968, 1971, 1972; Caplan and Schwartz 1973; Schwartz and Benditt 1976; Kunz et al. 1978; Taylor and Lewis 1986). Most evidence suggests that the proliferating cells are retained in the endothelial intimal layer; however, some circulating endothelial cells thought to be sloughed from the vascular endothelium may also possess proliferative potential. Because human adult aorta and cord blood artery and vein endothelial cells have been determined to possess the clonal hierarchical proliferative potential similar to the circulating ECFCs derived from cord blood and adult peripheral blood (Ingram et al. 2005), it is plausible that the circulating ECFCs may be derived from vascular endothelium. Only further study will permit a detailed clarification

of the relationship between resident and circulating ECFCs.

ROLE OF EPCs IN VARIOUS HUMAN CLINICAL DISORDERS

A summary of the role of EPCs in human disease is complicated by the fact that so many different EPC definitions have been used. In many instances, circulating EPC concentrations have been enumerated and correlated to a disease state in an effort to serve as a biomarker for disease detection or staging (Alaiti et al. 2010). In some cases, the functional role of the EPCs has been elucidated when the cells were infused as a reparative therapy (Loomans et al. 2004). Defects in EPC function have also been identified in some patients with diabetes and potential therapies to restore certain aspects of EPC function have been proposed (Fadini et al. 2007). Despite the ambiguity in fully characterizing an EPC, numerous clinical trials have been conducted in patients with heart disease, diabetes, peripheral arterial disease, pulmonary disease, and cancer in which putative EPCs have been examined as a biomarker or used as a cell therapy to treat human subjects (see www.clinicaltrials.gov).

DEFINING A HUMAN EPC

A growing consensus is emerging that there are many circulating blood cells that participate in the process of new blood vessel formation and vascular repair. Controversy persists as to whether cells that display numerous features of the hematopoietic lineage but participate in new blood vessel formation should be called an EPC or not. If the term EPC is reserved for a progenitor cell for the endothelial lineage, we would propose that there are fundamental properties that this cell should display: a circulating cell that gives rise to progeny displaying clonal proliferative potential and differentiation restricted to the endothelial lineage, ability to form lumenized capillary-like tubes in vitro (cells must display cytoplasmic vacuolation capacity), and ability to form stable human blood vessels (cells must secrete a basement

membrane) when implanted into tissues (with or without a scaffold) that become an integrated part of the host circulatory system and display potential to undergo remodeling to form the intima of arterial, venous, and capillary structures. At present, the rare circulating ECFCs display these features while most other bone marrow–derived cells currently called EPCs fail to do so (Hirschi et al. 2008). Finding a unique cell surface marker that would permit prospective isolation and enrichment of cells displaying the above activities would certainly clarify the EPC identity and must remain a focus for the field.

ACKNOWLEDGMENTS

We would like to thank Tiffany Lewallen for her expert administrative assistance in preparation of the article. This work was supported in part by funds from the Riley Children's Foundation. The author has no competing financial interests to disclose.

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Cold Spring Harb Perspect Med 2012; doi: 10.1101/cshperspect.a006692 originally published online September 28, 2011

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