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Critical re-evaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use

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Abstract

Diverse subsets of endothelial progenitor cells (EPCs) are used for the treatment of ischemic diseases in clinical trials and circulating EPCs levels are considered as biomarkers for coronary and peripheral artery disease. However, despite significant steps forward in defining their potential for both therapeutic and diagnostic purposes, further progress has been mined by unresolved questions around the definition and the mechanism of action of EPCs. Diverse culturing methods and detection of various combinations of different surface antigens were used to enrich and identify EPCs. These attempts were particularly challenged by the close relationship and overlapping markers of the endothelial and hematopoietic lineages. This article will critically review the most commonly used protocols to define EPCs by culture assays or by FACS in the context of their therapeutic or diagnostic use. We also delineate new research avenues to move forward our knowledge on EPC biology.

Keywords

progenitor cells; stem cells; angiogenesis; endothelial progenitor cells; risk factor

Introduction

Since the initial discovery of endothelial progenitor cells (EPCs),[1] significant steps forward have been taken in order to reach a better definition and a detailed functional characterization of these cells. However, the outcome and success of several studies have been limited by the lack of unambiguous and consistent definitions of EPCs.[2] EPCs have been effectively used to stimulate angiogenesis and vascular repair in several experimental settings. Moreover, human autologous cell therapies using EPC-containing products (such as bone marrow or mobilized peripheral blood) are feasible and effective in the treatment of coronary and peripheral ischemic syndromes.[3, 4] Despite these undisputable evidences, the translation of basic research into the clinical practice has been dampened by unresolved

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questions around EPC definition and functions. In parallel, it has been recognized that measurement of circulating EPC levels can provide clinical information on the atherosclerotic burden and even on the future cardiovascular risk. Despite multiple studies showing associations of circulating EPC phenotypes with patient characteristics and prognosis, the pathophysiological impact of circulating EPC levels is still unclear. In this article, we critically review the most commonly used culture methods and surface antigen-based definitions of EPCs in the context of their use as a therapy or diagnostic aid.

2. Characterisation of EPCs

The identification and characterization of EPC has been most challenging and controversial. In general, two approaches have been used to isolate EPCs: a) culture and colony assays and b) selection of subpopulations based on surface markers. Despite the fact that multiple cultured or selected subpopulations improve neovascularization in animal models of ischemia, the true nature and mechanism of action may differ between the various cell populations. Of note, all current methods for identifying or quantifying the endothelial lineage potential of circulating cells suffer from limitations in that none has been shown to reliably predict the behavior of the circulating cells in a relevant in vivo context. Furthermore, it is not known whether cultured cells exist in the bloodstream as such or whether they mainly represent an artificial phenotype generated by specific culture conditions.[5] Furthermore, as we learn more about the plasticity of the cell phenotype, earlier statements indicating that the expression of one or another particular marker “proves” that a cell in culture is not an EPC in vivo, seem to be stronger than the evidence.

2.1. Cultured endothelial progenitor cells

2.1.1. In vitro culture assays—Most culture assays were used to obtain circulating EPCs from peripheral blood for identification of EPCs as biomarkers for cardiovascular disease, for analysis of intracellular signaling pathways, or for enriching cells for therapeutic angiogenesis.[6] Overall, the protocols differ mainly regarding the culture time and most of the short term protocols (4–7 days) yield cells with myeloid/hematopoietic characteristics. Particularly, the “early EPCs” which are generated by culturing peripheral blood mononuclear cells on fibronectin for 4 days in VEGF containing medium[7–9] express CD45 and typical myeloid markers such as CD14 and CD11b. Although several groups reported the co-expression of endothelial markers by these cells, it has been debated whether the detection of endothelial markers might result from a contamination with microparticles deriving from other elements in the culture (such as platelets) leading to false positive events in the FACS analysis.[10] Recent studies show that transfer of epigenetic material, including proteins and nucleic acids, is a previously unappreciated way of intercellular communication.[11–13] This can occur by direct cell-to-cell contact, as shown by Koyanagi et al., who reported that transdifferentiation of early EPCs co-cultured with neonatal cardiomyocyte was dependent upon transfer of complex cellular material through nanotube connections.[11] Moreover, microRNAs were shown to be transported via Gap junctions from cardiomyocytes to cardiac stem cells thereby inducing cardiac commitment.[14] Although not proven for EPCs or bone marrow-derived cells, such direct cell-to-cell communication pathways might modulate cell fate decisions. Transfer of information can also be achieved by cell-.-contact independent mechanisms: for instance, microvesicles have been shown to mediate intercellular communications of bone marrow cells with other cell types, with modification of the transcriptional profile mediated by microRNA and mRNA.[15] Thus, one may speculate that contaminating cells or microparticles transfer RNA or microRNAs to the cultured cells thereby truly modifying gene expression patterns and the cellular phenotype of “candidate” EPCs. This may even leave open the possibility that cellular reprogramming through transfer of nucleic acids occurs in vivo and accounts for hemato-endothelial

"transition" and overlapping phenotypes. However, the lack of a mature endothelial cell phenotype in cultured "early EPCs" is supported by the recent finding that the endothelial gene promoters are silenced.[16] Along this line of evidence, we suggest that evaluation of the epigenetic mechanisms governing phenotypic regulation of the cell represents an important area of investigation that can provide incremental knowledge on EPC biology in vitro and in vivo (Table 1).

Since the proliferative capacity might be one criterion to define a progenitor cell, several groups established colony assays. The most prominent assay was developed by Hill et al, who selected only non-adherent peripheral blood mononuclear cells (with the intention to remove monocyte/macrophages) for 5 days.[17] These colony forming units were named CFU-EC and were initially characterized to express endothelial markers. However, similar to "early EPCs", these cells also express myeloid and hematopoietic markers and subsequent studies have suggested that CD3+ CD31+ CXCR4+ T cells (referred to as angiogenic T cells) are forming the core of these colonies[18] and that a combination of purified T cells and monocytes form CFU-EC structures.[19] Likewise, isolated CD14+ cells were shown to give rise to "early EPCs"[7] and depletion of CD14+ monocytic cells prevented the formation of CFU-ECs.[20] Together, these findings document that short term cultured EPCs constitute a heterogenic population that mainly originates from myeloid hematopoietic cells and share features with immune cells, particularly monocyte/macrophages. Therefore, naming these cells "EPCs" has been criticized and we would prefer the term "circulating angiogenic cells" as already suggested, based on their ability to promote angiogenesis in vivo, not necessarily related to endothelial commitment.[21, 22]

Interestingly, long term culture of "early EPCs" yielded outgrowing cells with a more mature endothelial cell phenotype, which are often referred to as "late" or "outgrowing" EPCs.[23] In addition, Ingram, Yoder and coworkers showed that, when culturing peripheral blood mononuclear cells on collagen for >14 days, mature endothelial cells with a high proliferative capacity can be obtained, which were named "endothelial colony forming cells" (ECFCs).[24, 25] This colony assay clearly reveals that, among the cells composing the "early EPCs" or CFU-EC culture, a small minority has true endothelial differentiation potential. From a clinical perspective, generation of ECFC in culture seems to be a on/off phenomenon, implying that ECFC cannot be efficiently obtained from all donors, especially in relation to age and presence of CVD.[26, 27] However, the protocols to culture ECFCs has been further refined, and humanized larger scale culture assays have been developed.[28] These outgrowing cells appear clonally unrelated to CFU-ECs and, due to the absence of hematopoietic and myeloid markers and the capacity to form vascular networks in implanted matrigel plugs in vivo, these long term cultured ECFCs were considered to be the "true" EPCs. ECFCs, however, lack progenitor markers and are (at the level of endothelial marker expression) indistinguishable from mature endothelial cells. The exact origin of these cells remains to be elucidated, but several authors speculate that these cells are deriving from the vascular wall.[25] If this hypothesis is true, these cells may not be true progenitor cells but a selection of highly proliferating shed endothelial cells.

To reduce the complexity of the cell composition and to define the origin of the EPCs, several groups have used selected bone marrow-, umbilical cord- or peripheral blood-derived CD34+ or CD133+ hematopoietic cells instead of peripheral blood mononuclear cells as the starting material. Whereas some authors were unable to gain colonies of mature endothelial cells,[29] others showed that the culture of selected cells yields co-expression of endothelial cell markers.[30–32] Possibly, cells may react differently depending on the culture conditions. For instance, hypoxic stress or pharmacological modulators of epigenetic enzymes were shown to change the epigenetic signature of endothelial marker genes in cultured EPCs.[16] Moreover, Asahara's group recently further refined the colony assays and

identified two different types of colonies that can be gained from cultured CD133+ single or bulk cells.[30] Cultured adherent CD133+ cells formed CFUs of either small or larger cells; while the small cell-colonies showed a more primitive hematopoietic stage and a highly proliferative activity, the larger cell-colonies exhibited vasculogenic properties. A hierarchical relationship between primitive small cell CFUs and definite large cell CFUs in vitro was also established, indicating that the EPC phenotype in culture is dynamic over time.[30] The systematic use of colony assays represents an important methodological clue to dissect the various steps in the process of EPC generation in vitro and to study cell origin, clonal expansion, hierarchical organization, as well as positive and negative selection (Table 1). However, the existence of in vivo counterparts of cell types defined by colony assays is far from being demonstrated. Generally speaking, the definition of EPCs based on culture protocols has some issues that have not been fully addressed. For example, once a culture process is started, the cells derived are probably no longer representative of what is functioning in the body. Although the cultured cells may as well be an useful product and some culture procedures can be even used to engineer blood vessels in vitro, it is unclear whether the cells obtained after the culture are indeed the same cells that are circulating in the body. The cultured cells are clearly derivative of the circulating cells, but what seems to be lacking so far is to show that the circulating cell(s) that lead to these manufactured cells are, more than other cells, functioning as EPCs in vivo, without being cultured. Even the use of single cell cultures with carefully selected cells does not exclude the possibility that the cells are “reprogrammed” in vitro by the artificial environment.

2.1.2. In vivo features of cultured EPC—Overall, most of the short term or long term culture methods yielded cells with the capacity to improve neovascularization in pre-clinical models.[23, 24, 3334] However, the cells obtained by the protocols differ with respect to their capacity to differentiate in endothelial cells and to physically form new blood vessels. Most studies suggest that the cell gained by the short term culture assays (“early EPCs”, CFU-ECs) predominantly enhance vessel formation by providing a potent mixture of growth factors that support angiogenesis.[35–3834] Rather, the cells obtained after long term culture (“outgrowing EPCs”, ECFCs) may generate endothelial cells and thereby physically contribute to formation of new capillaries.[23, 34] It should be noted that the functional assessment of ECFCs is more in its early stages than the study of short-term cultured EPC, which have undergone a thorough critique and re-evaluation. The use of advanced 3D confocal imaging has significantly re-dimensioned the extent to which bone marrow derived cells appear to contribute to the peripheral endothelium in different settings.[39–42] The ability of ECFC to differentiate into mature endothelium and to replace the peripheral endothelium in vivo should be viewed under the same rigorous scrutiny. Another typical feature of long term outgrowing cells is that they form vascular structures in vitro in the absence of co-culture, whereas the short term cultured cells require the interaction with endothelial cells and particularly promote vascular network formation of mature endothelial cells in vitro.[43] Although it appears as if the discrimination of cells in “early” versus “late” EPCs has reached a consensus in the scientific community, the complex mixture of cells gained particularly in the mononuclear cell culture assays and the fact that “late” EPCs originate from a rare population of cells hidden in the “early EPC”, some protocols may yield cells with overlapping activities e.g. some cultured cells may have both activities. Additionally, the environment may influence the cell fate and therapeutic benefit, and the specific culture conditions may promote endothelial differentiation of myeloid cells.[19, 20] Moreover, the in vivo environment may influence cell fate and function. Thus, under ischemic conditions, injection of fully mature endothelial cells failed to improve neovascularization,[8, 44, 45] although they formed vessels when implanted into Matrigel plugs.[46] Additional complexity is added by the finding that bone marrow-derived or circulating cells can be incorporated in the perivascular area thereby indirectly promoting vessel growth and potentially vessel

stability without forming new endothelium. This feature has first been demonstrated for hematopoietic cells,[47] including the so-called Tie2-expressing monocytes (TEMs),[48] and has been confirmed for human EPCs, which can exist in a quiescent perivascular state in the absence of ischemia, whence they are recruited to the intimal layer after an ischemic stimulus.[49] Consistently, suicide gene studies documented that cultured early EPCs injected in mice with myocardial infarction physically incorporated in vessels in vivo for several weeks without necessarily forming new endothelial cells, yet being needed to support post-ischemic angiogenesis.[50]

2.2. Selection of subpopulation by surface markers

Culture assays have the advantage to expand the cells for therapeutic or diagnostic purposes. However, as discussed above, it is unclear to what extent the artificial milieu changes the cell phenotype and, particularly when starting with preparation of total mononuclear cells, the interaction of the different cells in the mixture may influence the cellular phenotype. Therefore, the direct isolation of cell populations by using surface antigens has the advantage to select defined populations of cells without the necessity of ex vivo manipulation. Several surface antigens have been used to enrich EPCs.

2.2.1 CD34+ and CD133+ cells—The scientific foundation of EPCs is based on the use of isolated hematopoietic CD34+ cells that were shown to give rise to endothelial marker expressing cells in vitro and in vivo.[1] Since CD34 can be also expressed by endothelial cells, other groups have used the more immature marker CD133 to select for putative EPCs.[51] However, these studies have been criticized, and Case et al were unable to confirm that CD34+CD133+KDR+ cells are giving rise to an endothelial progeny and were generally questioning the concept that bone marrow-derived cells can acquire an endothelial cell fate.[29] When analyzing the epigenetic status of CD34+ and CD34+KDR+ cells, indeed a high level of DNA methylation of the eNOS promoter and silencing histone modifications of several endothelial marker genes suggests that these cells are not predisposed to acquire an endothelial cell fate, in the absence of adequate reprogramming stimuli.[16] However, multiple groups have convincingly documented that peripheral blood-, bone marrow- and umbilical cord blood-derived CD34+ or CD133+ cells are enriched for endothelial lineage potential and can express endothelial marker genes and form endothelial structures in vitro and in vivo.[52453053] Moreover, human CD34+ cells physically contributed to angiogenesis in a zebrafish model.[54] The critical role of the CD34+ cells is further supported by the finding that the pro-angiogenic activity is lacking in selected CD34-negative cells.[40, 41] Overall, the interpretation of these discrepant findings is difficult and suggest that not all CD34+ cells can act as EPCs and/or the conditions used to isolate the cells may influence their epigenetic state and functional properties. Indeed, some studies suggested that the subpopulation of CD34+ cells which co-express the VEGF-receptor 2 (KDR) is more enriched in endothelial progenitor cells.[42] Friedrich et al. reported that CD133+CD34-KDR + cells (which have a frequency in peripheral blood similar to CD34+KDR+ cells) are more vasoregenerative and represent a more immature EPC phenotype, which further matures into endothelial cells.[43] The parallel analysis of CD45 expression has been also proposed to distinguish EPCs. Most (90%) CD34+ progenitor cells express CD45 at low intensity (CD45dim), while less than 10% are CD45-negative. Case et al. showed that cord blood and G-CSF mobilized peripheral blood CD34+KDR+ and CD34+CD133+KDR+ cells develop into hematopoietic but not endothelial colonies and that, rather, the CD34+CD45-population forms endothelial colonies in vitro.[29] Given that cord blood and mobilized peripheral blood are enriched in hematopoietic progenitors, it is not clear to what extent these results apply also to the steady state peripheral blood.[55] In addition, the relationship between CD34+CD45- cells and mature circulating endothelial cells (CECs) remain to be established; by analyzing blood samples from male-to-female bone marrow transplantation,

we found that only 5–10% of circulating CD34+ cells are of non-bone marrow origin, and may correspond to CECs (Fadini GP, unpublished data). Finally, Schmidt-Lucke et al. recently reported that CD34+KDR+ cells showed better relationships with coronary artery disease and response to statin therapy if restricted to the CD45dim gate.[45]

2.2.2 Other surface antigens—In order to better define and further enrich for EPCs, several groups used other markers or combinations of several antigens. The SDF-1 receptor CXCR4, which is required for homing of hematopoietic cells, was used to isolate cells with a high migration capacity and improved neovascularization capacity.[56] However, the improved functional activity was mainly attributed to the enhanced homing of CXCR4+ cells and the release of multiple pro-angiogenic cytokines. In addition, cells expressing CD31, a surface antigen that on present in monocytes and endothelial cells, were isolated from peripheral blood and bone marrow and these cells showed a high pro-angiogenic and vasculogenic activity.[57, 58]

2.3. Difficulties in defining EPCs

The first evidence supporting the existence of a common precursor to blood and endothelial lineages stems from the last century. However, even in embryonic development it is unclear whether this concept holds true (for review see [59]) and formal proof of such a common precursor in adult life is still missing. A bilineage potential of bone marrow-derived cells has been documented by various in vitro and in vivo studies.[60] Moreover, by studying patients carrying the BCR/ABL fusion gene in their bone-marrow-derived cells, Gunsilius et al demonstrate that a variable proportions of endothelial cells is generated by bone marrow-derived cells in vitro and in vivo.[61] While, as discussed above, some studies failed to demonstrate a clonal contribution of bone marrow-derived cells to the endothelial lineage, accumulating evidence support a new concept that interconnects hematopoietic and endothelial cells:[62] at least during development, a hemogenic endothelium was shown to give rise to hematopoietic cells.[63] The hemogenic capacity of developmental endothelial cells progressively decreases and ceases as the endothelium matures. Yet, if a specific endothelium had hemogenic potential during development, it may be possible that mature endothelial cells de-differentiate and re-establish an overlapping endothelial-hematopoietic phenotype, including CD45 expression, also in adulthood. Reactivation of antenatal gene expression profiles and cellular phenotypes has been shown in several adult organs and tissues subjected to injury, including the myocardium and blood vessels.[64, 65] Therefore, the discovery of the embryonic endothelial-to-hematopoietic transition (EHT) has important implications for interpretation of the EPC phenotype and vascular biology in general.[66] Genetic and epigenetic studies of the hemato-endothelial origin and fate of putative EPC phenotypes, as well as of the EHT in vitro are important future challenges in this research area (Table 1). Indeed, since several antigenic combinations have failed to distinguish between hematopoietic and endothelial progenitors, lineage tracing studies in mice are mandatory to determine the lineage relation of endothelial and hematopoietic cells. However, these studies might be complicated by the fact that many marker proteins are expressed by both endothelial and hematopoietic cells; e.g. even VE-cadherin, which is considered as one of the best and most specific markers for mature endothelial cells, can be detected on subpopulations of hematopoietic cells in the bone marrow. Provided a common hemangiogenic progenitor exists in the adult organism, even the expression of CD45, which is generally considered a specific pan-leukocyte marker, might not be a reliable watershed between the hematopoietic and endothelial lineage.[62]

3. EPCs as biomarkers of cardiovascular disease

Besides their pathophysiological and therapeutic implications, EPCs have been extensively studied as a novel prototype of cardiovascular risk biomarkers. Various biomarkers (such as for instance, C-reactive protein) are not necessarily involved in the ongoing pathologic processes in the cardiovascular (CV) system. In contrast, although the mechanism by which EPC subtypes control neovascularization and vascular repair remain unclear, various studies suggest that the therapeutic application of the cells affects the recovery of blood flow after ischemia and atherosclerosis. These data indicate that EPC are not innocent biomarkers but active players in maintaining a healthy CV system. Cellular biomarkers were previously limited to classic leukocyte subpopulations, namely neutrophils and monocytes, which correlate with the prevalence and incidence of cardiovascular disease (CVD). Although EPCs are several orders of magnitude less frequent in the bloodstream, the study of these cells has widened the spectrum of cellular biomarkers and has supported the concept that circulating EPCs may affect the CV system.

3.1. EPC quantification in the clinical setting

The measurement of EPCs as cardiovascular biomarkers in large clinical trials requires simple, rapid, and reproducible methods. Flow cytometry is the gold standard for this aim but, as discussed above, none of the proposed antigenic combinations can be considered fully specific for EPCs. Based on the definition of EPCs, the minimal antigenic profile should include at least one marker of stemness/immaturity (usually CD34 and/or CD133 in humans; CD34, c-kit, or Sca-1 in mice), plus at least one marker of endothelial commitment (usually KDR [also known as VEGFR-2 and Flk-1]). Following the original characterization by Asahara,[1] circulating EPCs have been defined as CD34+KDR+ by several investigators, as it was confirmed that this phenotype identifies cells capable of stimulating angiogenesis in vivo.[40] The number of circulating CD34+KDR+ cells is around 50–100/1 million WBC (0.005–0.01%), equal to about 350–700 cells/mL. Subsequently, it has been criticized that this phenotype overlaps in part with hematopoietic stem/progenitor cells (which are CD34+ and can also express KDR) and with CECs (which express KDR and may be CD34+).[67] Although the contamination of the CD34+KDR+ population by CECs (defined as CD45-CD31+CD146+Syto16+)[46] should be very low or negligible, some authors suggest that the co-expression of the stem cell antigen CD133 increases specificity for EPCs, as it is not expressed by mature endothelial cells.[47] Unfortunately, the frequency of CD34+CD133+KDR+ cells in peripheral blood is about 20-fold lower, making quantification less reliable. Indeed, according to the Poisson distribution of rare events, the coefficient of variation is inversely proportional to the number of positive events. Therefore, increasingly complex antigenic phenotypes might be more specific for EPCs, but have lower reproducibility, thus limiting their use in daily clinical practice. One way to circumvent this limitation is to acquire a very large amount of events (1.000.000 to 2.000.000) to gather a higher number of positive events. Some studies used CD31, vWf, or VE-cadherin as markers for the endothelial commitment. While there is no comparative analysis to recommend the use of KDR instead of other markers, it should be noted that CD31, vWf, and particularly VE-cadherin may identify cells in a more advanced stage of maturation along the endothelial differentiation process[68]. However, increased complexity of the antigenic combination, despite providing additional information about the cells under investigation, does not necessarily improve the performance of the cells as clinical biomarkers. Indeed, a disease biomarker does not necessarily have to be highly biologically informative, but it is required to have strong statistical associations with several clinical aspects of the disease. Instead of widening the antigenic phenotype to increase specificity, some investigators aimed at simplifying the identification and quantification of circulating EPCs. For instance, Povsic et al. showed that cells expressing aldehyde dehydrogenase

(ALDH) at high intensity ($ALDH^{bright}$) are enriched in EPCs and have clinical correlates.[49] This strategy may ease to spread the use of EPC-like biomarkers in the clinical practice, especially if the analysis can be performed in a standardized fashion at different laboratories and, possibly, on stored samples. Despite FACS analysis should be regarded as the most appropriate methodology to quantify EPCs in the clinical setting, several studies used culture methods with short-term protocols (early EPCs, CFU-EC, or CACs), which also allowed to study functions of EPCs in vitro. Therefore, the variety of methods used to study EPCs in published clinical studies is so wide that, when it comes to review the literature, specification of the exact method used in each work becomes critical. Indeed, even when looking at the same clinical condition, studies using different methodologies could come to opposite conclusions.[69] Overall, the findings should be considered stronger when the results are confirmed by using different methods to identify or isolate EPCs and, possibly, by different investigators.

3.2 Influence of cardiovascular risk factors on circulating EPCs

Several studies have demonstrated that circulating EPCs are reduced in the presence of classic cardiovascular risk factors, independently from established CVD. Even non-modifiable risk factors appear to impact on EPCs: aging subjects and males have lower CD34+KDR+ EPC levels than young individuals[70] and females,[71] respectively. While this phenomenon reflects the progressive stem cell pauperization occurring with age, the gender difference in EPCs is likely one determinant of the cardiovascular protection of fertile women. It is still not clear whether family history for CVD associates with low EPCs independently of other risk factors,[7273] but the genetic background plays a role.[74] Among classic CVD risk factors, smoking,[75–79] hypertension, [80–86] hypercholesterolemia,[87, 88] obesity[89–91] and diabetes[92–95] have been consistently associated with reduced circulating EPCs, even when using disparate methodologies, ranging from FACS analysis to cell culture. In several occasions, linear correlations were also found between severity of the risk factor and degree of EPC level that support a causal link between risk factors and EPC reduction (Table 2). Regarding emerging risk factors, associations or correlations have been shown between reduced EPCs and hyperhomocysteinemia,[96] microalbuminuria,[97] inflammation,[98] and insulin resistance.[99] In several occasions, the mechanisms whereby a given risk factor affects EPC biology have been identified (reviewed elsewhere, e.g. [100]). It should be noted that CV risk factors most often occur in combination in the same patients. Despite several clinical studies showed the independent effect of the risk factor under investigation in multivariable analyses, it is still unclear to what extent the presence of single risk factors or their combinations negatively affect EPCs. There is indeed evidence from multiple studies that EPCs are progressively reduced as the number of risk factors in the same patient increase.[72, 95] In the setting of metabolic syndrome, clustering of risk factors synergistically impaired the number of circulating CD34+ cells.[101] Importantly, some reports demonstrated that specific treatments of the risk factors are able to restore circulating EPCs toward normal levels. For instance, smoking cessation increased CD45dimCD34+CD133+KDR+ EPCs.[75] Blood pressure lowering with different classes of drugs has shown ability to counteract EPC reduction in hypertensive patients.[102, 103] Blood glucose lowering with insulin therapy in diabetic patients was able to increase circulating CD133+KDR+ and CD34+CD133+KDR+ EPCs,[104] while other anti-diabetic medications may be active on EPCs.[105] LDL-apheresis in patients with familial hypercholesterolemia increased CD34+KDR+ EPCs (although CD34+CD133+KDR+ cells remained unchanged),[106] while statin therapy is among the best characterized intervention to increase EPCs.[107] Finally, weight reduction increased CD34+ and CD34+c-kit+ cells in obese patients, linearly dependent on the degree of lost weight.[91] Taken together, these data consistently show that EPC levels are reduced in the presence of CV risk factors, especially when they cluster together, and that this alteration is partly reversible.

3.3 EPCs and prevalence of cardiovascular diseases

Currently, EPC reduction is considered one mechanism whereby risk factors negatively affect cardiovascular function and promote CVD. It can be speculated that patients with lower EPC levels, at means of risk factors, are more susceptible to the development or progression of CVD, because of the defective endothelial repair and compensatory angiogenesis. Indeed, several manifestations of CVD are associated with reduced EPCs in the bloodstream, after correction for confounding factors.[108] Among the earliest stages of CVD, subclinical signs of vascular damage are indeed marked by further reduced EPCs. Hill et al. were the first to demonstrate a direct correlation between EPCs (CFU-EC) and endothelial function, measured as brachial artery flow mediated dilation (FMD).[17] Subsequent studies have confirmed the relationship between FMD and EPCs, identified as either CFU-EC,[109] CD34+KDR+,[110, 111] CD133+KDR+,[112, 113] or CD34+CD133+KDR+ cells[114] in different population of subjects. These consistent findings obtained using different methodologies substantiate the concept that EPCs represent a biomarker of endothelial function. Furthermore, the earliest anatomical sign of atherosclerotic remodeling, increased intima-media thickness (IMT), has been associated with reduced CD34+KDR+ EPCs in healthy subjects, independently of CRP and the Framingham risk score;[115, 116] similar associations were found for CD34+ cells.[117] Importantly, the level of circulating EPCs further decline in the later stages of atherosclerosis in different districts, as demonstrated for CD34+KDR+ cells in coronary, [118–120] carotid and cerebral,[116, 121–123] and peripheral atherosclerosis,[116121]; again, similar results were obtained with CD34+ cells.[117, 124, 125] Correlations were also found between severity of the atherosclerotic burden and EPC levels,[119–121] indicating that low EPCs represent a biomarker of the systemic atherosclerotic involvement. In the literature, there are remarkable exceptions to the widespread concept that EPCs are reduced in patients at risk of or with established CVD. For instance, using the ECFC culture protocol, Guven et al. paradoxically reported progressively higher EPC numbers in parallel with increasing severity of CAD.[22] In the population-based Bruneck Study, the number of early EPCs in culture was found to be lower in patients with high carotid IMT, but showed a paradoxical direct correlation with the Framingham risk score.[126] The extent to which these discrepancies are related to the method used or to the characteristics of the study population remains to be determined. In the setting of acute CV events, such as myocardial infarction[127–129] and stroke,[130131132] CD34+KDR+ EPCs levels and/or CD34+ cells are increased, because they are mobilized from the bone marrow into the bloodstream (for a review of the mechanism see Aicher et al).[133] This is supposed to be a compensatory attempt to provide vasoregenerative cells and limit residual ischemia and/or achieve better reperfusion. Indeed, a stronger EPC mobilization response is associated with a better outcome in terms of left ventricular function[128, 134] and neurological disability or lesion area/growth[123] after acute MI or stroke, respectively. On the opposite, in pathological conditions characterized by impaired EPC mobilization, such as diabetes mellitus,[135] the angiogenic response to ischemia may be compromised by the insufficient supply of EPCs to the ischemic tissue. Quantitative EPC alterations have been found also in the presence of heart failure (HF).[136] Levels of CFU-EC, CD34+ cells and CD34+CD133+KDR+ EPCs may display a biphasic trend during the various stages of congestive HF, with elevation and depression in the early and advanced phases, respectively.[137] However, as HF is characterized by stage-dependent changes in body fluids, it is critical to determine to what extent these changes in circulating progenitor cells are attributable to hemodilution and hemoconcentration. While this may be unmasked by the correlation between EPC levels and brain natriuretic peptides (BNP),[137] progenitor cell counts should be expressed as fractional, to avoid this artifact. The fractional count of CD34+CD45dim cells appears to be reduced in HF irrespectively of etiology, while it was found that reduction and dysfunction of CFU-EC is typical of ischemic HF.[138] CD34+ and CD34+CD133+ cells were also shown to inversely correlate with NYHA class.[139]

Interestingly, exercise activity was able to increase CD34+ cells and CD34+KDR+ EPCs in HF patients,[140, 141] although HF patients have a reduced ability to mobilized CD34+KDR+ EPCs after physical exercise, compared to control subjects.[142]

3.4 Prognostic impact of EPCs

Besides correlations with prevalent CVD, one of the most important requirement for a candidate biomarker is to be predictive of future CVD events. Only a few studies have evaluated the independent ability of circulating (endothelial) progenitors to predict incident CV events. Paucity of these studies is partly attributable to the fact that FACS analysis performs much better on fresh samples and there is no standardization of FACS for rare events quantification of frozen blood cells. In a sample of 120 individuals at different CV risk (including 43 control subjects, 44 patients with stable coronary artery disease, and 33 with acute coronary syndromes), Schmidt-Lucke et al. found that a CD34+KDR+ EPC level below the median value was associated with a higher incidence of a composite CV endpoint suggestive of atherosclerotic disease progression.[143] Werner et al. enrolled 519 patients with angiographically confirmed CAD, who were followed-up for 12 months: the CD34+KDR+ EPCs, as well as CD133+ cells and CFU-EC were predictive of a first major cardiovascular event, independently of potential confounders.[144] In a population of 216 patients with chronic renal failure on hemodialysis followed-up for an average 23 months, CD34+ cell count was an independent determinant of both cumulative cardiovascular event-free survival and all-cause survival;[145] this finding in hemodialysis patients was subsequently confirmed using early EPC culture.[146] Similarly, low CD34+ cells were predictive of cardiovascular events and total mortality in a population of 214 subjects at different CV risk (including 114 healthy controls), followed for a median of 34 months. Interestingly, the reduced progenitor cell count was associated with increased CV risk especially in patients with metabolic syndrome.[147] Despite all these studies attribute prognostic relevance to the level of circulating progenitor cells, it was not determined to what extent this new biomarker could be used to improve cardiovascular risk stratification in the clinical practice. To answer this question, crude data from the 4 aforementioned longitudinal studies have been pooled to generate a cohort of 1,057 patients with an average follow-up of 1.7 years, who were at moderate to high baseline CV risk (Figure 2). Using statistical metrics specifically designed to assess the performance of a candidate risk biomarker, it was found that progenitor cell quantification (either CD34+ or CD34+KDR+) helps identifying more patients at higher risk of future events over the short term.[148] While these data provide further support to the role of EPCs as CVD biomarkers, replication is needed, especially in lower-risk populations, because biomarkers always tend to perform better in high-risk subjects.[149] Reduced levels of circulating EPC, defined as cultured pro-angiogenic cells (early EPCs) independently predicted cardiovascular deaths and hospitalizations for cardiovascular reasons in a cohort of 111 patients with HF followed for 2.5 years.[150] In a comparative study among cell phenotypes, Schwartzenberg et al. reported that CD34+CD133+ cells, but not CD34+KDR+ and CD133+KDR+ EPC phenotypes, were predictive of future adverse cardiovascular outcomes in 76 patients during a 24-months follow-up.[151] However, this result cannot be considered definitive, as the cohort was small and all patients had ACS, which is known to mobilize EPCs,[152] and can affect the prognostic ability of the measure. Moreover, in the pooled analysis,[148] there was no difference between CD34+ cells and CD34+KDR+ EPC in terms of the prognostic capacity in a much larger cohort of patients. Therefore, comparative analyses are needed to define the best progenitor cell phenotype that predicts future CVD. By now, based on a critical review of available studies looking at different EPC phenotypes as disease biomarkers, the CD34+KDR+ antigenic combination (with or without gating on CD45dim events) appears to be the best compromise in terms of sensitivity, specificity and reliability to quantify EPCs in the clinical setting. In addition, the simple quantification of CD34+ cells has proven as a valid alternative CVD biomarker. In a cohort of patients, levels

of CD34+ cells were more strongly correlated to the Framingham risk score than of CD34+KDR+ cells,[101] and were able to predict incident CV events in the follow-up,[147] while CD34+KDR+ cells were not. At present, the pathophysiological meaning of changes in the total CD34+ cell population is not entirely clear, as they contain ~80% HSC, ~15% EPC, plus a small amount of progenitors for other lineages[153] and CECs. Additionally, pauperization of circulating CD34+ cells may be more linked to a generalized biological aging process than specifically to CVD. Nonetheless, as quantification of CD34+ cells is already performed in most hematology laboratories in a standardized fashion, it may be more easily introduced in the clinical practice for CVD risk estimation than complex EPC phenotypes. To sum up, (E)PCs represent a valuable biomarker of cardiovascular risk that mirrors the natural history of the entire atherosclerotic process. Even if no study has so far determined the changes in EPC levels over such a long period of time to span the entire atherosclerotic disease, integrating results from multiple studies suggests that EPC levels identify patients at different risk for adverse outcomes (Figure 3). Moreover, clinical association data comparing different EPC phenotypes helps in attributing significance to some antigenic combinations, while some others may not even exist in vivo and represent mainly in vitro artifacts.

4. Conclusions

This critical re-evaluation of EPC phenotypes for therapeutic and diagnostic purposes reveals that, as our understanding of cellular plasticity improves, the definite EPC identity becomes even more elusive. While this is to some extent attributable to our limited armamentarium for precise lineage tracing analysis, it may also reflect that the endothelial progenitor is a dynamic phenotype in space and time. Indeed, the endothelial differentiation potential of circulating progenitors varies according to the local environment and changes over time, as can be recapitulated in the culture dish. Furthermore, as embryo studies reveal that a EHT is a naturally occurring phenomenon, persistence of hematopoietic feature may not diminish the interest around “circulating angiogenic cells” (CACs, previously termed early EPCs or monocytic EPCs), which may be hierarchically related to ECFC (true EPCs). As long as therapeutic applications are concerned, a detailed functional characterization of the cells under investigation using preclinical models appears to be more relevant than their antigenic phenotype. For diagnostic purposes, clinical biomarker studies should seek a compromise in terms of specificity of the antigenic combination for EPC definition and reliability of their quantification, possibly analyzing multiple phenotypes simultaneously. So far, definition of EPCs is a work-in-progress and remains a challenge that is continuously providing insights on the relationships between the vascular and hematopoietic systems.

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Non-standard abbreviations and acronyms

ACS	Acute coronary syndrome
CEC	Circulating endothelial cells
CFU	Colony forming unit
CV	Cardiovascular

CVD	Cardiovascular disease
EC	Endothelial cells
ECFC	Endothelial colony forming cells
EHT	Endothelial –to-hematopoietic transition
EPC	Endothelial progenitor cells
FACS	Fluorescence activated cell sorter
FMD	Flow mediated dilation
G-CSF	Granulocyte colony stimulating factor
KDR	VEGF receptor 2
TEM	Tie2 expressing monocytes
VEGF	Vascular endothelial growth factor
vWF	von willebrand factor

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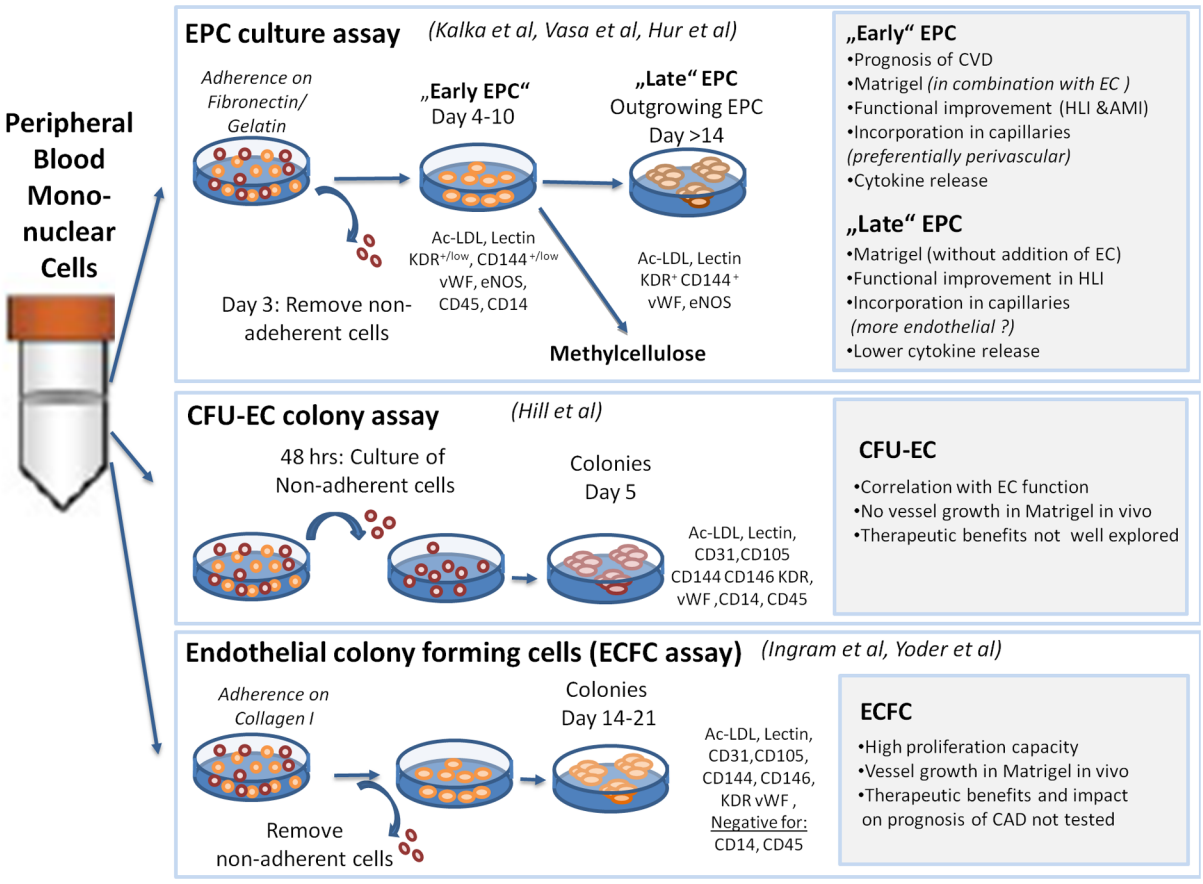

Figure 1.
An overview of the most common methods used to isolate EPCs.

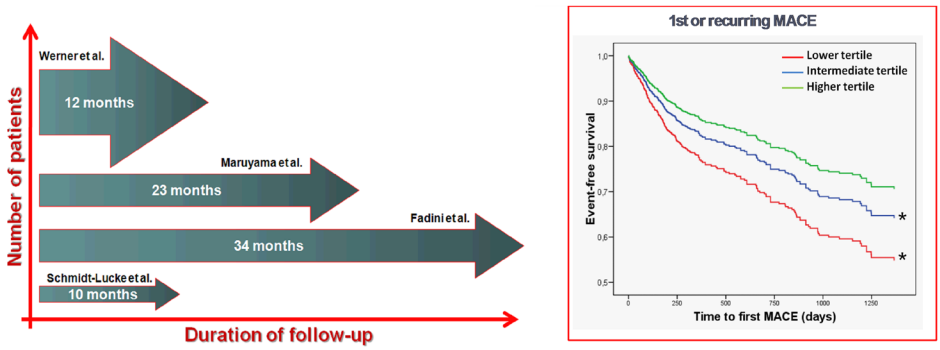

Figure 2.

A pooled analysis of crude data from 4 longitudinal studies of circulating progenitor cells for cardiovascular risk stratification.[128–130,132] In the left panel, characteristics of the study are presented: thickness of the arrows are proportional to the number of patients (y-axis), while length of the arrow is proportional to duration of follow-up (x-axis). The right panel shows Kaplan-Meier curves of occurrence of 1st or recurring major adverse cardiovascular event (MACE) in patients categorized as belonging to the higher, intermediate or lower tertile of circulating progenitor cell levels.

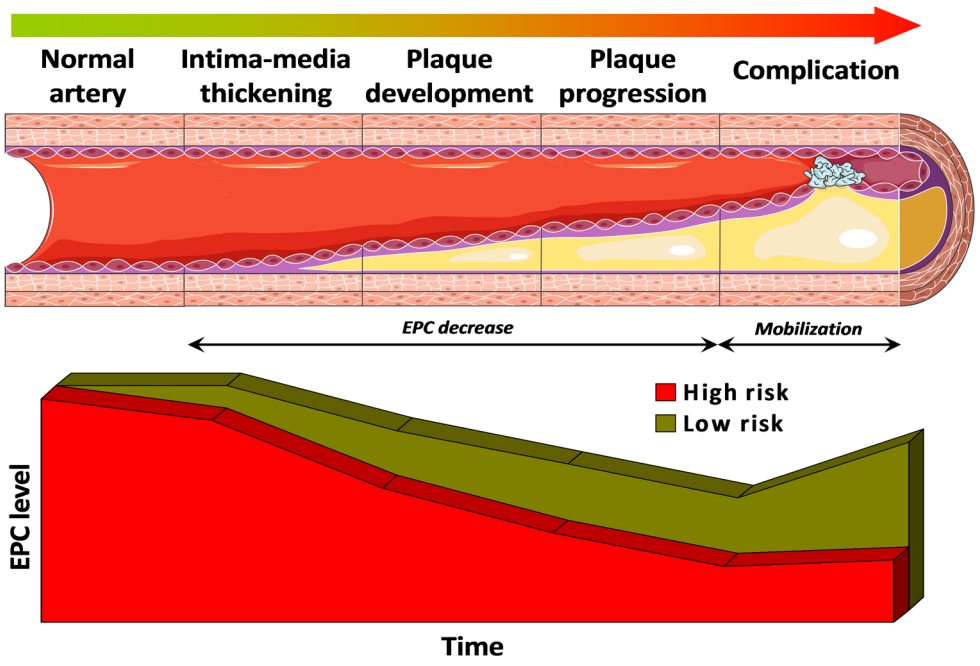

Figure 3.

EPC mirror the natural history of atherosclerosis. The level of EPC (set at maximal in patients with normal arterial anatomy and function) starts to decline when cardiovascular risk factors appear in high risk patients. Further, EPC progressively decline with initial vascular remodeling (IMT), plaque development and progression. Lower EPCs are markers of high risk for future cardiovascular events. Finally, when complications occur (such as AMI or stroke) EPCs should be increased by bone marrow mobilization. When this mechanism is perturbed, a worse outcome can be predicted.

TABLE 1

New avenues in the study of EPC origin and fate

In this table, we describe a few relatively novel important concepts that would significantly expand our current knowledge on EPC biology in the next future.

Current concepts	Future developments
In vitro colony assays	• Lineage tracing of single colonies • Clonal origin of EPC phenotypes • Hierarchical organization of EPC phenotypes
In vivo lineage tracing	• Lineage tracing of the endothelial origin under conditions of tissue homeostasis and disease
Epigenetic regulation	• Transfer of epigenetic material • Phenotypic modulation of EPCs by miRNAs and modifiers of the histone code
Intercellular communications	• Phenotypic modulation by direct cell-to-cell contact • Transfer of proteins and nucleic acids via exosomes and microparticles
Hemato-endothelial transition	• Overlapping phenotypes between endothelial and hematopoietic phenotypes • Parallelisms with the developmental hemangioblast

TABLE 2

Summary of clinical studies reporting EPC alterations in relation to classic cardiovascular risk factors.

Risk factor	Finding/EPCs phenotype	Correlations/Observations
Smoke		
Kondo et al.[75]	Reduced CD45dimCD34+CD133+KDR+ cells.	Inversely correlated with the number of cigarettes smoked. Increase after smoke cessation, and decreased again after resumption.
Ludwig et al.[77]	Reduced CFU-EC, and CD34+/CD133+ cells in smoking women	Independent of hormone cycle.
Yue et al.[78]	Reduced CD34+KDR+ and CD133+KDR+ cells	Inversely correlated with pulmonary arterial pressure and vascular resistance.
Michaud et al. [79]	Reduced early EPCs in healthy smokers vs controls	Directly correlated with plasma anti-oxidant capacity and nitrite concentrations.
Hypertension		
Pirro et al.[80]	Reduced levels of CD34+KDR+ cells in patients with essential hypetension	Correlation with expression of the differentiation factor HOXA9
Oliveras et al.[81]	Reduced CD45dimCD34+CD133+ cells and early EPCs in patients with refractory hypertension.	Independent of confounding factors.
Umemura et al.[82]	Reduced CD45dimCD34+CD133+ cells in patients with hypertension	Hypertension and age independent predictors of low EPCs
Giannotti et al.[83]	Reduced CD34+KDR+ cells in prehypertensive and hypertensive patients	Dysfunction of cultured EPCs.
Delva et al.[84]	Normal number of CFU-EC in patients with essential hypertension.	No correlation with blood pressure.
Yang et al.[85]	Normal number of CD34+KDR+ cells in patients with essential hypertension, but reduced function of early EPCs in vitro.	Proliferatory and migratory activities of circulating EPCs closely correlated with arterial elasticity
Lee et al.[86]	Reduced CD34+KDR+ cells in hypertensive patients with left ventricular hypertrophy	Reduced adhesiveness of EPCs in hypertensive patients with left ventricular hypertrophy
Hypercholesterolemia		
Rossi et al.[87]	Reduced CD34+CD133+ cells and impaired function of early EPCs in patients with high LDL cholesterol.	Inverse correlation between EPCs and LDL and direct correlation with HDL
Chen et al.[88]	Reduction of early cultured EPCs in patients with hypercholesterolemia	<retracted>
Obesity		
Muller-Ehmsen et al.[91]	Reduced CD34+KDR+, CD34+CD133+ and CD34+c-kit+ cells in obese vs overweight subjects.	Negative correlation between EPCs and BMI
Tobler et al.[90]	Reduction of CD34+KDR+, CD34+CD133+KDR+ cells in obese patients	Negative correlation with BMI.
Heida et al.[89]	Reduced function of cultured early EPCs in obese patients.	Eversible by inhibition of p38 MAPK
Diabetes mellitus		
Fadini et al.[95]	Reduced CD34+ and CD34+KDR+ cells in type 2 diabetes	Direct correlation with ABI.
Fadini et al.[94]	Reduced CD34+KDR+ cells in newly diagnosed diabetes and reduced CD34+ cells in pre-diabetes	Negative correlations with fasting and post-challenge glucose.
Loomans et al.[93]	Reduced cultured early EPCs in type 1 diabetes	Negative correlation with HbA1c.
Egan et al.[92]	Reduction of several FACS EPCs phenotypes in type 2 diabetes	Negative correlation with the presence of multiple diabetic complications.