

Endothelial progenitor cells: identity defined?

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Abstract

In the past decade, researchers have gained important insights on the role of bone marrow (BM)-derived cells in adult neovascularization. A subset of BM-derived cells, called endothelial progenitor cells (EPCs), has been of particular interest, as these cells were suggested to home to sites of neovascularization and neoendothelialization and differentiate into endothelial cells (ECs) *in situ*, a process referred to as postnatal vasculogenesis. Therefore, EPCs were proposed as a potential regenerative tool for treating human vascular disease and a possible target to restrict vessel growth in tumour pathology. However, conflicting results have been reported in the field, and the identification, characterization, and exact role of EPCs in vascular biology is still a subject of much discussion. The focus of this review is on the controversial issues in the field of EPCs which are related to the lack of a unique EPC marker, identification challenges related to the paucity of EPCs in the circulation, and the important phenotypical and functional overlap between EPCs, haematopoietic cells and mature ECs. We also discuss our recent findings on the origin of endothelial outgrowth cells (EOCs), showing that this *in vitro* defined EC population does not originate from circulating CD133⁺ cells or CD45⁺ haematopoietic cells.

Keywords: endothelial progenitor cells • haematopoietic cells • CD34 • CD45 • CD133

Introduction

The development of the vascular system is an essential event during embryonic development of many animal species. During this process, local mesodermal precursors differentiate into vascular and endothelial cells (ECs) to form a primary vascular plexus, a process referred to as vasculogenesis [1]. Until the description of circulating endothelial progenitors (CEPCs), this process of vasculogenesis was thought only to occur during embryonic develop-

ment, but not in postnatal life. Indeed, a decade ago, two groups reported that human CD34⁺ cells isolated from circulating peripheral blood (PB), umbilical cord blood (UCB) and bone marrow (BM), could differentiate into ECs *in vitro* and *in vivo* in mouse models, thereby contributing to neoendothelialization and neovascularization in the adult organism [2, 3]. Additional studies in mice suggested incorporation of BM-derived cells into blood vessels at

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tumour sites [4, 5], healing wounds [6, 7], areas of endothelial denudation [8, 9], induced hindlimb ischaemia [10] and following experimental myocardial infarction [11].

These landmark studies on endothelial progenitor cells (EPCs) thus challenged the traditional concept that endothelial regeneration and angiogenesis occurs exclusively *via* the proliferation of pre-existing resident vessel wall ECs, and thus it appeared that vasculogenesis also occurs in postnatal life. Moreover, this novel concept that EPCs enter the blood stream *via* the BM and provide a pool of CEPCs in postnatal life was even more striking, because there is no clear evidence that circulating vascular precursors contribute to vasculogenesis during murine or human embryonic development.

In patients that had undergone BM or PB stem cell transplantation, evidence was also presented that some ECs lining the blood vessels in the recipient were chimeric in origin, (*i.e.* of both recipient and donor origin), pointing to the existence of circulating cells in blood and BM that contribute to endothelial turnover of blood vessels in humans [12, 13]. Indirect evidence for the concept that EPCs act as a back-up system for maintaining vascular homeostasis in humans was suggested by showing an inverse correlation between the number of PB CEPCs and presence of atherosclerosis, an adverse cardiovascular risk score, level of cardiovascular dysfunction, or cardiovascular morbidity and mortality risk in human subjects [14–18]. Also, the functional properties of EPCs such as cell adherence, migration, invasion and vessel formation appear to be attenuated in patients with increased cardiovascular risk factors and established cardiovascular disease [15, 19–21]. Therefore, EPCs could also serve as a biomarker to predict cardiovascular outcomes and might help in monitoring the effects of primary and secondary cardiovascular prevention strategies.

In spite of the growing number of reports on EPCs as a potential tool in regenerative medicine or a therapeutic target in oncology, contradicting results have been reported at the pre-clinical and clinical level, and several issues such as the definition, identification, characterization and the role of EPCs in vascular homeostasis and disease remain controversial [22–36]. In this review, we will discuss these issues based on published results conducted at the pre-clinical phase.

The proof-of-concept *in vivo*: the cell, the read-out and the animal model

After 10 years of vigorous research in EPC biology, we have not yet reached a consensus on the definitive appearance and function of an EPC. What the field is currently suffering from is the use of a single term (EPC) to refer to BM-derived or circulating cells of diverse lineages. It is apparent that progress in the use of EPCs to treat human disorders may not reach its ultimate potential unless we can specifically identify all of the component cell types that contribute to neovasculogenesis, define the role that each cell lineage plays in the process, and identify strategies to assess the

in vivo function of these diverse cell types in patients with vascular disorders, thus defining which cell type may need to be replaced to reinvigorate the vascular repair process. Therefore, if an EPC is defined as an immature precursor cell that individually displays postnatal vasculogenic activity, then any cell called an EPC should be capable of forming new ECs and blood vessels *in vivo*. Unfortunately, attempts to illustrate this defining property of a (candidate) EPC have often yielded conflicting results, which probably relates to the highly variable approaches undertaken in different research venues with regard to *cell source, cell purification, the animal model or assay used, method of detection and analysis, and data interpretation.*

The CEPC: still a putative cell

In many studies addressing the identity of EPCs in vascular biology, unselected PB mononuclear cells (MNCs) were used, or the purity of the (starting) CEPCs population was low, making it difficult to identify the CEPCs within the MNC population that gave rise to the ECs (*i.e.* the exact precursor (CEPC)–product (EC) relationship in these studies was not addressed). Many investigators have identified or designated putative CEPCs with flow cytometry using a single surface marker such as CD34 or CD133 in humans, or various combinations of surface markers were used, which has actually resulted in a complicated list of putative CEPC immunophenotypes in man and mice (Table 1). As shown in Table 1, most of the surface marker combinations used in flow cytometry studies included the marker CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2), because initial studies in the field have reported that CD34⁺ and VEGFR-2⁺ cells purified from various sources (UCB, PB and BM) are able to generate ECs *in vitro*, suggesting that CD34⁺ cells contains CEPCs [2, 3, 37]. One specific subset of CD34⁺ cells, designated as CD34⁺VEGFR-2⁺CD133⁺ cells are widely accepted to identify 'true CEPCs' in humans but yet these cells were never directly tested for generating new ECs *in vitro* or *in vivo*, which is essential to validate CD34⁺VEGFR-2⁺CD133⁺ cells as true CEPCs [38–40]. Recently, however, Case *et al.* reported for the first time that isolated human UCB or mobilized adult PB CD34⁺VEGFR-2⁺CD133⁺ cells in fact represent an enriched population of CD45⁺ haematopoietic precursors using *in vitro* haematopoiesis assays, but CD34⁺VEGFR-2⁺CD133⁺ did not contribute to the formation of ECs *in vitro* [41]. Similarly, CD34⁺CD45⁺CD146⁺ cells previously reported to represent CEPCs, were not directly assayed *in vitro*, nor *in vivo* for contribution to newly formed endothelium, and thus, it is difficult to know whether this cell type acts as a true CEPC [42]. Therefore, the scientific foundation for using the variable surface marker combinations and CD34⁺ subsets depicted in Table 1 remains elusive. Moreover, the use of these diverse combinations to define a singular entity (the CEPC), makes the significance of flow cytometric studies difficult to interpret, creates obstacles to the direct comparison of data between laboratories, and may result in discrepancies in the interpretations of study results among different laboratories. Therefore, investigators should strongly consider that any 'putative' CEPC, whatever its phenotype, be carefully assessed

Table 1 Surface immunophenotype of human and murine CEPCs

Immunophenotype in humans	Reference	Immunophenotype in mice	Reference
CD34 ⁺ CD31 ⁺	[135]	Sca-1 ⁺	[10]
CD34 ⁺ CD62L ⁺	[135]	Sca-1 ⁻ Lin ⁻ cKit ⁻	[145]
CD34 ⁺ CD133 ⁺	[136]		
CD34 ⁺ CD11b ⁺	[137]	Lin ⁻ cKit ⁺ Flk1 ⁺ CD13 ⁺ CD133 ⁺ VE-Cadherin ⁺	[50]
CD34 ⁺ CD45 ⁺	[110]		
CD34 ⁺ CD133 ⁺ CD45 ⁺	[138]		
CD34 ⁺ FGFR1 ⁺	[111]	CD45 ^{dim} CD34 ⁺ Flk-1 ⁺	[9]
CD34 ⁺ VEGFR-2 ⁺	[112]	CD31 ⁺ Flk-1 ⁺ CXCR4 ⁺	[146]
CD34 ⁺ CD133 ⁺ VEGFR-2 ⁺	[38]	Sca-1 ⁺ cKit ⁺ Lin ⁻	[56]
CD34 ⁻ CD133 ⁺ VEGFR-2 ⁺	[55]	CD45 ⁻ CD34 ⁺ Flk-1 ⁺	[98]
CD34 ⁺ VE-Cadherin ⁺ CD3 ⁻	[139]	cKit ⁺ CD31 ⁺	[147]
CD34 ⁺ CD133 ⁺ VEGFR-2 ⁺ CD45 ⁺	[40]	cKit ⁺ CD34 ⁺ Flk-1 ⁺	[148]
CD34 ⁺ CD45 ⁺ CD146 ⁺	[42]	CD45 ⁻ cKit ⁺ CD13 ⁺	[149]
CD34 ⁺ CD45 ⁻	[140]		
CD133 ⁺	[17]		
CD133 ⁺ CD45 ⁻	[141]		
CD133 ⁺ VEGFR-2 ⁺	[142]		
CD14 ⁺ VEGFR-2 ⁺	[71]		
CD14 ⁺ CD34 ⁺	[70]		
ALDH ^{bright}	[143]		
CD31 ⁺	[144]		

by validating its postnatal endothelial differentiation capacity *in vitro* and *in vivo*.

A critical set of variables to consider when performing endothelial differentiation studies is that putative CEPCs might only give rise to ECs depending on the exact combination of growth factors to which the cells have been exposed *in vitro*, and/or depending on the animal model used to assess postnatal vasculogenic activity, and/or the nature, extent, or method of delivery of the angiogenic stimulus applied *in vivo*. These important caveats, in what should be the most important criterion to validate EPCness, makes the field of EPCs intriguing, but at the same time complicated. Therefore, further efforts should be focused on the development of a straightforward standard assay, or set of assays, that are accessible to all investigators, to specifically define and validate the function of (candidate) EPCs, so that investigators would have a benchmark for comparison, and a rationale for the examination and clinical translation of selected cell subsets in targeted clinical disorders.

To validate the EC differentiation capacity of a candidate CEPC, *single cell studies* offer a rigorous approach to address the precursor (CEPC)–product (EC) relationship. However, numerous technical

and biological limitations must be overcome in performing a clonal assay. For instance, one of the potential limits of single cell experiments is that the isolated single cell may behave differently in the absence of other cells. Therefore, transplanting or culturing a single cell may not be an appropriate EPC assay, but may require the presence of (supportive) cells with a different phenotype or function. However, one of the greatest obstacles to date in the field of EPC biology is the lack of a unique marker, or combination of markers, that solely identifies the rare CEPCs in humans, making a prospective isolation and thus single cells studies impossible. In the murine BM transplantation system, two single cell transplantation studies have been reported, showing that a single mouse haematopoietic stem cell (HSC) could give rise to endothelial progeny after retinal injury [43, 44]. However, these studies have been challenged by other groups that failed to detect endothelial differentiation from transplanted HSCs *in vivo* using different donor cell marking strategies [23, 45].

Considering the remarks discussed in this section, two important questions remain with regard to the phenotype of CEPCs: (i) If CD34⁺VEGFR-2⁺CD133⁺ appear not to be true CEPCs, what

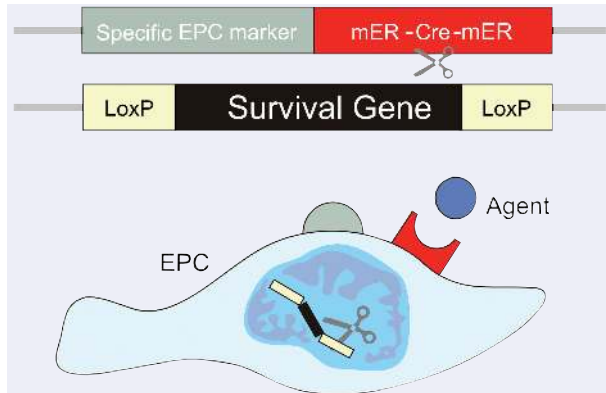


Fig. 1 Conditional EPC knockout mouse model based on a unique CEPC marker. A modified estrogen receptor (mER; in red) is co-expressed on CEPCs that expresses a unique marker (indicated in grey). This unique marker may be either a membrane (as shown) or a cytoplasmic marker. Upon challenge with a receptor specific agent, the mER activates the Cre enzyme (indicated by scissors) in CEPCs. The Cre-enzyme recognizes the specific LoxP sequences (white) that flank a survival gene (black) resulting in its deletion and eventually CEPC death.

then is the exact surface phenotype of the CEPCs? and (ii) How can we explain the statistical (inverse) correlation between the several cell subsets depicted in Table 1 and cardiovascular indices and outcome, even if these cell types would not represent true CEPC? As we will discuss later in this review, the phenotypes shown in Table 1 mostly represent haematopoietic-derived cells, rather than true CEPCs, similar to the $CD34^{+}VEGFR-2^{+}CD133^{+}$ cells discussed above. Nevertheless, haematopoietic-derived cells may contribute to vascular repair and homeostasis in an indirect manner, as suggested by several investigators [25, 31, 46–48]. Indeed, haematopoietic lineage cells might be recruited to injured or angiogenic sites and secrete regulatory cytokines that promote vessel homeostasis and repair by local cells, including local vessel wall ECs. It is possible that cardiovascular risk factors and established cardiovascular disease decrease the circulating number and properties of the haematopoietic-derived cells. Hence, low levels of these circulating cells might correlate with adverse cardiovascular outcome. However, this concept is entirely different from the EPC concept proposed 10 years ago where EPCs were suggested to function as a structural backup from the BM [2].

The question as to what the exact phenotype is of true CEPCs will be discussed at the end of the review after we have outlined several crucial issues related to the identification, definition and differentiated progenies of putative CEPCs.

Do CEPCs play an essential role in vascular (patho)physiology?

Although many papers have been reported on EPCs in man and mice, the direct proof-of-concept or true relevance of these cells in

vascular homeostasis, vascular growth and disease (atherosclerosis) is still uncertain. Perhaps one of the most solid approaches to address the relevance of CEPCs in vascular homeostasis is to look at the natural history of vascular aging or the extent of vascular disease in animal models lacking these CEPCs. This could be achieved by selectively eliminating the CEPCs in mice with pharmacological agents, or by means of genetic knockdown. The vascular outcome in mice with knockedout EPCs could then be compared with control mice in diverse pathological settings. However, (*genetic*) knock-down studies to assess the mechanistic underpinning between CEPCs and vascular (patho)biology, requires that one possess the ability to prospectively identify and thus specifically target the CEPCs, without targeting the mature vessel wall ECs. Indeed, the current approaches used to abolish or enhance the function and/or number of endogenous CEPCs might not have been selective enough, and are likely to have targeted mature vessel wall ECs, and other (BM-derived) cells involved in vascular repair as well, thus complicating the interpretation of the study results of CEPCs in modulating the process of vascular repair [49]. Recently, some studies have focused on the role of CEPCs in tumour angiogenesis in murine models using several knockdown tools [5, 50, 51]. In these studies, murine BM-derived CEPCs were immunophenotyped as $cKit^{+}Flk-1^{+}CD13^{+}CD133^{+}$ cells. Moreover, it was suggested that these candidate CEPCs express Id-1 proteins and a monomeric form of the surface markers VE-Cadherin (*i.e.* CD144) in a unique fashion, whereas the resident mature ECs did not. This allowed the investigators to selectively inhibit the CEPCs without targeting the resident mature vessel wall ECs, and thus address the relevance of CEPCs in tumour angiogenesis. However, studies have shown that local, mature vessel wall ECs also express the monomeric VE-Cadherin epitope upon vessel disassembly and tumour angiogenesis [52]. Therefore, it is possible that the neutralizing antibody used to target the CEPCs might also have tackled the proliferating mature vessel wall ECs in this study [52].

In another study, it was suggested that murine CEPCs selectively express Id-1, a protein previously shown to be involved in angiogenesis [4, 51]. Using genetically manipulated BM cells, the expression of this Id-1 gene could be turned off 'à la demande', resulting in a profound reduction of tumour angiogenesis and tumour growth. However, because Id-1 is also expressed in haematopoietic lineage cells, it remains uncertain whether the inhibition of tumour angiogenesis is merely because of reduced tumour-recruitment and/or paracrine angiogenic function of haematopoietic lineage cells, instead of reduced CEPC function, differentiation or incorporation [50, 53]. Therefore, although these studies illustrate the role of BM cells in tumour angiogenesis, because of a lack of specificity, they do not formally prove the unique or dominant position of putative CEPCs in the modulation of tumour angiogenesis. Therefore, at the end, the identification of a unique marker gene that specifically identifies CEPCs will allow us to unambiguously address the 'proof-of-concept' or causal role of CEPCs in cardiovascular (patho)physiology and tumour pathology.

In Figure 1, we propose a genetic knock-down model based on the expression of a unique cell marker in CEPCs (being either a

Table 2 Immunophenotype of haematopoietic cells and endothelial cells

	Embryonic HPC	Postnatal HPC	ECs	References
CD14	–	–	–	[58, 85]
CD31 (PECAM-1)	+	+	+	[58, 85, 106, 150]
CD34	+	+	+	[41, 106, 109, 150, 151, 152, 153]
CD38	–/+	–/+	–	[152, 153]
CD43	+	+	–	[109, 151]
CD44	?	+	?	[153]
CD45	–/+	+	–	[41, 58, 106, 108, 109, 153]
CD54 (ICAM-1)	?	+	+	[154]
CD90	+	+	(+) ^a	[109, 153]
CD105 (Endoglin)	?	+	+	[58, 155]
CD106 (VCAM-1)	?	?	(+) ^b	[133]
CD117 (C-kit)	+	+	(+) ^a	[58, 79, 151, 153]
CD133	(?) ^c	+	–	[58, 153]
CD143	+	+	?	[156]
CD146 (MUC-18)	?	?	+	[58]
CD164	+	+	–	[157]
VEGFR-2	+	(+) ^d	(+) ^e	[58, 106, 132, 151]
Tie-2	+	+	+	[106, 158]
VE-Cadherin (CD144)	+	–	+	[58, 106, 109]

Note: The surface marker profile of human embryonic haematopoietic precursors (HPC), postnatal HPCs and ECs is depicted. The surface marker profile of the EC markers shown is based on the analysis of both *in vivo* ECs and *in vitro* cultured vessel wall ECs.

(+)^a indicates that we identified a small CD90⁺ or CD117⁺ population on non-passaged vessel wall ECs.

(+)^b indicates that CD106 is upregulated following endothelial activation [133].

(+)^c indicates that the haematopoietic potential (T cells) of embryonic stem cell-derived CD133⁺ HPCs has been demonstrated only *in vivo* [134].

(+)^d refers to a report on the expression of VEGFR-2 on a small HSC population [132].

(+)^e indicates that the precursor–product relation between circulating VEGFR-2⁺ cells and EOCs generated *in vitro* has not yet been proven.

surface or intracellular marker). As illustrated, in this conditional knock-down model, only CEPCs that express a unique marker gene will be targeted and die after challenge with the specific agent, whereas mature vessel wall ECs and haematopoietic cells that do not express this unique cell marker, will not be targeted. Comparative studies between knockedout mice and control mice will then accurately answer the question whether CEPCs are truly causal or act as an essential backup for vascular disease and physiology, respectively.

The *in vivo* read-out and animal model

Perhaps the most important but often disregarded issue in the assessment of the precursor–product relationship are the *criteria*

used to identify the EPC progeny as ‘true ECs’ in vivo. In fact, identifying a cell as a bona fide EC is crucial for classifying its precursor as a true EPC. To identify ECs generated from CEPCs *in vivo*, most reports have relied on the anatomical position of the donor cell population within the context of a perfused vascular structure in combination with a single marker or combination of cell surface markers that typically are expressed on normal vascular ECs such as CD31 or CD34, or the uptake of acetylated low density lipoprotein (LDL), as well as the binding of certain plant lectins (Ulex Europaeus Agglutinin-1 or UEA-1) to identify ECs [54–56]. However, most of the characteristics, or cell markers used in previous studies such as CD31, CD34, lectin binding or LDL ingestion, are not specific for the endothelial lineage, but also characterize haematopoietic lineage cells (Table 2) [57, 58]. This notion is important because vascular homeostasis and repair are

multi-cellular processes, recruiting several cellular participants such as local vascular wall cells (vessel wall progenitors, smooth muscle cells, pericytes, ECs and macrophages) and circulating BM-derived cells, including putative CEPCs, but also haematopoietic (derived) cells [22, 25, 31, 32, 59, 60–63].

Mature haematopoietic cells include red blood cells, platelets, myeloid cells such as monocytes/macrophages and granulocytes, dendritic cells and lymphoid cells including B cells, T cells, NK cells and NKT cells. Haematopoietic cells derive from HSCs and haematopoietic progenitor cells (HPC respectively) that reside within the BM. Importantly, haematopoietic-derived cells such as monocytes, granulocytes, platelets and even HSCs/HPCs have been shown to be involved in vascular repair [48, 64]. However, because both endothelial lineage cells and haematopoietic cells are present at sites of neovascularization and co-express a host of similar surface markers, it might be difficult to discriminate them from each other at sites of vascular repair, and appreciate their individual contribution to the healing or regenerative process. Therefore, the diverse recruited cell types now known to populate sites of neovascularization are highly likely to have previously been lumped into the single term 'EPC' in many early studies of postnatal vasculogenesis, explaining some of the apparent controversy in the field.

One of the strategies that have been used to directly assay donor cell differentiation into ECs *in vivo* has been the use of transgenic mice that express a fluorescent marker (*e.g.* green fluorescent protein (GFP)) only in cells expressing an endothelial specific gene, such as Tie-2 [65]. Therefore, transplantation of BM cells from transgenic mice into wild-type mice allows the tracking of the cells of interest, and their fate during mobilization from the BM into sites of vascular injury, and discriminates them from other cell types and host cells involved in vascular repair and regeneration. However, even these sophisticated approaches have often yielded contradictory results, probably because expression of Tie-2 is not entirely restricted to the endothelial lineage, and is also expressed by pericytes and haematopoietic (derived) cells such as monocytes, that also migrate to sites of vascular repair [22].

On the other hand, it has been argued that the failure to retrieve genetically labelled BM-derived Tie-2⁺ ECs in the paper of De Palma *et al.* might be because of the fact that the exogenous Tie-2 promoter used, may not mark all the mature ECs, or that during the random genetic manipulation of the cells, the CEPC population might not have been targeted with the viral Tie-2 vector [5]. Also, it is possible that the failure to detect few, if any BM-derived ECs in the neovasculature of experimental models may be related to poor engraftment of the EPC compartment following their transplantation [22, 66]. Although the latter pitfall can be circumvented by the use of a parabiosis model (where two mice are surgically connected and share a common circulatory system allowing exchange of circulating cells), conflicting results have also been reported in the parabiosis model [36].

To unequivocally illustrate *in vivo* that the EPC-derived progeny in the newly formed vasculature is truly endothelial in nature, a more direct or convincing approach would be the extraction of putative EPC progeny from the tissues (by means of a genetic tracer such as GFP) and FACS analyse (with and without previous

culture) these cells using a wide panel (CD11, CD45, VE-Cadherin, CD146, CD31, CD13, CD105, etc.) of antigens and combinations of these markers to illustrate their true endothelial lineage and discriminate them from other cell lineages, especially the haematopoietic lineage. In addition, these extracted cells could also be tested functionally by means of proliferative capacity or tube forming capacity. So far, only one group has used such a FACS strategy to identify EPC progeny *in vivo* [5], but the marker combination used (CD31⁺Lectin⁺GFP⁺) does not allow a clear discrimination from haematopoietic (derived) cells that also display these properties [5, 57].

Other methodological issues, such as the number of tissue sites sampled, or the time frame of the study intervals, and the microscopic technique employed in the tissue analysis, may explain the variable results of EPCs to contribute to sites of neovascularization in different animal model systems. For instance, while the use of confocal microscopy may permit a complete volumetric three-dimensional rendering of the donor cell contribution to new blood vessels in a damaged tissue, the use of light microscopy might impose difficulty in discriminating whether cells are incorporated at the luminal layer and are integrated within the endothelial layer, or are located at peri-luminal sites, just beneath the endothelial layer [22, 28]. Therefore, differences in the microscopic technique, and/or method of analysis of the imaging data, may also contribute to the highly variable reported rate of incorporation of EPCs in repairing vessels (0–90%). Furthermore, it remains uncertain how many of the 'luminal integrated cells' (*e.g.* monocytes, that phenotypically overlap with ECs) are in fact passenger cells participating in an inflammatory reaction to the vascular injury, or cells in the process of transmigrating deeper into the vessel wall and interstitial tissues. Thus, whenever possible, the use of three-dimensional imaging may provide the most sensitive and specific information on the location and contribution of the donor cells to tissues under repair (given the use of appropriately tested antibodies that are specific and validated for detection of cells, cell nuclei or organelles, basement membrane and extracellular matrix proteins).

Other variables that must be considered when using animal models include the genetic background of the mouse, the type of vascular injury to be incurred and the organ system to be challenged. In the case of a tumour model system where one wishes to examine donor cell contributions to tumour endothelium, the tumour type, stage and therapy may be important [5, 49]. Also, the nature of the angiogenic stimulus may be critical, because the cellular and molecular mechanisms of (neo)vascularization differ, depending on whether a tumour, a denuded artery, a myocardial infarction, hindlimb ischaemia or a traumatic injury is used to assay a candidate EPC [67, 68]. Therefore, specific cell subsets may be recruited preferentially, and conflicting results may ensue, depending on the animal or tissue model.

Also, the degree of EPC contribution may also depend on the type of vascular bed that has been studied. Indeed, the degree of CEPC incorporation and contribution to neovessels may be influenced by competition between CEPCs and the resident ECs during vascular repair and regeneration.

Table 3 Characteristics of human EC-like cells, EOCs and CECs

EC-like cells	EOCs	CECs
(EPCs, ECs, CFU-ECs, CACs, ATs, early out-growth CE-EPCs, CMMCs and early EPCs)	(EPCs, ECs, CFU-ECs, BOECs, EFCs, EPDCs, EC-like, late EPCs, late endothelial outgrowth)	(Circulating endothelial cells)
1. Generated after 4–21 days in culture	1. Appear after > 7 days in culture	1. Low proliferative ECs, shed from the vascular wall into the circulation
2. Round (pancake) to spindle shaped appearance; no typical confluent monolayer	2. Typical polygonal cells in a confluent cobblestone monolayer	2. Have a similar phenotypic profile compared to EOCs
3. Express endothelial and haematopoietic markers (e.g. CD45, CD14)	3. Express CD31, CD34, CD105, CD146, VE-Cadherin, VEGFR-2, but not the haematopoietic surface markers CD133, CD14 or CD45	3. Do not express haematopoietic markers and have no apparent haematopoietic potential or function
4. Bind UEA-1 lectin and take up LDL	4. Bind UEA-1 lectin and take up LDL	
5. Maintain haematopoietic potential and/or functions	5. Have no apparent haematopoietic potential	
6. Have low proliferative potential	6. Bear high proliferative potential	
7. Do not generate vascular tubes <i>in vitro</i> in matrigel	7. Generate vascular tubes <i>in vitro/in vivo</i> in matrigel	
8. Improve neovascularization <i>in vivo</i>	8. Improve neovascularization <i>in vivo</i>	
9. Originate from CD45 ⁺ haematopoietic lineage cells (CD34 ⁺ CD45 ⁺ , CD133 ⁺ CD45 ⁺ , CD34 ⁻ CD45 ⁺ , CD14 ⁺ CD45 ⁺)	9. Originate from CD45 ⁻ CD133 ⁻ CD34 ⁺ cells, bone marrow (*) and probably the vascular wall	

CFU-ECs [77, 159, 160]: colony forming unit of endothelial cells; ATs [161]: attaching cells; CACs [47]: circulating angiogenic cells; CE-EPCs [76]: culture expanded endothelial progenitor cells; CMMCs [162]: culture modified mononuclear cells; EOCs [58]: endothelial outgrowth cells; BOECs [163]: blood EOCs; EFCs [79]: endothelial colony forming cells; EPDCs [37]: endothelial progenitor-derived cells; CECs [163]: circulating endothelial cells,

(*) [94]: bone marrow multipotent adult progenitor cells (MAPCs) have been suggested to be the earliest EOC precursors.

References: [30, 37, 41, 42, 47, 57, 58, 74–76, 79, 80, 82–86, 95, 117, 119, 131, 163].

Summarizing this section, it is imperative that improved approaches are developed to reconcile the previous conflicting reports on the role of EPCs in vascular repair, to further define the exact immunophenotype of CEPCs, and to define other participating cell types in adult vessel repair and regeneration.

EPCs defined *in vitro*: the Achilles heel in EPC biology

EOCs and EC-like cells

Most investigators have focused on the study of 'EPCs' obtained after the *ex vivo* culture of unfractionated MNCs that contain putative CEPCs. This is because CEPCs appear to be present in the

circulation at very low numbers, requiring '*ex vivo* culture' to obtain sufficient numbers to characterize and study them in disease models. Numerous assays have been developed to plate MNCs in specific conditions to 'make putative CEPCs differentiate into ECs *in vitro*' [2, 3, 37, 38, 55, 69–72], 'to expand CEPCs *ex vivo*' [73–76], or to 'assay CEPCs for colony forming capacity' [17, 77–79]. Irrespective of the assay or the purposes of the EPC cultures reported so far, two major cell types have been shown to emerge out of these MNC cultures: (1) cells that display a mixed endothelial-monocytic/haematopoietic phenotype [47, 57, 58, 74, 76, 80–86] which we refer to as EC-like cells, and (2) cells with high proliferative potential that display typical endothelial characteristics, reminiscent to vessel wall ECs, which we refer to as endothelial outgrowth cells (EOCs) [37, 58, 75, 79, 81, 85–88].

The nomenclature used to describe each major cell type has been widely varying and often intermixed (Table 3), which has contributed to the confusion in this field. Also, in many of the early studies of EPC biology, it has been impossible to appreciate the exact

cell type (EC-like cells versus EOCs) obtained *in vitro*, owing to the limited characterization of the cells described in the reports. Indeed, both EC-like cells and EOCs express several identical surface markers such as CD31, lectin binding, vWF and uptake of LDL [57]. However, recent studies have shown that EC-like cells do not represent true ECs and derive from heterogeneous CD45⁺ haematopoietic cells, including CD34⁺CD45⁺(CD133⁺) HSCs/HPCs and CD45⁺CD14⁺ monocytic cells that both co-express a set of 'endothelial' surface markers [57, 58, 85]. Therefore, we encourage the use of extensive criteria, as depicted in Tables 2 and 3 that allows for an accurate discrimination between these two cell types *in vitro*. This discrimination is based on phenotypical, morphological and functional characteristics typical for cultured vessel wall ECs. However, it is important to note that the markers depicted in Tables 2 and 3 are not uniformly expressed by all ECs lining the blood vessels *in vivo*, and ECs may change their surface marker expression profile upon *ex vivo* culture [89, 90]. Nevertheless, ECs *in vivo* have many characteristics in common similar to the *in vitro* defined EOCs, as described in Tables 2 and 3 [37, 79, 85, 91, 92]. Therefore, apart from their vessel forming ability, it has been stipulated that cells can be ascribed as 'true EPCs' only if they generate true ECs sharing most of these characteristics [57, 86, 87, 93–95]. Conversely, the circulating cells that generate EC-like cells *in vitro* cannot be considered as true CEPCCs [57].

In the murine system, similar EOCs and EC-like populations have been described *in vitro*, or at least the same nomenclature has been used to describe similar endothelial progenies. Similar to the human situation, early EPCs have been shown to derive from haematopoietic precursors [96]. So far, murine MNCs have been plated to obtain EOCs, but it is unknown what the exact phenotype is of the circulating cell (see the putative murine CEPCCs phenotypes in Table 1) that gives rise to these murine EOCs *in vitro* (*i.e.* a precursor–product relationship is completely lacking) [97, 98].

With regard to the role in neovascularization *in vivo* of both *in vitro* generated EOCs and EC-like cells, conflicting schools of thought exist. There is evidence that injected (*in vitro* generated) EC-like and EOCs act in synergy during vascular repair. EOCs appear to structurally contribute to neovessels, whereas EC-like cells do not directly contribute to the neovessels, but rather, act in an indirect paracrine fashion by locally secreting angiogenic substances that promote structural healing by resident ECs and incorporated EOCs. Because the incorporation and direct structural contribution to neoendothelium has been claimed to be a major criterion for defining a cell as an EPC, some have redefined EC-like cells as 'angiogenic cells' instead of EPCs, because they do not directly participate in neovascularization, and therefore, do not act as true EPCs in the literal sense (*i.e.* structurally contribute to neovessels) [47, 57, 99]. In fact, this redefinition is in agreement with studies in the embryo [46] and adulthood [23, 25, 45], showing that (non-cultured) haematopoietic lineage cells (that give rise to EC-like cells *in vitro*) do not structurally contribute to growing vessels, but rather, act in an indirect, instructive manner to help in neovascularization. However, disagreement persists in the scientific community with respect to this redefinition and a consensus in the field is far from unanimous.

What are potential caveats with *in vitro* defined cells?

An important drawback in the study of cultured cells is that 'EC-like cells' and 'EOCs' may have acquired or lost properties during culture that influence experimental outcomes (in a positive or negative way) compared to their non-cultured counterpart (*i.e.* the circulating cells from which they originate, which are in fact the cells of true interest, the EPC). In other words, the function (*e.g.* vasculogenic and incorporation properties) and phenotype (*e.g.* surface marker profile) of *in vitro* propagated endothelial lineage cells may be culture artefacts compared to their *in vivo* counterpart. In fact, this question is relevant to any *in vitro* defined cell population and should cause one to be careful in directly comparing the role, phenotype and function of cultured cells with respect to their original *in vivo* (non-passaged) precursors [90, 100]. More specific to the field of EPC biology, two reports showed that injected, non-cultured CD14⁺ monocytes did not enhance or incorporate into neovessels, whereas their *in vitro* derivatives did [76, 84]. This might indicate that native monocytes are already present abundantly at sites of tissue healing [25, 63] or that monocyte-derived EC-like cells are culture artefacts with altered properties (not normally present *in vivo*) induced by the culture conditions, and might not exist as such *in vivo*.

The search for the EOC precursor: lessons from embryonic development

The close developmental association, surface marker, and molecular overlap between haematopoietic cells and ECs supports the concept of the haemangioblast as being the bipotent predecessor for both haematopoietic cells and ECs (see Fig. 2) [101]. Although many studies support the haemangioblast concept *in vitro*, whether this cell exists as a distinct entity during embryonic development *in vivo* and whether it persists into adulthood remain a subject of great debate [102–105]. In fact, some groups have shown evidence for the existence of 'haemogenic endothelial cells' (HECs) (that express the markers CD31, CD34, VEGFR-2 and VE-Cadherin) [106], which provides an alternative explanation for the developmental overlap between haematopoietic and ECs (*i.e.* these 'unipotent' transitional ECs instead of haemangioblasts differentiate into haematopoietic progenitor cells (HPCs) in the embryo, see Fig. 2) [107].

We have used some of the strategies previously utilized to define the embryonic 'haemangioblast' and 'HECs' to address the phenotype of the cell(s) that give rise to EOCs in the human adult, with specific emphasis on the common leucocyte marker CD45, as a defining marker [58]. Both the embryonic haemangioblast and HECs express VEGFR-2 and CD34, explaining why these markers have been popular to identify CEPCCs in the human adult [106, 108, 109]. Importantly, studies in haemato-endothelial development have shown that CD34⁺ embryonic haemangioblasts or HECs do not express the common leucocyte antigen CD45, but acquire this marker only during differentiation into HPCs (and thus become CD34⁺CD45⁺), but not during commitment into the endothelial lineage (Fig. 2) [106, 108,

109]. Therefore, CD45 expression marks haematopoietic specification from foetal life into adulthood, while it is not expressed on endothelial lineage cells (Table 2). The developmental overlap, the common surface marker expression on HSCs/HPCs and ECs, and the findings that CD34⁺ cells can generate both haematopoietic and endothelial progeny in the adult [37] has led investigators to believe that CD34⁺CD45⁺ HSCs/HPCs display a 'haemangioblastic' or 'EPC capacity' in human postnatal life [3, 110]. However, analysis of the marker CD45 was not performed in the original description of CD34⁺ CEPCs [2, 3, 38, 111, 112]. This is a major limitation because CD34⁺ cells in the adult not only contain CD34⁺CD45⁺ HPCs but also a small CD34⁺CD45⁻ cell fraction, similar to the embryonic situation. Importantly, we showed that the CD34⁺CD45⁻ cell fraction, but not CD34⁺CD45⁺ haematopoietic cells within CD34⁺ cells generates EOCs [41, 58]. Therefore, if EOCs are considered to be derived from true CEPCs, these cells should be contained within the CD34⁺CD45⁻ cell fraction, but not within the CD34⁺CD45⁺ haematopoietic cell fraction. Indeed, we showed that the CD34⁺CD45⁺ HSC/HPC cell fraction did not generate EOCs, but differentiated into EC-like cells through a CD14⁺ monocytic pathway [58].

We also detected the marker VEGFR-2 in the CD34⁺CD45⁻ cell population, a tyrosine kinase receptor indispensable for both endothelial and haematopoietic lineages [113, 114]. However, CD34⁺VEGFR-2⁺ cells within the CD34⁺CD45⁻ cell fraction may represent 'mature' ECs [39], or CD34⁺VEGFR-2⁺ cells that co-express CD133 may represent the pool of CEPCs [38, 39]. Indeed, because the CD34⁺VEGFR-2⁺ phenotype may also represent mature ECs, Peichev and co-workers have proposed that CD34⁺VEGFR-2⁺ cells co-expressing CD133 represent true immature CEPCs. Upon differentiation into mature ECs, CD34⁺VEGFR-2⁺CD133⁺ CEPCs are believed to lose expression of CD133. This is corroborated by the fact that mature ECs lining the blood vessels in the adult organism neither express the CD133 protein [38]. However, we showed that the EOC-generating CD34⁺CD45⁻ cell fraction does not express CD133 [58]. In fact, several other investigators have been unable to detect the CD133 antigen on CD34⁺CD45⁻ cells, which confirms our findings [42, 115, 116]. As such, the surface antigen CD133 cannot be used to identify putative circulating EOC-precursors. In fact, CD34⁺VEGFR-2⁺CD133⁺ cells were recently shown to be CD45⁺ haematopoietic progenitors instead of true CEPCs and do not generate EOCs [41, 58]. Therefore, previous reports showing that CD34⁺ (CD45⁺) HPCs contain EPCs or haemangioblasts, and that CD133⁺ cells can differentiate into 'ECs' can be challenged with respect to the specific CD34⁺ subpopulation studied, and/or the criteria used to identify endothelial progeny as bona fide ECs (see Table 3).

Do EOCs derive from an immature CEPC?

The developmental and maturation from 'stem cells' or 'progenitors' into 'mature cells' is well established in many developing sys-

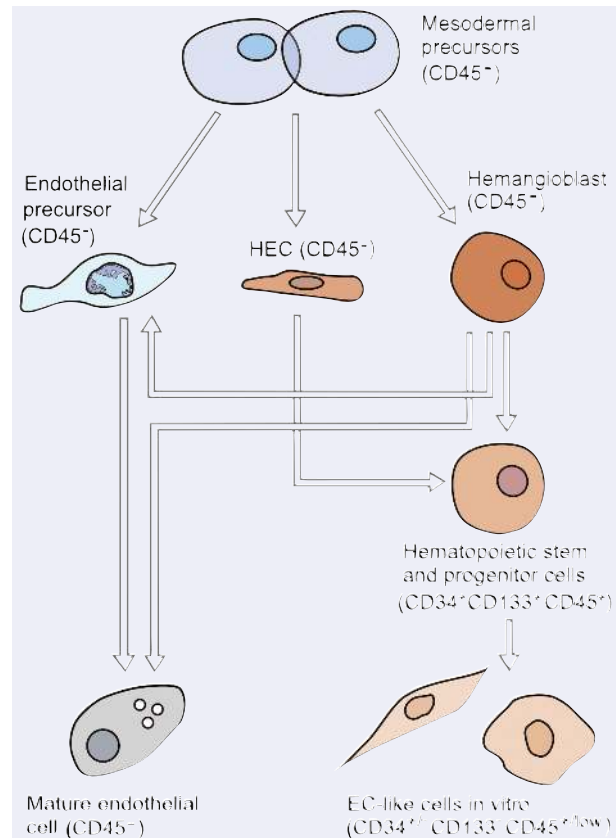


Fig. 2 Haemato-endothelial developmental pathways and their relation to the expression of CD45.

In the embryo, CD45⁻ mesodermal precursors give rise to CD45⁻ endothelial precursors (EPCs), haemangioblasts and/or haemogenic endothelial cells (HECs). The CD45⁻ EPCs differentiate into functional and mature ECs. Embryonic haemangioblasts are CD45⁻ and differentiate to both CD45⁻ endothelial lineage cells and CD45⁺ HSCs/HPCs *in vitro*. Alternatively, or in addition to haemangioblasts, CD45⁻ HECs give rise to CD45⁺ haematopoietic stem/progenitor cells. HSCs/HPCs can give rise to EC-like cells *in vitro* and retain expression of CD45, whereas expression of the CD133 antigen is downregulated.

tems, and is characterized by a stepwise loss and gain of specific markers, and/or cell functions. However, because of the lack of a marker that discriminates circulating EOC-precursors from *in vitro* cultured EOCs, the exact identity of circulating EOC-precursors remains uncertain. As a consequence, it is unknown whether CD34⁺CD45⁻ EOC-precursors are distinct from EOCs generated *in vitro* with respect to phenotypical and functional characteristics. In other words, it is unknown whether the EOCs 'generated' or 'expanded' *ex vivo* represent the differentiated progeny of a distinct, undifferentiated precursor and whether EOCs are just circulating mature ECs.

Nevertheless, it has been claimed that EOCs are a CEPC-derived population based on functional differences between EOCs

and 'control' mature vessel wall ECs such as human umbilical vein endothelial cells (HUVECs). In these studies, EOCs displayed a higher proliferative potential, angiogenic cytokine release and stress resistance compared to HUVEC and other vessel wall ECs [37, 88, 117]. However, conflicting results have been reported on these characteristics and these findings do not allow us to conclude that EOCs are an EPC-derived cell population [118]. Indeed, to address EPCness of EOCs, the most straightforward approach would be the direct assessment of EOCs (head-to-head with mature vessel wall ECs) in *in vivo* vasculogenic assays in mice such as experimental myocardial infarction, hind limb ischaemia and other clinically relevant conditions.

To date however, only one study has reported enhanced vessel forming ability of EOCs compared to 'mature' vessel wall ECs in an ischaemic mouse model [119]. All other studies have categorized EOCs as EPCs or an EPC-derived population based on vasculogenic properties assayed in *in vivo* and *in vitro* matrigel assays [57, 120]. In the matrigel assay, endothelial lineage cells are incubated in an extracellular matrix derived from murine sarcoma cells that supports vascular tube formation. Vascular tube formation in matrigel is thought to recapitulate *in vivo* vasculogenesis and angiogenesis. However, the relevance of matrigel assays for addressing EPCness of EOCs is cumbersome, because 'mature ECs' such as HUVECs were also shown to robustly incorporate and generate vascular tubes using a matrigel assay *in vivo* [57, 120].

Therefore, more experimental data are required to document incorporation and regeneration of durable blood vessels by injected EOCs (and compared with mature vessel wall ECs such as HUVECs) in several relevant *in vivo* models. Moreover, because *in vitro* cultured EOCs may have decreased vasculogenic properties ('decrease of EPCness') compared to their *in vivo* EOC-precursors, the identification of a specific EOC-precursor marker would allow the isolation of EOC-precursors and test them in these assays and directly compare them with cultured EOCs and mature vessel wall ECs.

Do EOCs derive from high proliferative vessel wall ECs?

Because EOCs display striking similarities with 'mature vessel wall ECs', one may speculate whether blood-derived EOCs are just 'culture-expanded' mature ECs that have dislodged from the vessel wall (*e.g.* at angiogenic sites, during increased shear stress, vein-puncture related etc. [121, 122]), rather than being the differentiated progeny from a CD34⁺CD45⁻ BM-derived immature CEPC. Indeed, it is known that ECs can detach from the vessel wall into the circulation in normal physiology and during several clinical disorders [123]. These vessel wall dislodged ECs, designated as circulating ECs ('CECs'), have been claimed to be 'mature ECs', but the general view is that these CECs do not give rise to EOCs *in vitro* because CECs bear no or low proliferative potential, whereas EOCs bear high proliferative potential [123]. In fact, in a seminal report studying sex-mismatched BM transplant patients, Lin *et al.* claimed that EOCs originate from putative BM precursors, whereas CECs with low proliferative potential originate from the vessel wall [87].

However, it is possible that the myeloablative procedure in this study might have attenuated the proliferative capacity of the recipient's vessel wall ECs, therefore neglecting other sources for EOCs than the BM, such as the vessel wall. Indeed, vessel wall ECs such as HUVECs have been shown to expand to similar levels compared to blood-derived EOCs, indicating that 'mature' vessel wall ECs do have a robust proliferative potential and may be at the origin of EOCs [37, 124–126]. This has been corroborated by a recent single cell study showing that ECs of the adult vessel wall display a proliferative hierarchy ranging from non-replicating ECs, to very high proliferative ECs [127]. Notably, we also found that 2% of freshly isolated HUVECs have a similar expansion potential compared to UCB-derived EOCs at the single cell level (F. Timmermans, unpublished observation). Although these findings suggest that EOCs cultured from circulating blood derive from high proliferative vessel wall ECs or true resident vessel wall EPCs (that both can enter into the bloodstream), this issue needs to be clarified. Furthermore, it remains to be seen whether high proliferative vessel wall ECs originally derive from incorporated BM precursors or not. Finally, it would be interesting to know whether BM EOC-precursors are located in the haematopoietic niche of the BM, or reside with the BM vessel wall.

CEPCs and CECs: functionally different cells having the same identity?

It is common use to indicate CECs as vessel wall ECs with low proliferative potential that preferentially slough off from the vessel wall during normal and pathological conditions [123]. Although several studies have suggested that the number of CECs is increased in some disorders [123], there has been a parallel debate on how 'CECs' should be defined from an immunophenotypic point of view (see reference [128] versus [129]), an obstacle reminiscent to the field of CEPCs. Moreover, our data showing that putative CEPCs (EOC-precursors) reside in the CD45⁻ cell fractions, and do not express CD133, further complicates the immunophenotypic discrimination between 'CECs' and 'CEPCs', because to date, the markers CD133 and CD45 were thought to discriminate putative CEPCs from CECs [39, 130, 131]. Also, the claim that CEPCs tend to be smaller cells compared to CECs, has not yet been validated given the lack of a clear precursor–product relationship that included this parameter *in vitro* as well as *in vivo*, as discussed in the first section of this review.

To date, the literature in the field of EPCs supports the marker combination CD34⁺VEGFR-2⁺ to identify CEPCs in humans [112]. However, circulating CD34⁺VEGFR-2⁺ cells may also represent haematopoietic progenitors [132] and mature circulating ECs [39]. Moreover, to date, it has not been carefully addressed whether the CD34⁺VEGFR-2⁺ phenotype contains the CEPCs that generate EOCs *in vitro*. Therefore, the search for a novel marker, or unique combination of markers and parameters that accurately discriminates CECs from CEPCs or high proliferative vessel wall ECs with flow cytometry is mandatory.

Summary

We have highlighted several areas of controversy that persist in the study of EPC biology. While significant progress has been made in our understanding that neovasculogenesis is a multicellular event, and is unlikely to occur from a single recruited progenitor cell type, further progress will require the development of novel assays and conscientious read-out criteria that specifically identify the functional ability of putative EPCs to directly participate in postnatal vasculogenesis. Without a standard set of *in vitro* and *in vivo* assays that allow one to allocate specific cell phenotypic profiles with specific activities, the field of EPC biology may stagnate. One can only look at the tremendous clinical advancements made in the field of HSC transplantation that occurred once both *in vitro* and *in vivo* assays were established, that strictly defined the hierarchical staged development of haematopoietic precursors, as a paradigm for the progress in clinical applications

of EPCs to cardiovascular disorders that may follow the development of similar assays to define neangiogenesis.

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