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# Endothelial progenitor cell: A blood cell by many other names may serve similar functions

**Mervin C. Yoder, MD**
Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN

## Abstract

The field of endothelial progenitor cell (EPC) biology is approaching a decade and a half since generating substantial promise as a potential reparative cell therapy for a spectrum of human clinical disorders. With considerable speed, scientists and clinicians moved from basic studies of isolating and characterizing the biologic properties of EPCs, to pre-clinical EPC treatment studies in rodent model systems of cardiovascular disease, and to the delivery of EPC or marrow-derived cells into selected human subjects (reviewed in [1, 2]). In some disease settings, patient benefits from the infused EPC or marrow-derived cells have been documented, though perhaps not to the extent hoped for or predicted by the results in the preclinical animal model systems [3]. In most human clinical trials, autologous bone marrow mononuclear cells have been infused into patients with cardiovascular disease in an attempt to provide certain presumed EPC subsets to ameliorate ischemic insult [4-7]. To provide some perspective on the advances to date, this review will begin by highlighting the major clarifications in EPC definitions that have occurred over the past 10 years and how this information has instructed changes to the selection of bone marrow subsets for patient use [8-11]. To bring perspective to the increased appreciation of the roles played by hematopoietic cells in vascular repair, we will provide an overview of the hematopoietic hierarchy in mouse and man and identify those subsets that display proangiogenic activities. This perspective may help the reader consider crucial milestones in the discovery and application of HSC and progenitor cells as a cell therapeutic that have not been well explored in the EPC field. The review will conclude with a list of issues that need to be addressed to permit a more quantitative and definable nomenclature for the cells that participate in vascular endothelial repair and replacement. This review will not address the role of those EPC comprised of resident or circulating endothelial cells or endothelial colony forming cells involved in vascular repair and regeneration under normal or pathological conditions (reviewed in [8-15]).

## Introduction

The field of endothelial progenitor cell (EPC) biology is approaching a decade and a half since generating substantial promise as a potential reparative cell therapy for a spectrum of human clinical disorders. With considerable speed, scientists and clinicians moved from basic studies of isolating and characterizing the biologic properties of EPCs, to pre-clinical EPC treatment studies in rodent model systems of cardiovascular disease, and to the delivery of EPC or marrow-derived cells into selected human subjects (reviewed in [1, 2]). In some disease settings, patient benefits from the infused EPC or marrow-derived cells have been documented, though perhaps not to the extent hoped for or predicted by the results in the preclinical animal model systems [3]. In most human clinical trials, autologous bone marrow mononuclear cells have been infused into patients with cardiovascular disease in an attempt

Mervin C. Yoder, MD Richard and Pauline Klingler Professor of Pediatrics Director, Herman B Wells Center for Pediatric Research Vice-Chair for Basic Research, Department of Pediatrics Indiana University School of Medicine 1044 W. Walnut Street, R4-W125 Indianapolis, IN 46202 USA Phone: 317-274-4738 Fax: 317-274-8679 myoder@iupui.edu.

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to provide certain presumed EPC subsets to ameliorate ischemic insult [4-7]. To provide some perspective on the advances to date, this review will begin by highlighting the major clarifications in EPC definitions that have occurred over the past 10 years and how this information has instructed changes to the selection of bone marrow subsets for patient use [8-11]. To bring perspective to the increased appreciation of the roles played by hematopoietic cells in vascular repair, we will provide an overview of the hematopoietic hierarchy in mouse and man and identify those subsets that display proangiogenic activities. This perspective may help the reader consider crucial milestones in the discovery and application of HSC and progenitor cells as a cell therapeutic that have not been well explored in the EPC field. The review will conclude with a list of issues that need to be addressed to permit a more quantitative and definable nomenclature for the cells that participate in vascular endothelial repair and replacement. This review will not address the role of those EPC comprised of resident or circulating endothelial cells or endothelial colony forming cells involved in vascular repair and regeneration under normal or pathological conditions (reviewed in [8-15]).

## Clarifications in the definition of endothelial progenitor cells

As originally identified by Asahara and co-workers in 1997 [16], circulating blood cells derived from the bone marrow could migrate to the site of vascular injury and promote recovery of blood flow via formation of vessels in a process called postnatal vascularization. These blood cells which could also be demonstrated to upregulate numerous cell surface markers thought to be endothelial specific in vitro, were identified as endothelial progenitor cells (EPC). However, some of the earliest cell surface markers used to identify the putative EPC included markers that were co-expressed by endothelial and hematopoietic cells such as CD34, CD117, CD133, CD105, CD144, CD184, CD309, acetylated low density lipoprotein, and various plant lectins [16-20]. Work over the past decade has clarified that most of the cells identified as EPC have been shown to represent hematopoietic cells of various stages of differentiation [8, 21] that could be shown to play largely paracrine roles in providing vascular reparative functions in mice and man and in fact, EPC have recently been re-classified as hematopoietic or non-hematopoietic [9]. The reports of bone marrow derived cells that integrate as EPC into injured vasculature or tumor vessels [22-26] has been countered by other studies using more specific transgenic reporter systems and confocal microscopy to indicate that donor putative EPC if they are present in vessels, reside in a peri-endothelial location [27-31]. While numerous studies have attempted to utilize autologous bone marrow cells as a cell therapy to treat various forms of cardiovascular disease or peripheral arterial disease, only very modest improvements in patient outcomes have been reported in meta-analyses and none of the therapies have become standard of care [6, 7]. In fact, increasing evidence suggests that many cardiovascular disorders impair the function of the marrow-derived cells [32, 33] and thus, one would need to find a method to repair the function of the autologous cells before one could re-infuse them as a vascular reparative therapy [1]. Thus, additional work is required to learn more about the roles of hematopoietic cells in vascular repair and the mechanisms through which they promote vascular endothelial cell survival, proliferation, migration, and angiogenesis.

## Introduction to hematopoietic cells as a clinical blood cell therapy

More than 50,000 HSC transplants are performed world-wide annually, making this one of the most common forms of tissue or organ transplantation. Successful translation of murine bone marrow transplantation techniques into human subjects has occurred over more than 5 decades of intense research and has been marked by significant changes in terminology and definitions over time as new methodologies and assays have evolved in the field. Much of

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the earliest work in the field rose out of basic research that attempted to understand the consequences of total body irradiation on host survival.

## Murine HSC identification and the stem cell theory of hematopoiesis

Whole-body irradiation of animals was noted to depress hematopoiesis and cause life-threatening deficiencies in all circulating blood cells (pancytopenia) in exposed mice. However, shielding the spleen of the experimental animal from the radiation beam (or transplanting a normal spleen into an irradiated animal) resulted in recovery from an otherwise lethal radiation dose [34, 35]. In 1956, Ford et al. [36] provided definitive evidence that donor infused murine marrow cells gave rise to all the blood cells of the irradiated host. Soon after, Till and McCulloch [37] provided evidence that single multipotent hematopoietic progenitor cells could be identified *in vivo* by injecting donor marrow cells into a lethally irradiated recipient animal and examining the recipient spleen for hematopoietic colonies 8-12 days later; each cell clone forming a colony was called a colony-forming unit in spleen cell (CFU-S) [38]. This assay provided the first compelling evidence that hematopoietic cells were clonally derived, although CFU-S were later found not to represent a HSC assay [39].

Pluznik and Sachs [40] and Bradley and Metcalf [41] reported that murine hematopoietic cells could be quantified in vitro in clonal assays of hematopoietic progenitor cell differentiation. Furthermore, these investigators reported that each myeloid colony developing *in vitro* arose from a single precursor cell called the colony-forming unit in culture or CFU-C. Later, Axelrad and co-workers [42] succeeded in demonstrating that red blood cell colonies were also clonally derived. These assays subsequently proved useful in isolating CFU specific hematopoietic growth factors [43, 44] and proving that hematopoietic progenitor subsets segregate into specific lineage producing precursors [45]. The specific hematopoietic progenitor subsets are described in several recent reviews of the topic [46-48].

Murine HSC continue to be definable only as cells that proliferate and differentiate into all lineages of circulating peripheral blood cells for more than 4 months after transplantation into recipient animals and cells that possess the capacity for self-renew *in vivo* (testable by performing secondary or tertiary transplantation)[49]. The frequency of human and murine hematopoietic stem cells has been estimated to be $1/10^5$-$10^7$ bone marrow cells. [50-55] The rarity of this population presented considerable impediments to HSC isolation until the later 1980's and 1990's with the availability of an array of monoclonal antibodies, fluorescence activated cell sorters (FACS), and a variety of congenic mouse strains.

Murine HSC and progenitor cells have been enriched (negative selection) using FACS by selecting bone marrow cells that fail to express cell surface antigens ($lin^-$) typically displayed by mature B and T lymphocytes, neutrophils, macrophages, natural killer cells, and red blood cells [56]. Three commonly used phenotypic markers expressed by murine HSC (positive selection) include stem cell antigen-1 (Sca-1), c-kit, and Thy-1 [57]. Sca-1 is a cell surface molecule that is required for normal stem cell self-renewal and progenitor cell proliferation and lineage maturation in mice [58.] C-kit is a cell surface receptor tyrosine kinase that is necessary for murine HSC survival [59] and *in utero* murine embryo survival [60]. Thy-1 is a cell surface molecule expressed by stem and lymphoid cells [61]. CD34 is a cell surface sialomucin expressed by hematopoietic and endothelial cells [62]. CD34 is expressed on proliferating adult marrow stem cells but may be down-regulated and not detectable on the surface of quiescent marrow stem cells [63, 64]. Other antigens or enzymes that have been utilized to enrich (positive or negative) murine bone marrow stem cells include AC133, CD31, CD38, CD41, CD43, CD105, CD150, aldehyde dehydrogenase, and leukocyte function antigen-1 (LFA-1) (Figure 1) [46]. HSC may also be enriched from

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other marrow cells by the fact that quiescent stem cells retain the least amount of a DNA-binding dye (Hoechst 33342) and appear as a "side-population" distinguishable (by cell sorting) from other hematopoietic cells [47]. Use of several different combinations of these cell surface or metabolic markers has permitted enrichment of stem cells to homogeneity as evidenced by long-term repopulation of all blood lineages in recipient mice from a single transplanted cell [48]. It is important to stress, that the cell surface phenotype of most populations of cells fails to achieve HSC purity; most populations only enrich for those cells that functionally demonstrate multi-lineage repopulating ability upon transplantation. Therefore, one cannot quantitate the presence of a HSC by counting cells with a particular phenotype, but must transplant and enumerate the in vivo competitive repopulating ability of limiting dilutions of test cells in host animals to confirm HSC numbers that may be present in that population.

The importance of functional HSC and progenitor cell (HSPC) niches in the murine bone marrow has been well described [65, 66]. While some HSPC are circulating throughout the systemic blood and lymphatic systems at homeostasis [67, 68], most HSC are maintained in quiescence within a niche in close approximation to the endosteal surface of bone and also near blood vessels that provide key survival factors [59, 65, 69]. Research over the last decade has led to the use of several growth factors and chemokine receptor antagonists to mobilize the HSPC into the systemic circulation and allow for collection of functional HSPC for stem cell transplantation [70-72]. Even if infused at high numbers, human and murine HSC will not substantially engraft in the host unless the host has received some form of bone marrow myeloablative conditioning to eradicate endogenous HSPC within their niches. Advancements in minimizing the bone marrow conditioning has permitting transplantation of HSC with minimal risks to the host and may provide new strategies for human stem cell transplantation for patients with genetic hematopoietic disorders (ie. sickle cell disease, thalassemia, Fanconi anemia) [73-75].

## Human HSPC identification and assays for detection

Human HSPC have been isolated using monoclonal antibodies and flow cytometric cell sorting. (reviewed in [46, 76, 77]. Putative human HSC are enriched in and have been isolated from bone marrow, umbilical cord blood, fetal liver, and mobilized peripheral blood as cells expressing CD34, Thy-1, AC133, and c-kit, but not CD45RA, CD38, or mature blood cell lineage markers ($lin^-$) (Figure 1). While CFC assays have proven useful in identifying the functional properties of flow cytometric sorted human progenitor cells, *in vivo* testing of selected HSPC populations in human subjects has not been possible for ethical reasons. Nonetheless, $CD34^+$ selected hematopoietic cells have been proven to support patients long-term after bone marrow transplantation [77] and the dose of CD34+ cells collected for transplantation can be useful in predicting the time for donor cell repopulation of the neutrophil and platelet counts in the patients [78, 79].

In an attempt to develop an *in vivo* system for detection and quantitation of human HSC, several groups have developed xenotransplantation murine models. Non-obese diabetic (NOD) mice bred with severe combined immunodeficient (*scid*) mice result in NOD/*scid* mice that accept human hematopoietic grafts [80] and this model has permitted calculation of the frequency of human repopulating cells present in a donor sample [81]. Similarly, *scid* mice can be implanted with fragments of human fetal thymus and bone that will survive and function as a hematopoietic microenvironment [82]; sublethal irradiation of these *scid*-hu mice permits engraftment of intravenously administered human cells [83]. NOD-*scid*-$IL2R\gamma c^{-/-}$ female mice, lacking all endogenous B, T, and NK cells, display the highest levels of human donor cell repopulation with an 11-fold greater level of reconstitution of all blood cell lineages upon transplantation of limiting dilutions of HSC than in male littermates and many fold greater than NOD/*scid* hosts [84]. Human cord blood

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$lin^{-}CD34^{+}CD38^{-}CD45RA^{-}Thy\text{-}1^{+}Rhodamine^{lo}$ $CD49f^{+}$ cells infused at a single cell level were recently reported to give rise to multi-lineage human blood cell reconstitution in female NOD-*scid-IL2Rγc*$^{-/-}$ mice in primary and secondary transplants and at marrow and tissue sites other than the site of injection (suggesting systemic migration and engraftment) [85]. This remarkable finding suggests that progress may have been made in optimizing a murine xenogeneic transplant system that is capable of identifying the engraftment and multi-lineage differentiation of even a single injected human HSC (using limiting dilution infusions of donor cells), although this human HSC subset has not yet been validated in human subjects.

### Summation of advances in HSC identification and understanding that have enhanced HSC use as a blood cell therapy

Progress over the past 5 decades has laid a foundation for quantitative functional analysis of HSPC in mouse and man. Once it was clear that the source of HSC was greatest in adult bone marrow and that infusion of donor marrow cells could rescue a lethally irradiated host, tools were available to progressively define the hematopoietic hierarchy. Wide availability of specific hematopoietic growth factors, clonal in vitro colony assays, monoclonal antibodies and flow cytometry sorting techniques permitted segregation of the hematopoietic hierarchy into discrete subsets of cells with characteristic cell surface markers and functional stem cell and progenitor stages with the capacity to give rise to specific lineages of cells. Translation of many of the concepts from the murine to human system has permitted establishment of a human hematopoietic hierarchy, though there are numerous differences in the specific markers used to define HSPC. In fact, there is not a single cell surface antigen that is expressed on the long term HSC in the mouse that is also present on the human long-term engrafting HSC. Given the availability of clonal in vitro hematopoietic progenitor assays and immunodeficient mouse models for human HSC engraftment, human HSPC can be functionally quantified following infusion and the differentiation potential of the cells followed over several months posttransplant. The availability of these tools has permitted practical study of the optimal reagents to mobilize human HSPC, cryopreserve the cells, and thaw adequate numbers of HSPC to engraft patients [84, 85].

## Overview of the proangiogenic roles played by hematopoietic cells

### HSC display proangiogenic activity

In the murine system, isolation of bone marrow populations enriched in HSC and transplantation of these cells into syngeneic irradiated recipient mice not only has been shown to regenerate all the blood cell lineages, but in some reports, donor HSC progeny upregulate endothelial cell antigens and take up residence as endothelial cells within numerous blood vessels in multiple tissues [86, 87]. HSC also readily engraft and are recruited to sites of endothelial injury following experimental retinal ischemia [87-90]. In some reports, the donor HSC appeared to form the endothelial lined vessels in the recovering retina [87, 89] while in other reports the HSC appeared to directly recruit the ingrowth of viable retinal endothelial cells or enhance retinal endothelial cell survival and regeneration via differentiation into microglia and secretion of growth promoting molecules for associated astrocytes [88, 90, 91]. Differences in the contributions of transplanted mouse HSC to endothelial lined vessels may be due to differences in the phenotypic subsets of cells used to enrich for the donor HSC, type of vascular injury, time of analysis (examination within the first two weeks after injury is more likely to show HSC contributions while examination months later is less likely to show HSC-derived resident endothelial cells), and type of transgenic reporter mouse used to identify the donor HSC [29, 92-94]. These and other variables are also relevant to the controversy as to why bone marrow cells may or may not integrate as endothelial cells in experimental tumor implants [95, 96]. Whether murine

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HSC actually change their fate to become endothelial cells may not yet be definitively resolved, however, it is clear that HSC can serve paracrine roles to enhance the process of neovascularization [97].

In the human system, transplantable HSC are typically collected as a mobilized peripheral blood fraction with or without further CD34 selection before administration to the transplant recipient. Nearly all of these CD34 expressing cells are also $KDR^+$ and $CD45^+$ but only some of the cells express AC133 [98-100]. As noted above, $CD34^+AC133^+$ cells engraft in immunodeficient mice and are enriched in hematopoietic CFU-C activity [99, 101, 102].Thus, the choice to use CD34 and AC133 and/or KDR as markers to identify circulating EPC [16, 103] was essentially an exercise in determining whether this population of cells known to contain human HSPC also displayed proangiogenic activity. Many reviews have highlighted the ability of human CD34 cells (with or without further enrichment into those subsets expressing AC133 and KDR) to play important roles as biomarkers for coronary disease, peripheral arterial disease, and tumor angiogenesis, as well as, potential therapy to treat ischemic cardiac and limb disorders (reviewed in [1, 8, 9, 13, 15]). Thus, there is ample evidence to define human HSPC as proangiogenic cells. Reports of transplanted human HSC contributing to cells of the endothelial lineage in vivo are limited [104, 105]. Several recent papers have highlighted novel methods of polychromatic flow cytometry that permit analysis of human $CD14^-CD235a^-$ $CD31^+CD45^+CD34^+$ cells into one subset expressing AC133 and another subset not expressing this antigen (Figure 2). Remarkably, the $CD14^-CD235a^-CD31^+CD45^+CD34^+AC133^+$ cells display proangiogenic activity while the $CD14^-CD235a^-CD31^+CD45^+CD34^+AC133^-$ cells do not display angiogenic activity [99]. Of interest, patients with peripheral arterial disease who suffer from deficient angiogenic activity displayed a diminished ratio of the proangiogenic to the nonangiogenic circulating hematopoietic subsets [99] (Figure 3), while pediatric patients bearing solid tumors displayed a significantly higher ratio of proangiogenic to nonangiogenic hematopoietic cells compared to age-matched control subjects but, the patient proangiogenic to nonangiogenic ratio normalized to age-matched control levels after tumor treatment [106]. These data are of interest, as they suggest that patient $CD34^+$ cells are heterogenous in proangiogenic activity and that the $AC133^+$ subpopulation of the $CD34^+$ cells may vary in frequency with human disease states. Would this also suggest that for optimal therapeutic benefits, the $CD34^+$ cells should be further fractionated to enrich for proangiogenic cell effects [1]?

## Myeloid progenitor cells display proangiogenic activity

As reviewed above, use of specific monoclonal antibodies and cell sorting permits isolation of subsets of murine bone marrow cells that are enriched for HSC or for various individual lineage progenitor stages (devoid of HSC activity) that can be validated using functional assays [46]. Wara and colleagues [107] recently reported significant differences in the ability of murine common myeloid progenitor (CMP) and granulocyte macrophage progenitor (GMP) cells to differentiate into proangiogenic cells (PAC) compared to murine HSC, megakaryocyte erythroid progenitor (MEP), or common lymphoid progenitor (CLP) cells. The murine CMP and GMP displayed significantly more upregulation of the vascular endothelial growth factor 2 receptor (VEGFR2) and a panel of other endothelial cell surface antigens upon in vitro culture, greater proliferation and migration on fibronectin, greater stimulation of umbilical vein endothelial cells to form capillary-like structures and enhanced adhesion to those endothelial tubes, and greater rescue of blood flow restitution in mice with experimentally induced hindlimb ischemic injury. Further analysis revealed a role for Kruppel-like factor 10 (KLF10) in promoting PAC generation from murine CMP and GMP. KLF10 expression in PAC was regulated by transforming growth factor-beta 1(TGF-β1) and upon TBF-β1 stimulation, KLF10 directly bound to a consensus nucleotide sequence within

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the VEGFR2 promoter to enhance VEGFR2 expression in PAC. Stimulated PAC displayed significantly greater adhesion, migration, and augmentation of recovery of blood flow in limbs of mice with experimentally induced ischemia compared to PAC generated from KLF10 deficient mice [108]. The significant recovery of the injured vessels and microvascular bed in the instrumented mice from the wild-type (WT) PAC was associated with 4% co-localization of PAC with $CD31^+$ capillaries and no co-localization was seen in animals that received PAC derived from the KFL10 deficient mice. Thus, the benefit from infused PAC in the animals with hindlimb ischemia was largely related to paracrine effects of the KLF10 expressing CMP- and GMP-derived PAC. Of interest, PAC isolated from patients with peripheral arterial disease displayed lower levels of KLF10 expression similar to diminished KLF10 expression in mice undergoing hindlimb arterial ligation [108]. These largely paracrine effects displayed by the KLF10 expressing PAC are reminiscent of the vast number of publications reporting beneficial effects of murine bone marrow-derived EPC in various animal models of cardiovascular or skeletal muscle ischemic injury (reviewed in [3, 8, 9, 17, 20, 23]). It will be interesting to compare potency of the murine PAC derived from the sorted CMP and GMP compared to unsorted PAC derived in published “EPC” protocols to determine whether there are any differences in proangiogenic effects derived from these cells. This may be an important point since PAC tend to be non-proliferative cells while the CMP and GMP can be dramatically expanded in vitro and may serve as a method to generate far greater numbers of PAC than currently possible [9].

A detailed comparison of PAC generation and function from human HSC and progenitor subsets has not been reported. Wara and colleagues [107] have reported that human umbilical cord blood and adult bone marrow CMP and GMP gave rise to significantly more VEGFR2 expressing progeny than the HSC enriched subset, but no analyses of CMP or GMP-derived PAC progeny for in vitro or in vivo angiogenic activities were reported. Since human HSC and progenitor cell ex vivo expansion has now been reported by several groups [109-113], it would appear that focused analysis of the various hematopoietic subsets to determine the most optimal stem or progenitor cell precursors of the PAC would be appropriate in moving toward the cell numbers required for clinical applications.

## Monocyte-macrophages display proangiogenic activity

Much has been published on the role of monocytes and macrophages in regulating the process of angiogenesis. A simple PubMed keyword search (12/09/12) for monocytes and angiogenesis resulted in 733 retrievals and a keyword search for macrophages and angiogenesis yielded 2134 results. A sampling of recent work reveals that different monocyte subsets appear more readily specified to proangiogenic macrophage fates [114-116], are key components of the tumor microenvironment [117, 118], can be targeted for specific anti-tumor angiogenic responses [119-121], employ both Wnt [122] and Notch [123-125] signaling pathways to interact with sprouting endothelial cells, and play key roles in some central nervous system pathophysiologic angiogenic processes [126]. It is now well accepted that many of the early outgrowth EPC isolation protocols enrich for monocytes and macrophages which under the conditions of the tissue culture medium and growth factor additives, upregulate endothelial cell surface proteins and downregulate typical activate macrophage antigens [127]. However, these monocytes and macrophages induced to display putative EPC characteristics never fully achieve the epigenetic changes in gene expression that permit the cells to display full endothelial cell gene expression patterns or functions [128] and therefore, putative EPC can be induced to display macrophage functions with standard forms of provocation [129, 130]. Thus, there is ample evidence that both mouse and human monocytes and macrophages display specific functions that promote angiogenesis during development, homeostasis, and disease. The capacity of monocytes and

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macrophages to upregulate endothelial antigens and appear to “look like EPC” may be a function of their microenvironment in vitro and in vivo [131-134].

## Summation and recommendations

The hematopoietic hierarchy is well described in mice and man. Numerous in vitro and in vivo assays have been developed to specifically define the many discrete steps in differentiation from the HSC to the eight major derivative lineages. The detailed analysis of murine HSC homing, engraftment, differentiation, and self-renewal following transplantation have permitted the translation of HSC transplantation into human clinical practice despite obvious differences in some of the specifics for each of these hematopoietic hierarchies that differ between mouse and man. The field continues to move forward with advances in methods to mobilize cells, enhance HSC expansion, and minimize host conditioning to broaden the patient populations amenable to this therapy.

Unlike the hematopoietic system, the field of EPC biology continues to suffer from a lack of key research tools and concepts that include:

1. lack of recognition that most putative EPC are simply proangiogenic hematopoietic subsets that may be better studied through use of already established hematopoietic hierarchical subset phenotypes rather than attempting to define novel markers,
2. no discrete in vivo quantitative gold standard assay for any putative EPC population to be measured by (difficult to measure the quantitative end points of paracrine effects of an infused population since multiple molecules may be producing numerous effects concomitantly),
3. lack of detailed pathway analysis for the molecules through which the various hematopoietic subsets (putative EPC) interact with the host vascular endothelial cells (it is not clear whether all the cells work through similar pathways or if different putative EPC subsets use different pathways),
4. inadequate ability to measure the angiogenic status of the host vascular endothelium which represents the responding population to the infused putative EPC (cannot isolate the cells and infuse into a host to measure endothelial repopulation or regeneration and thus cannot quantitatively measure host responder status which may vary by age, sex, tissue, treatment, or disease),
5. inadequate ability to define the proangiogenic status of circulating putative EPC in patients with suspected angiogenic deficiency state or to sense the presence of excessive angiogenesis that may be occurring in a patient with a malignancy,
6. incomplete information to confirm a hematopoietic to endothelial lineage switch as is suspected to occur in instances when putative EPC are thought to integrate as qualified participants to regenerate vascular endothelial monolayer properties (need for unique lineage specific reporter systems to permit fate mapping studies to be performed in mice to permit identification of a hematopoietic to endothelial transition).

The field of EPC study is relatively new and numerous advancements may facilitate better understanding of how the hematopoietic and vascular endothelial systems interact to produce, maintain, and repair functional vessels during human development, throughout our lifespan, and under the duress of a host of pathophysiologic stressors that culminate in cardiovascular disease.

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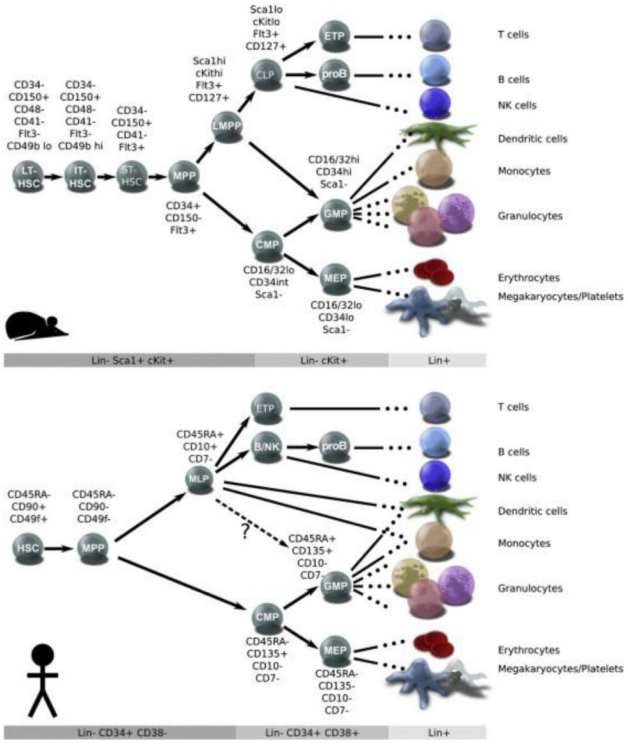


**Figure 1.**

Models of Lineage Determination in Mouse and Human Hematopoietic Hierarchies. The major classes of stem and progenitor cells are defined by cell surface phenotypes, which are listed next to each population and in the gray bars below each schematic. Terminally differentiated cells are shown on the right, and inferred lineage relationships are depicted with arrows. In mice (A), HSCs can be separated into long-term (LT), intermediate-term (IT), and short-term (ST) classes based on the duration of repopulation. In humans (B), HSCs are defined by the expression of CD49f and other markers, but their heterogeneity has not been investigated. In mice, differentiation of HSCs gives rise to transiently engrafting multipotent progenitors (MPPs), and a series of immature lymphoid-biased progenitors (such as LMPPs) that undergo gradual lymphoid specification. In humans, MPPs can be identified by the loss of CD49f expression; however, only one population of immature lymphoid progenitors (MLPs) has been described. Both mice and humans have well-defined populations of common myeloid progenitors (CMPs), granulocyte macrophage progenitors (GMPs), and megakaryocyte erythroid progenitors (MEPs). Lin: cocktail containing cell surface markers for all terminally differentiated populations (B cell; T cell; NK; dendritic cell, monocyte, granulocyte, megakaryocyte, and erythrocyte). Modified from Doulatov S, et al. Hematopoiesis: A human perspective. Cell Stem Cell 10:124, 2012.

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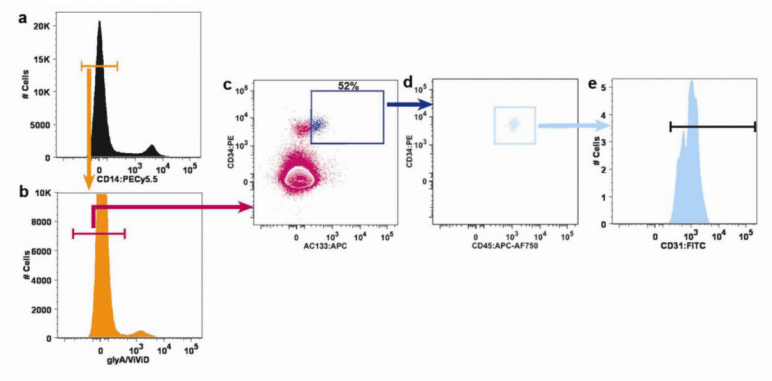


**Figure 2.**

Frequency analysis of $CD31^{+}CD34^{bright}CD45^{dim}AC133^{+}$ cells. Uncompensated raw data was collected on a digital flow cytometer, compensated after acquisition by using FlowJo software, and visualized in plots with bi-exponential scaling. Mononuclear cells were identified on a forward versus side scatter plot and then $CD14^{-}$ cells (orange gate in a) were identified. All $CD14^{-}$ cells were then assessed for viability and glycophorin A (CD235a) expression (b). $CD14^{-}$ $glyA^{-}ViViD^{-}$ (viable cells do not retain this molecule) cells (pink gate in b) were subgated onto a bivariant antigen plot to identify $CD14^{-}glyA^{-}ViViD^{-}CD34^{bright}AC133^{+}$ cells (dark blue gate in c). Viable $CD14^{-}glyA^{-}CD34^{bright}AC133^{+}$ cells are further subgated to identify the $CD45^{dim}$ subpopulation (light blue gate in d). CD31 expression on the resulting viable cells was confirmed on a CD31 histogram (e). Figure modified from Estes ML, et al. Application of polychromatic flow cytometry to identify novel subsets of circulating cells with angiogenic potential. Cytometry Part A 77A:835, 2010.

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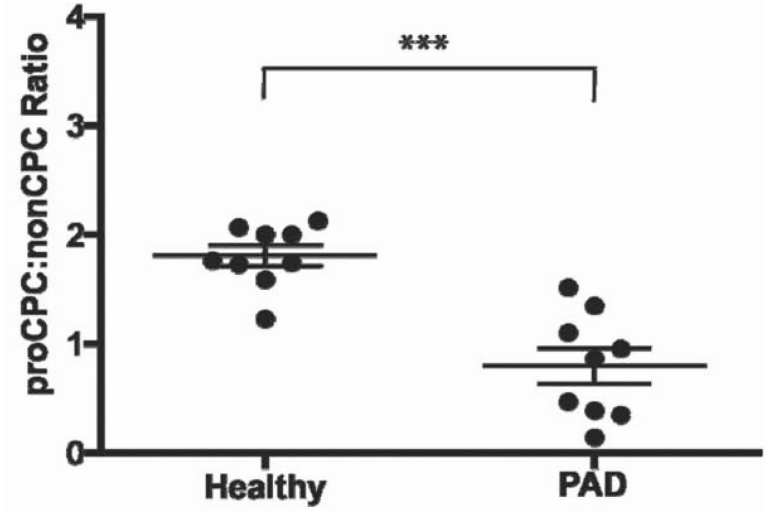


**Figure 3.**

Ratio of circulating progenitor subsets denotes disease state in peripheral arterial disease patients. The ratio of proangiogenic ($CD31^{+}CD34^{bright}CD45^{dim}AC133^{+}$) to nonangiogenic ($CD31^{+}CD34^{bright}CD45^{dim}AC133^{-}$) cells is depicted and patients with peripheral arterial disease (PAD) display a significant decrease when compared to age and gender matched control subjects. Figure modified from Estes ML, et al. Application of polychromatic flow cytometry to identify novel subsets of circulating cells with angiogenic potential. Cytometry Part A 77A:837, 2010.