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Endothelial progenitor cells: Quo Vadis?

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Abstract

The term endothelial progenitor cell (EPC) was coined to refer to circulating cells that displayed the ability to display cell surface antigens similar to endothelial cells in vitro, to circulate and lodge in areas of ischemia or vascular injury, and to facilitate the repair of damaged blood vessels or augment development of new vessels as needed by a tissue. More than 10 years after the first report, the term EPC is used to refer to a host of circulating cells that display some or all of the qualities indicated above, however, essentially all of the cells are now known to be members of the hematopoietic lineage. The exception is a rare viable circulating endothelial cell with clonal proliferative potential that displays the ability to spontaneously form inosculating human blood vessels upon implantation into immunodeficient murine host tissues. This paper will review the current lineage relationships among all the cells called EPC and will propose that the term EPC be retired and that each of the circulating cell subsets be referred to according to the terms already existent for each subset. This article is part of a special issue entitled, "Cardiovascular Stem Cells Revisited".

Keywords

Endothelial progenitor cell (EPC); Endothelial cells

1. Introduction

The continuous endothelial lining of the blood vascular system has generally been reported to be a slow or non-replicative population of cells in the adult organism [1]. However, some evidence exists to support the role of a limited local endothelial cell replicative response to an endothelial denudation injury, leading to rapid endothelial restitution [2–5]. In areas of large injury, multiple circulating blood elements (platelets, neutrophils, monocytes, and other blood cells) are recruited to the exposed basement membrane in the area of injury with a more delayed recruitment of resident endothelium to reestablish vascular integrity and blood flow [6–9] (Fig. 1). In 1997, circulating blood cells displaying a variety of cell surface proteins thought to be endothelial specific upon in vitro culture and the ability to localize and promote vascular regeneration at sites of ischemia upon transplantation, were identified and called endothelial progenitor cells (EPC) [10]. Since the EPC were derived from the systemic circulation, the role of these cells in promoting new blood vessel formation was considered to be an example of postnatal vasculogenesis. Subsequently, many studies performed in a variety of animal model systems appeared to support the contention that bone marrow derived cells played an important role in vascular repair and regeneration and could facilitate tissue recovery after experimental ischemic injuries [11–14]. Indeed, recruitment

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of bone marrow-derived EPC was being promoted as a primary mechanism for endothelial replacement in areas of vascular damage. In parallel, numerous studies conducted in human subjects with a host of cardiovascular, metabolic, malignant, autoimmune, inflammatory, or other disorders were reported to display changes in the circulating concentration of EPC that correlated with the severity or risk of adverse outcome for that particular disease state [15–18]. The ability of human EPC to rescue diminished blood flow in preclinical experimental animal models combined with the known deficiency of circulating EPC in some patients with cardiovascular disorders provided the rationale to initiate clinical trials of the infusion of various bone marrow or EPC populations into human subjects with myocardial or systemic vascular disorders. The results of these studies have largely proven the infusion of marrow cells or EPC to be safe and beneficial in certain circumstances, though the effects on organ recovery in human subjects have been less robust than the results obtained in the preclinical rodent studies [19–21]. Thus, questions have arisen as to whether the preclinical rodent models are not as predictive for human cardiovascular disorders as might have been speculated or whether the EPC examined in the preclinical rodent models do not function in similar ways upon infusion into human subjects. It has become clear over the past 3–4 years that a whole host of different types of blood cells and endothelial cells are somewhat ambiguously being included within the single term EPC [22,23]. Thus, at present, the term EPC fails to refer to a distinct or unique cell type with definable characteristics. The most extreme differences in cellular functional subsets among those cells called EPC are most apparent in studies conducted with human blood cells. This review will attempt to examine the commonly used methods to identify putative EPC in human subjects and present the most recent data that provides clear distinction as to what types of cells may be isolated within each of the specific methods. Given the analysis of these results, we will make recommendations as to the most accurate nomenclature that may be applied to the cells involved in angiogenesis and postnatal vasculogenesis.

2. Isolating putative EPC by cell adhesion to fibronectin-coated dishes with display of certain lectin and lipoprotein binding properties

In the original paper defining a progenitor endothelial cell, Asahara et al [10] reported that 15.7% of adult peripheral blood cells expressing CD34 could be isolated using immunomagnetic beads and culture of this population on fibronectin-coated tissue culture wells led to the emergence of spindle-shaped cells within 3 days. At 7 days of culture, 9–71% of the attached cells expressed CD45, CD34, CD31, Flk-1 (vascular endothelial growth factor 2 receptor), Tie-2, or E-selectin (some cell surface markers thought to identify cells of the endothelial lineage). In this same paper, the 7-day attached cultured cells also displayed the ability to take up the lectin *Ulex Europeaus* agglutinin-1 (UEA-1) and fluorescence labeled acetylated low density lipoprotein (acLDL). In other studies performed in that seminal paper, the adherent cell population recovered from the cultures expressing the above antigens was noted to co-localize with capillary vessels within the ischemic tissues of experimentally instrumented rabbits and mice and was associated with improved recovery of blood flow to ischemic limbs in these experimental animals. The ability of these cultured cells to enhance blood flow recovery, to co-localize with new vessels, and to display endothelial-like antigens in vitro gave confidence to the authors to proclaim this population as circulating progenitor cells for the endothelial lineage.

In a series of subsequent papers, EPC were defined as those cells that attached to fibronectin-coated culture dishes within 4–7 days and displayed the ability to take up UEA-1 and acLDL [24–26]. Use of this definition permitted the authors to isolate low-density peripheral blood mononuclear cells or bone marrow cells from rodents, rabbits, or human subjects and to compare their properties to rescue blood flow in ischemic states in animals with induced vascular injuries. Over time, this approach to an EPC definition has been

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utilized extensively in both human and rodent studies and has been translated to human clinical studies where the concentration of the adherent putative EPC circulating in the peripheral blood has been correlated with various clinical states [27–30].

If UEA-1 and acLDL uptake were unique to EPC and no other cells in this assay format, then this approach might lead to a viable definition. However, UEA-1, which recognizes L-fucosylated molecules on the surface of mammalian cells, is not restricted to binding to endothelial cells, but binds to many types of epithelial cells (transformed and non-transformed) and various hematopoietic cells including platelets [31–39]. This latter point is critical as Prokopi et al. [40] have recently reported that platelets are a common contaminant of peripheral blood mononuclear cells prepared for plating in the EPC adherence assay. The platelets were noted to readily disintegrate into microparticles and fuse with the adherent heterogenous mononuclear cells attached to the fibronectin-coated dishes. Of interest, many of the attached mononuclear cells displayed a variety of cell surface proteins that were contributed by the platelets (the attached mononuclear cells did not express the mRNA for the proteins being expressed on the cell membrane). Thus, the presence of any contaminating platelets in this adherence assay for EPC could lead to UEA-1 binding on the mononuclear cells, providing false-positive results [40]. In addition, peripheral blood monocytes are known to be highly enriched upon plating on fibronectin-coated dishes [41] and would be expected to display many proteins, including the scavenger receptors that bind acLDL, that are also expressed by endothelial cells (in the presence of the growth factors and serum used in the “EPC” assay culture medium) [42,43]. In a recent extensive mRNA expression profiling analysis of EPC derived from peripheral blood mononuclear cells, Medina et al. [44] reported that the adherent EPC displayed a pattern of mRNA expression that was enriched in hematopoietic specific pathways, particularly those that were immune related or inflammatory. In fact, proteomic comparison between the EPC and monocytes indicated that 77% of the proteins isolated by 2-D gels from EPC are also expressed by monocytes. In sum, neither acLDL nor UEA-1 binding are restricted in binding to a cell that could be called an EPC. Furthermore, platelets (or platelet microparticles) and monocytes would be expected to be bound to the fibronectin-coated dishes in this assay and would have to be depleted from the mononuclear cells under evaluation prior to defining any putative adherent cell as an EPC. Indeed, Prokopi et al. [40] reported that the highest correlation between any type of circulating blood cell and the concentration of in vitro adherent $UEA\text{-}1^{+}acLDL^{+}$ putative EPC was noted for monocytes and platelets in a large population based study of >500 human subjects. These findings call for significant scrutiny to be applied to papers in which putative EPC are being identified using UEA-1 and ac-LDL binding to adherent human mononuclear cells as sole definitive criteria.

3. Isolating putative EPC using the cell surface phenotype of $CD34^{+}CD133^{+}KDR^{+}$

As noted in the first EPC description, both CD34 and vascular endothelial growth factor 2 receptor (KDR [human subjects] or Flk-1 [rodents]) expression on human peripheral blood mononuclear cells could be used to enrich for the putative EPC phenotype and function [10]. Other investigators extended these observations to suggest that a circulating population of endothelial precursors could be identified in human subjects by the phenotype $CD34^{+}AC133^{+}KDR^{+}$ [45]. The authors noted that mature human umbilical vein endothelial cells did not express AC133 and thus, the ability of the $AC133^{+}KDR^{+}$ cells to give rise to adherent cells that bound acLDL and expressed KDR but not AC133 (a putative mature endothelial phenotype) confirmed the triple positive cells as an EPC. Further analysis of the cell surface proteins expressed on this subset included identification of CXCR4, CD31, CD13, and not CD14 or CD15 [45]. Surprisingly, the authors did not assay for expression of CD45, the common leukocyte antigen that is expressed on most nucleated circulating blood

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cells. Nonetheless, the cell surface phenotype $CD34^+AC133^+KDR^+$ has gained wide use as a means to measure putative circulating EPC in healthy and diseased human subjects. However, the question of whether a putative EPC defined as a $CD34^+AC133^+KDR^+$ cell, expresses CD45 has remained a controversial topic [23,46].

Several groups have subsequently examined the functional activities of the cells contained within the population of blood cell phenotype $CD34^+AC133^+KDR^+$. While this population of cells is enriched 300- to 400-fold for hematopoietic stem and progenitor cells (defined by morphology, hematopoietic colony forming assays, and in vivo engraftment in immunodeficient mice), $CD34^+AC133^+KDR^+$ cells do not spontaneously form capillary-like structures with lumens in vitro nor human blood vessels in vivo upon implantation in a collagen/fibronectin scaffold [47–49]. As noted by numerous authors, the $CD34^+AC133^+KDR^+$ cells do facilitate the growth of tumor microvasculature and overall tumor growth [50–52]; however, there is no evidence that these cells directly form the long-lasting endothelial cells of the vessels. While these cells may be recruited to denuded vessels in ischemic sites early in the process of wound repair and thus occupy the position of an endothelial cell [22], the host endothelial cells comprise the long-lived vascular endothelium and are not derivatives of the hematopoietic system in the mouse [53–57]. It is more probable that the proangiogenic hematopoietic cells promote the process of angiogenesis via paracrine mechanisms [44,48] (Fig. 2).

As to the question of whether or not the $CD34^+AC133^+$ cells also display CD45 expression, Estes et al. [48] have recently utilized polychromatic flow cytometry and functional assays to confirm that these double positive cells all express CD45. Since these cells also express the morphological and functional properties of hematopoietic stem and progenitor cells, it is apparent that $CD34^+AC133^+$ are proangiogenic hematopoietic stem and progenitor cells and not EPC [48]. Indeed, the lack of the ability of the $CD34^+AC133^+$ cells to directly form human blood vessels in vivo confirms that they do not possess the postnatal vasculogenic activity that was originally proposed for a cell with EPC properties [58].

4. Isolating putative EPC using colony forming assays

In the original EPC description, Asahara et al. [10] noted that the plated human peripheral blood $CD34^+$ cells rapidly formed cellular clusters in vitro, particularly if re-exposed to the $CD34^-$ cells during the co-culture. These clusters were comprised of an aggregate of round cells with spindle-shaped cells radiating from beneath the aggregate and away from the cluster. The spindle-shaped cells readily bound acLDL. Asahara et al. [10] described these clusters as resembling the blood island structures that are observed in chick embryos and are known to contain both blood and endothelial cells. Thus, these clusters were reported as evidence that the $CD34^+$ peripheral blood cells were turning into spindle-shaped acLDL binding putative endothelial cells.

Ito et al. [59] modified this original assay of Asahara et al. [10] for identification of EPC by attempting to deplete the human peripheral blood mononuclear cells of monocytes or circulating mature endothelial cells by allowing all of these mature cells to attach to the fibronectin-coated dishes for 24 h before removing the non-adherent cells. The non-adherent fraction was replated on the fibronectin-coated dishes and the clusters that emerged at 7 days were scored as EPC colony forming units. Subsequently, Hill et al. [60] further modified the assay to include a 48-h pre-plating period prior to replating the non-adherent cells. The colonies of putative EPC that emerged from the cultured non-adherent human peripheral blood mononuclear cells were called CFU-Hill. This assay was used to identify an inverse correlation of the concentration of CFU-Hill with cardiovascular risk status of human subjects.

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Other investigators have identified a different type of colony of cells emerging from the plated peripheral blood mononuclear cells. Endothelial colony forming cells (ECFC), also called late outgrowth endothelial cells (OEC) or blood outgrowth endothelial cells (BOEC), typically emerge from an adult blood sample in 14–21 days [61,62]. In contrast, ECFC emerge as early as 6 days from time umbilical cord blood cells are plated on type 1 collagen or fibronectin-coated dishes [61,63]. The ECFC emerge as tightly adherent colonies with a typical cobblestone appearance and are rare in adult human blood samples with approximately 1 colony/10^8 mononuclear cells plated. ECFC possess clonal proliferative potential that can be observed in single cell cultures [61]. Umbilical cord blood contains a higher frequency of ECFC than adult peripheral blood and the circulating concentration appears to decrease with advancing age in human subjects. The human cord blood cells display much greater telomerase activity than the ECFC derived from human subjects [61]. While both cord blood and adult peripheral blood ECFC spontaneously form human blood vessels when implanted in matrix scaffolds in immunodeficient mice, cord blood ECFC display a greater density of vessels than the ECFC from adult blood samples [64,65]. An important aspect of the human blood vessel forming ability of ECFC is the property of these vessels to be connected to the host immunodeficient murine vessels and to become a part of the systemic circulation of the host animals [65,66]. This functional capacity of the ECFC is certainly indicative of postnatal vasculogenesis; the ability to form a vessel in the absence of a pre-existing vessel proposed to be uniquely displayed by circulating EPC (Fig. 3).

While the cell surface phenotype of the ECFC progeny is nearly indistinguishable from the pattern of expression displayed by vascular endothelial cells, some progress has been made in enriching this population by first depleting the mononuclear cells of monocytes, red blood cells, dead cells, and CD45 expressing blood cells [48]. The highly expressing $CD34^+$ population of cells co-expresses CD146, CD31, and CD105. The $CD34^{hi}CD45^{neg}$ cells contain essentially all of the viable circulating endothelial cells and are enriched by 300- to 400-fold for ECFC activity [47]. At present, no specific antigen has been identified that can unequivocally discriminate the ECFC from other circulating blood elements. In fact, a recent comparison of the proteome of human peripheral blood OEC and dermal microvascular endothelial cells revealed a 90% overlap; though a few (8 indicated) protein spots on the gels of the OEC proteome remain as possible candidate OEC markers but must be sequenced for further verification [44].

Several research groups have directly compared the functional potential of the CFU-Hill with the ECFC derived from adult peripheral blood or cord blood. It is apparent that CFU-Hill is comprised of a variety of blood cells including monocytes, lymphocytes, and hematopoietic progenitor cells skewed toward the myeloid lineage [39,65,67–69]. The spindle-shaped cells emerging from the aggregate of hematopoietic cells are macrophages verified by the fact that the cells express the colony stimulating factor-1 receptor, display active phagocytosis of bacteria, express nonspecific esterase that is inhibited by sodium fluoride, and readily ingest acLDL. The macrophages in the CFU-Hill assay also express CD31, CD144, von Willebrand factor, endothelial nitric oxide synthase, CD105, and bind UEA-1 lectin (in these assay and culture medium conditions). Thus, by only searching for evidence of endothelial marker expression on adherent cells emerging from within the clusters and failing to exclude other potentially “contaminating” cell types, the original publications may have concluded they had identified a unique cell type, when in fact the cells belonged to already known hematopoietic lineages.

In contrast to the hematopoietic functions identified for the CFU-Hill, ECFC display functions such as in vitro clonal proliferative potential, in vitro self-renewal potential, in vitro incorporation into endothelial monolayers or capillary tube formation, in vivo human vessel formation with incorporation into the systemic circulation of immunodeficient mice,

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and in vivo chimeric vessel formation into areas of ischemia [65,66,70,71]. Thus, ECFC appear to function as a circulating precursor with in vivo human vessel forming ability and thereby among all current putative EPC, represent the cell displaying the most features of a human postnatal vasculogenic cell [22].

5. Quo vadis?

While numerous papers have been published discussing the role of EPC in various human disorders and in examining the role of various putative EPC in rescuing the blood flow to experimentally induce vascular lesions in rodents or in human clinical trials of cell therapy for cardiovascular disease, the field continues to struggle in defining the EPC. These changes in the field led us to add the Latin phrase, Quo vadis, which translates, “Whither goest thou” to the title of this review. A concise summary of all the above overview would be to consider that the cells originally identified as EPC in the various assays were and are in fact hematopoietic lineage cells (progenitors, monocytes, and/or platelets) that display proangiogenic properties (Table 1). The one cell type that displays postnatal vasculogenic activity upon transplantation in a matrix scaffold is the rare circulating ECFC or OEC (Table 1). Thus, the field may wish to delimit the use of the term EPC and simply describe the cells involved in vascular repair and regeneration with the specific terms already in existence; i.e. primary human cells promoting angiogenesis via paracrine effects include proangiogenic hematopoietic stem and/or progenitor cells, monocytes, macrophages, or platelets (or other blood cells) whereas the rare circulating cells that display vessel-forming ability are ECFC or OEC (Table 1). Because several different types of blood cells are implicated as proangiogenic, future studies will be required to determine the exact role that each cell plays in the process of vascular repair or regeneration, the mechanism of interaction with the host endothelium, and the molecular pathways engaged. For example, are the monocytes involved in angiogenesis uniquely specified from circulating monocyte precursors or do they exist as a distinctly separate differentiated subset [72,73]? Are the vascular anastomotic chaperoning properties displayed by tissue macrophages at sites of angiogenesis restricted to the Tie2 subset and do they involve direct contact with the endothelium [74]? Likewise, little is known of the in vivo functions of ECFC in the many preclinical models of human cardiovascular disease and increased studies in this area may be illuminating; for example, Dubois et al. [75] have reported that infusion of OEC into pigs following experimentally induced acute myocardial infarction resulted in significant improvement in myocardial infarct remodeling and heart function via direct incorporation of the cells into the host endothelium and Medina et al. [76] using a murine model of retinal ischaemia, reported that human OECs directly incorporate into the host murine vasculature, significantly decreasing avascular areas, concomitantly increasing normovascular areas and preventing pathologic pre-retinal neovascularisation. In the end, by better defining the cells that display functional roles in vessel repair, we may be better able to (1) consider cellular replacement of that subset if found to be dysfunctional (requires development of appropriate in vitro functional assay to assess the function of autologous cells) and (2) identification of the molecular mechanisms underlying the functions of the various hematopoietic and endothelial subsets to repair the vasculature and (3) development of small molecule effectors to mimic the beneficial effects of the efficacious cellular subsets (rescue the angiogenic functions of host cell subsets and augment and/or restore innate repair mechanisms).

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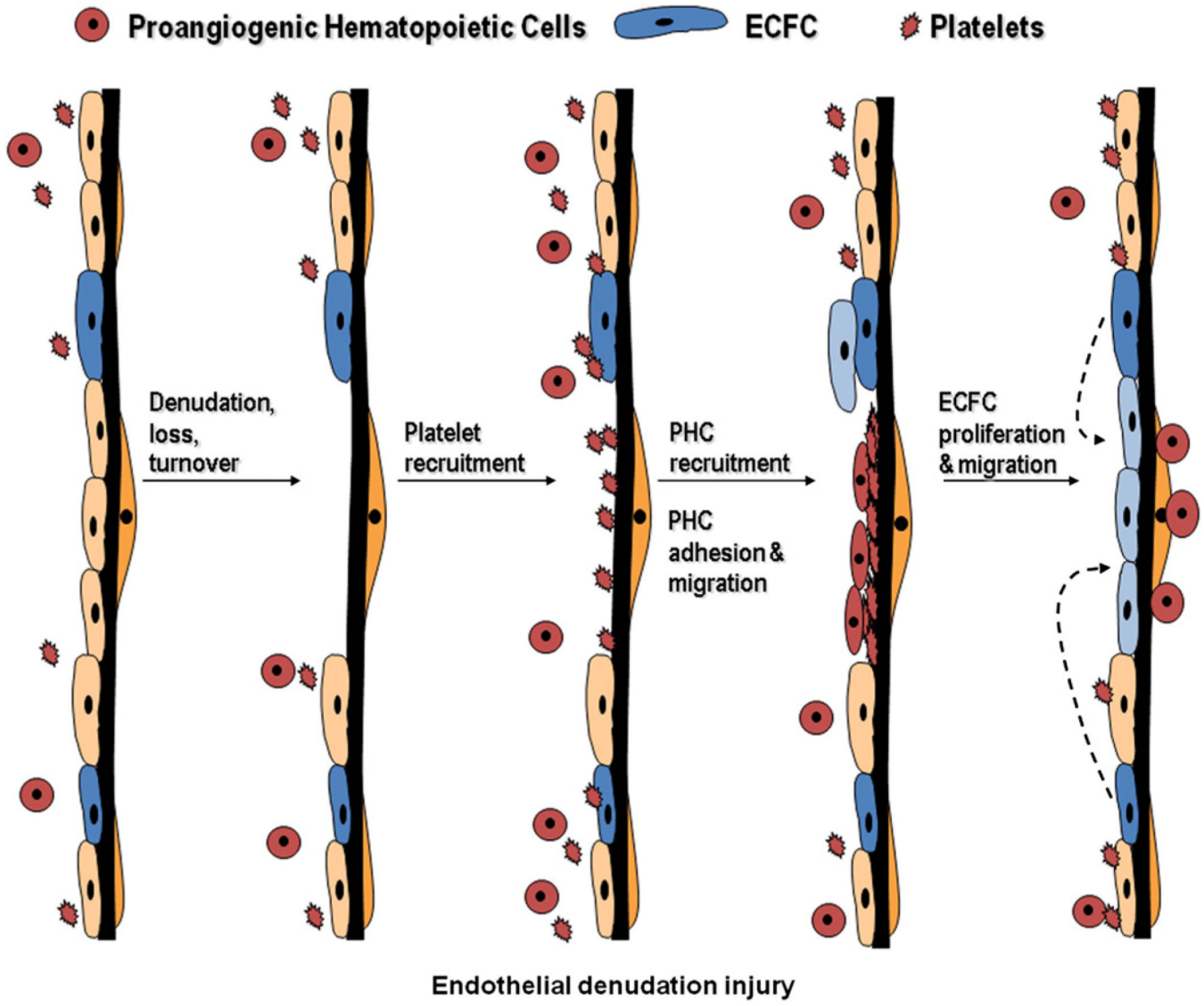

Fig. 1.

Role of various putative EPC in endothelial denudation injury. Following endothelial cell denudation injury, loss, or turnover, endothelial cells with proliferative potential (ECFC) divide and migrate to repair the injured area. This follows a series of events where platelets and proangiogenic hematopoietic cells are first recruited to the site of injury to facilitate repair.

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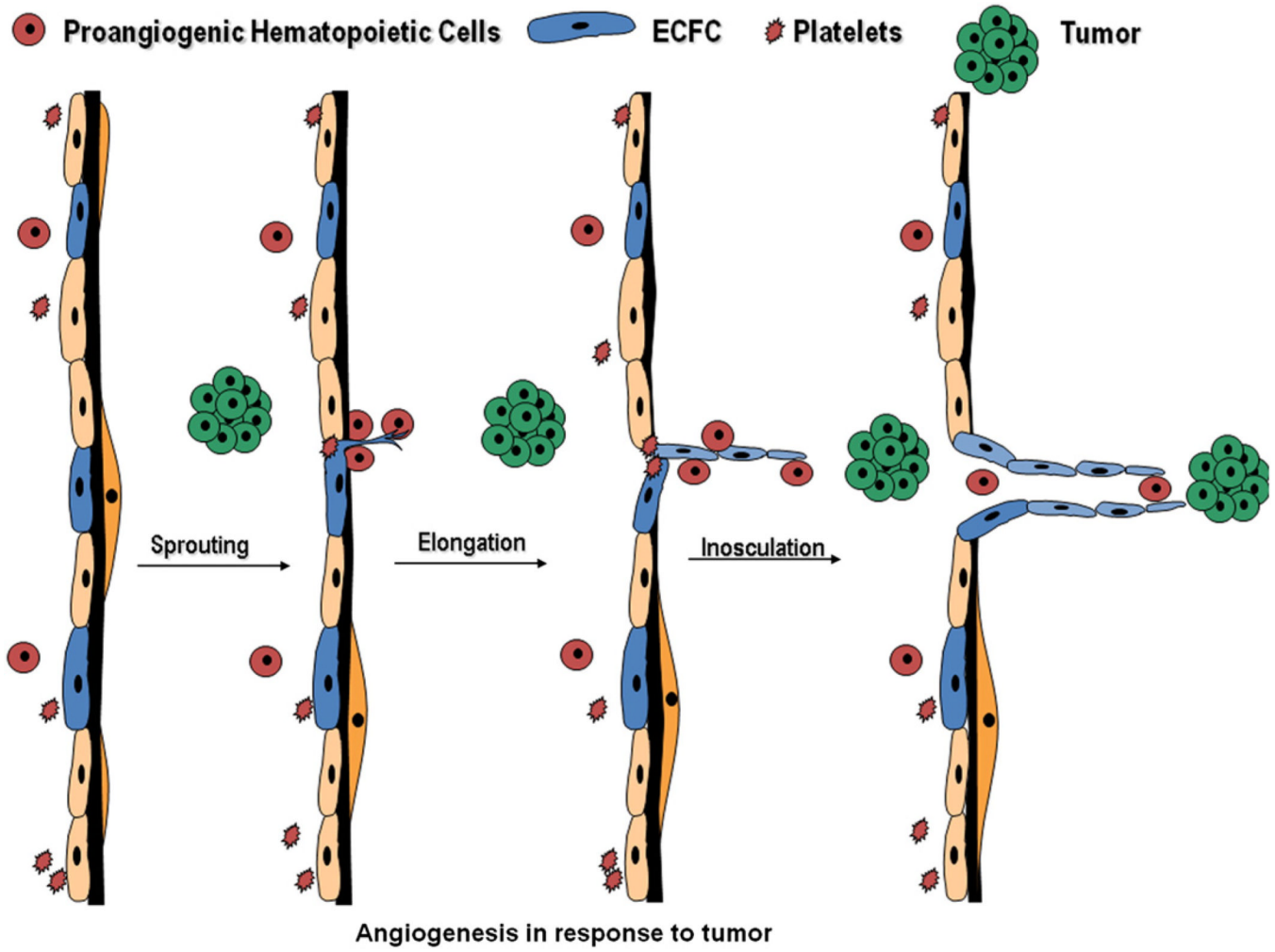

Fig. 2.

Putative EPC roles in angiogenesis. Resident endothelial cells with proliferative potential are likely to initiate sprouting, elongation, and inosculation leading to the formation of a new vessel in the presence of a stimulus such as a tumor. The circulating proangiogenic hematopoietic cells may play roles in disruption of the endothelial barrier properties, matrix degradation, promotion of endothelial cell sprouting, and endothelial capillary remodeling.

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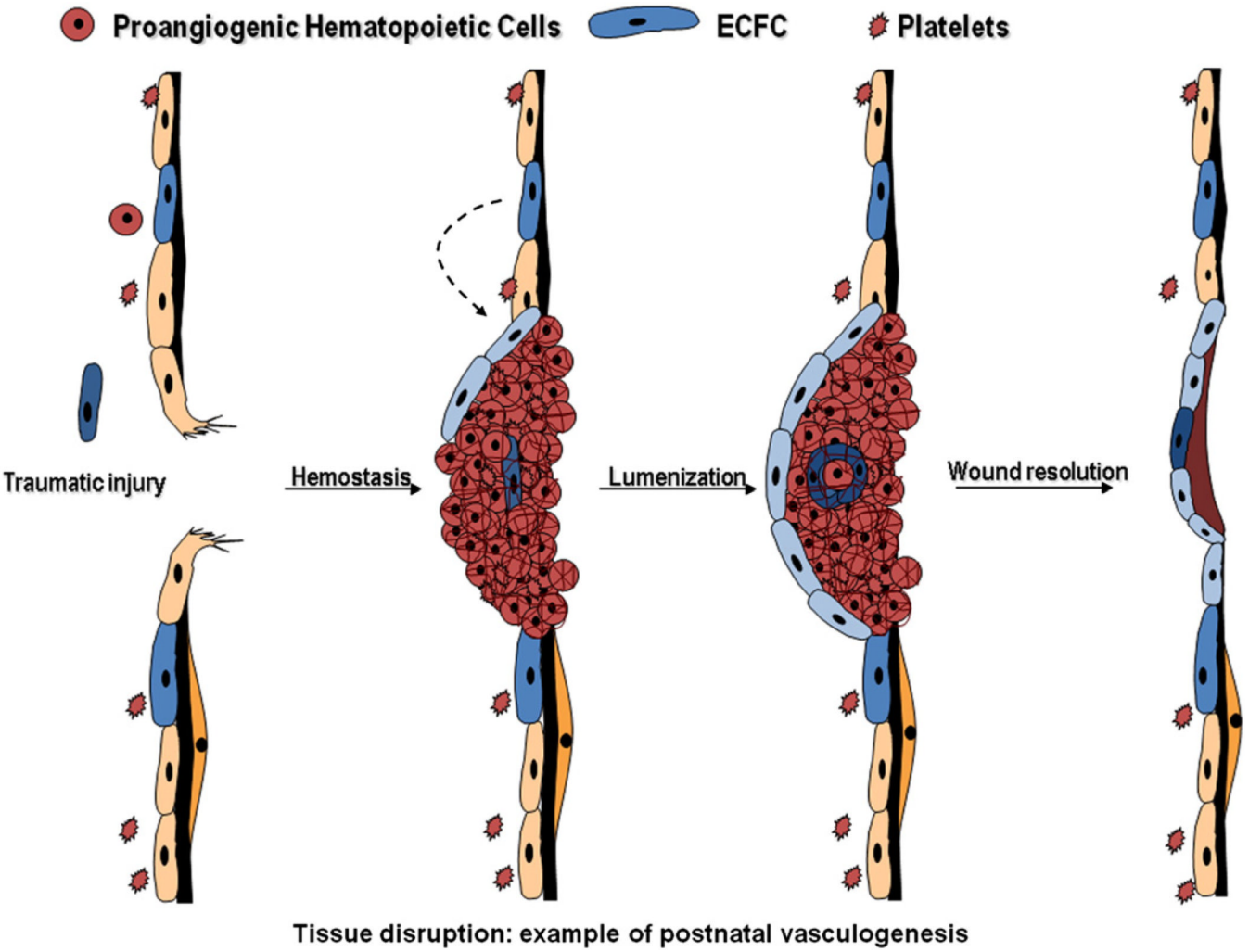

Fig. 3.

Circulating ECFC display postnatal vasculogenic activity. Following traumatic injury to a tissue vasculature, hemostasis is initiated to prevent extensive hemorrhage. Rare circulating ECFC may become trapped within the clot and initiate vasculogenesis as a first step toward recanalization of the thrombus with or without subsequent vessel remodeling.

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Table 1

Summary of hallmark studies in defining a human EPC.

Year	Lead author	Masker	Method Used to Isolate	Marker to define	Term	Weaknesses of the study	Proposed term
1997	Asahara	CD34 or KDR	Immunomagnetic bead separation of CD34 or KDR positive adult peripheral blood cells	CD45, CD34, CD31, Flk-1, Tie-2 E-selectin, UEA-1, acLDL	EPC	Failed to exclude the presence of hematopoietic cells and no direct demonstration of in vivo vessel forming ability	Proangiogenic hematopoietic cell
2000	Peichev	CD34, AC133, and KDR	Immunomagnetic bead separation of CD34 positive adult peripheral blood cells	CXCR4, CD31, CD13, acLDL, and not CD14 or CD15	CEP	Did not assay for CD45 expression – cells neither form vessels in vitro nor in vivo	Proangiogenic hematopoietic cell
2001	Lin	CD146 (P1H12)	Plating of mononuclear cell pellet or immunomagnetic bead separation of CD146 positive adult peripheral blood cells	CD34, CD144, vWF, Flk-1, and not CD14	BOEC	No evidence for clonal proliferative potential and no direct demonstration of vessel forming ability	BOEC
2003	Hill	Mononuclear cells	48 h pre-plating period prior to replating non-adherent blood mononuclear cells and colony formation 4–9 days later	CD31, Tie2, and KDR	CFU-Hill	Failed to exclude the presence of hematopoietic cells in the colonies and no direct demonstration of vessel forming ability	Pro-angiogenic hematopoietic cell
2004	Ingram	Mononuclear cells	In vitro colony emergence and clonal proliferative potential	CD34, CD146, CD31, CD105, and not CD45 and direct demonstration of vessel formation in vivo	ECFC	Lacks antigen to distinguish ECFC from other circulating EC	ECFC
2007	Rohde	Rohde Mononuclear cells	48 h pre-plating period prior to replating non-adherent blood mononuclear cells and colony formation 4–9 days later	(VEGF-R1, 2, and 3), CD31, CD34, CD146, vWF, and not CD14 CD45	CFU-EC	Could not identify all of the proangiogenic activity related to cultured hematopoietic cells	Proangiogenic hematopoietic cell
2007	Yoder	Mononuclear cells	In vitro colony emergence and clonal proliferative potential and 48 h pre-plating period prior to replating non-adherent blood mononuclear cells and colony formation 4–9 days later	CD34, CD146, CD31, CD105, and not CD45 and direct demonstration of vessel formation in vivo	ECFC Vs. CFUHill	Lacks antigen to distinguish ECFC from other circulating EC	ECFC and Proangiogenic hematopoietic cell

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Year	Lead author	Masker	Method Used to Isolate	Marker to define	Term	Weaknesses of the study	Proposed term
2009	Prokopi	Mononuclear cells	Plating of mononuclear cell pellet on fibronectin-coated dishes and at 72 h removed nonadherent cells	CD31, vWF, UEA-1, CD4, and acLDL	EPC	Lack detailed method for determining the extent of the contaminating platelet proteome to the adherent blood cells	Putative EPC and platelets
2010	Estes	CD34, AC133, CD45, CD31	Polychromatic flow cytometry and cell sorting	CD34, AC133, CD45, CD31	Proangiogenic hematopoietic cell	Lacked detailed examination of contribution of proangiogenic cells to the tumor microvasculature	Proangiogenic hematopoietic cell

UEA-1, Ulex Europeaus agglutinin-1; acLDL, acetylated low density lipoprotein; CEP, circulating endothelial precursor; ECFC, endothelial colony forming cells; BOEC, blood outgrowth endothelial cells; vWF, von Willebrand factor; CFU-EC, colony forming unit-endothelial cell; CFU-Hill, colony forming unit-Hill.