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## Forum Review

# Oxidative Stress Impairs Endothelial Progenitor Cell Function

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**Abstract**

Circulating endothelial progenitor cells (EPCs) in adult human peripheral blood were identified in 1997. Since their original identification, EPCs have been extensively studied as biomarkers to assess the risk of cardiovascular disease in human subjects and as a potential cell therapeutic for vascular regeneration. EPCs are exposed to oxidative stress during vascular injury as residents of blood vessel walls or as circulating cells homing to sites of neovascularization. Given the links between oxidative injury, endothelial cell dysfunction, and vascular disease, recent investigation has focused on the responses of EPCs to oxidant stress and the molecular mechanisms that control redox regulation in these specialized cells. In this review, we discuss the various cell and flow-cytometric techniques used to define and isolate EPCs from circulating blood and the current human and mouse genetic data, which offer insights into redox control in EPC biology and angiogenesis. Finally, we review how EPC responses to oxidant stress may be a critical determinant in maintaining the integrity and function of the cardiovascular system and how perturbations of redox control in EPCs may lead to various human diseases. *Antioxid. Redox Signal.* 10, 1895–1907.

### Introduction to Endothelial Progenitor Cells (EPCs)

Since their original discovery in 1997, circulating endothelial progenitor cells (EPCs) have emerged as an important biologic marker for a variety of human cardiovascular diseases and a potential cell therapeutic for restoration of damaged blood vessels (4, 34, 36, 40, 62, 87, 93, 110). One of the earliest events in organogenesis is the development of the vascular system. In mammals, the early blood vessels of both the embryo and yolk sac develop after differentiation of mesodermal cells or by aggregation of *de novo* forming angioblasts into a primitive vascular plexus (*i.e.,* vasculogenesis), which then undergoes an intricate remodeling process whereby growth, migration, sprouting, and pruning leads to the development of a functional circulatory system (*i.e.*, angiogenesis) (39, 85, 86). The role of EPCs in vasculogenesis and angiogenesis continue to be characterized. However, accumulating evidence strongly suggests that EPCs persist into adult life (4, 30, 78, 84). Endothelial cells (ECs) were first detected circulating in the bloodstream >30 years ago (5, 29, 41, 96), but only recently have they become incorporated into the paradigm of vascular neogenesis.

In 1997, a landmark study by Asahara *et al*. (4) challenged the traditional understanding of angiogenesis and vasculogenesis by suggesting that circulating cells in adult peripheral blood (PB) may also contribute to new blood vessel formation. In these studies, a population of human circulating $CD34^+$ cells was purified that displayed properties of both ECs and progenitor cells. These cells were termed "endothelial progenitor cells" and were purported to give rise to differentiated ECs in a process known as postnatal vasculogenesis. Furthermore, subsequent studies demonstrated that these cells were derived from the bone marrow, circulate in PB, and home to sites of new blood vessel formation that include tumor microenvironments and ischemic tissues (42, 51, 57, 72, 81, 90, 103). Based on this paradigm, and by using variations of basic EPC culture methods and flow-cytometric techniques, changes in EPC concentration have now been correlated to a wide variety of human diseases including cardiovascular disorders, diabetic vasculopathies, and progres-

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sion of angiogenesis in tumor microenvironments (10, 22, 23, 36, 40, 44, 68, 76, 87, 104, 107, 108, 110). Further, EPCs are emerging as potential cell therapeutics for repair of damaged blood vessels (4, 35, 36, 40, 62, 72, 81, 87, 93, 110). Therefore, it is clear that EPCs have a role in vascular homeostasis and pathology and potentially as a therapeutic agent for blood vessel repair.

### EPC Definition and Controversy

At present, no specific marker exists for EPCs, and thus the term EPC is routinely used to encompass a group of cells ranging from circulating ECs to hemangioblasts (*i.e.*, cells that give rise to both hematopoietic stem cells and ECs). EPCs actually represent a minor subpopulation of PB mononuclear cells (MNCs) that can be isolated from both adult PB (36) and human umbilical cord blood (CB) (49) via *in vitro* cell culture methods that were specifically developed to select or expand (or both) this cell population. In general, three culture methods for isolating EPCs have been described (Fig. 1). The original method developed by Asahara *et al*. (4) was subsequently modified (40, 50) and can be performed by using a commercially available kit (Endocult, Stem Cell Technologies, Vancouver, British Columbia, Canada). In this method, unfractionated MNCs isolated from adult PB or CB are plated on fibronectin-coated dishes. After a 48-h adherence to deplete the sample of adherent macrophages and mature ECs, the nonadherent cells are replated on fresh fibronectin-coated dishes. Over the next 5–9 days, *in vitro* clusters of cells, termed colonies, emerge. These colonies comprise round cells centrally and sprouts of spindle-shaped cells at the periphery and are often referred to as colony-forming unit–Hill cells (CFU-Hill) or CFU-ECs (Fig. 1; Method I). In addition, these cells are characterized by uptake of acetylated low-density lipoprotein (acLDL) and binding of the lectin *Ulex europeus* agglutinin-1 (UEA-1), phenotypes ascribed to ECs. Of interest, it was reported that CFU-ECs could be cultured from MNCs enriched for either the hematopoietic stem/progenitor cell marker CD34 or vascular endothelial growth factor receptor-2 (VEGFR-2, otherwise known as KDR) expression (4).

In another widely used and methodologically similar approach, unfractionated MNCs are cultured in supplemented endothelial growth media for 4 days, and the nonadherent cell fraction is then removed, thus resulting in an adherent target cell population (Fig. 1; Method II) (16, 17, 55). The resulting cultured cells display morphologic features similar to ECs, although discrete colonies are not formed. These cells are also characterized by uptake of acLDL and binding to UEA-1 (17, 55). These cells are widely referred to as circulating angiogenic cells (CACs) because they promote neovascularization in animal models of myocardial infarction

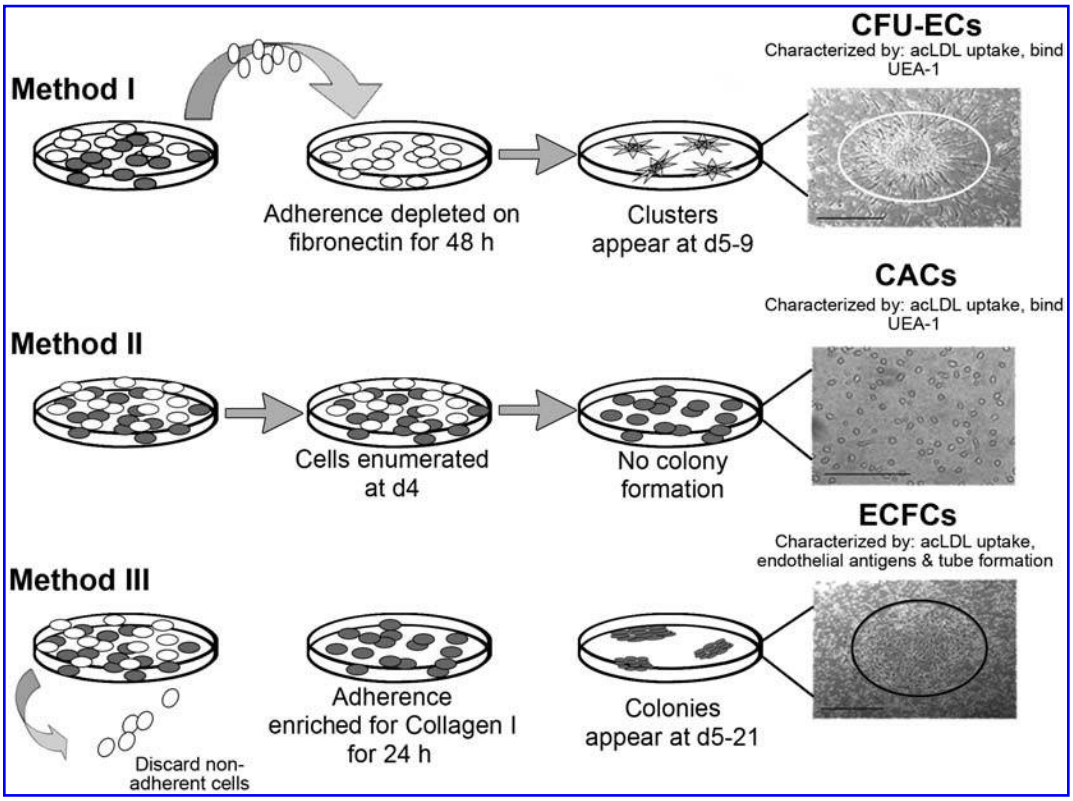


**FIG. 1. Endothelial progenitor cell culture methods.** Method I: Culture of CFU-ECs is a 5-day process in which nonadherent PB or CB MNCs give rise to an EPC colony (scale bar, 100 $\mu$m). Method II: CACs are the adherent PB or CB MNCs of a 4- to 7-day culture procedure. CAC cultures do not form colonies (scale bar, 200 $\mu$m). Method III: ECFCs are derived from adherent PB or CB MNCs cultured for 5–21 days in endothelium-specific conditions and display colonies with a cobblestone morphology (scale bar, 100 $\mu$m). Images were collected by using a Zeiss Axiovert 2 inverted microscope with 10×/0.25Ph1 CP-ACROMAT (CFU-EC and ECFC) or 32×/0.40Ph1 LD-ACROSTIGMAT (CAC) objectives. Images were acquired by using a SPOT RT color camera (Diagnostic Instruments, Sterling Heights, MI) with the manufacturer's software.

(MI) and critical limb ischemia (55, 56, 82, 83). CACs and CFU-ECs appear similar in both *in vitro* function and cell-surface antigen expression. Thus, in the literature, both distinct cell types are often grouped together under the term EPCs.

The third and least studied of all EPC types are referred to as endothelial colony-forming cells (ECFCs). ECFCs are derived from the adherent cells that attach when adult PB or CB MNCs are plated on collagen type 1–coated culture dishes in endothelial-specific growth media. In brief, nonadherent cells are discarded during gentle wash steps and 10–21 days after plating (5–7 days for CB), ECFC colonies emerge from the resulting adherent cell population and display a typical cobblestone EC appearance (Fig. 1; Method III) (48, 49, 65, 113). Furthermore, ECFCs are phenotypically indistinguishable from cultured ECs, uptake acLDL, bind to UEA-1, and possess *de novo* vessel-forming ability (48, 49, 65, 113). Importantly, our group identified a differentiation hierarchy of ECFCs based on colony-forming ability, proliferative potential, and self-renewal capacity of individual cells (Fig. 2). In short, high proliferative potential-ECFCs (HPP-ECFCs) form large, macroscopic colonies that are replatable, demonstrating self-renewal capacity. HPP-ECFCs give rise to all subsequent stages of ECFC progenitors in addition to secondary replated HPP-ECFCs. Low proliferative potential-ECFCs (LPP-ECFCs) form colonies that contain >50 cells, and EC clusters are colonies with two to 50 cells. Neither LPP-ECFCs or EC-clusters form colonies on replating (49). Interestingly, our group determined that ECFCs are enriched in CB and vascular endothelium such as human aortic ECs and human umbilical vein ECs (HUVECs) (48). Although these data are intriguing and support the concept that EPCs are resident in blood vessel walls, these findings make interpretation of previous studies challenging because cell lines derived from vessel walls are routinely used as a source of differentiated ECs in the literature. Because ECFCs appear later in culture compared with both CFU-ECs and CACs, they are also referred to as "late outgrowth" EPCs in the literature, whereas CFU-ECs and CACs are referred to as "early outgrowth" EPCs. However, this nomenclature is confusing and may no longer be appropriate, as it does not accurately identify the various EPC subpopulations being studied.

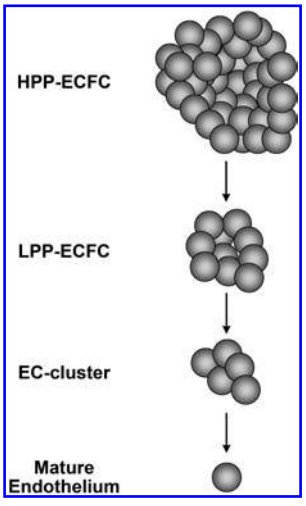


**FIG. 2. Endothelial colony-forming cell hierarchy model based on proliferative and clonogenic potentials.** High proliferative potential–endothelial colony-forming cells (HPP-ECFCs) are large colonies that, on replating, form secondary and tertiary colonies. In addition to replating into secondary HPP-ECFCs, they also give rise to all the subsequent stages of endothelial progenitors. Low proliferative potential–endothelial colony-forming cells (LPP-ECFCs) do not form secondary colonies or LPP-ECFCs after replating and contain >50 cells. EC-clusters contain <50 cells and can arise from a single cell. Mature terminally differentiated endothelial cells do not divide.

The input cell populations for the three culture methods used to isolate EPCs (Fig. 1) are typically heterogeneous (*i.e.*, low-density MNCs), making it difficult to determine the exact precursor cell that gives rise to the cultured EPCs. Conversely, EPCs can be prospectively identified without the need for culture by selecting subpopulations of MNCs based on cell-surface antigen expression. However, the definition of EPCs via this method is rather complex because of the absence of any restricted and unique markers for EPCs. In the majority of studies to date, EPCs are typically identified via flow-cytometric techniques that prospectively identify MNCs that coexpress the cell-surface antigens CD34, AC133, and/or VEGFR-2 (4, 30, 64, 71, 78, 84, 92, 110). In other work conducted to improve characterization of EPCs and differentiated EC antigenic markers, Peichev *et al.* (78) reported that nearly all $CD34^+VEGFR\text{-}2^+$ circulating EPCs in human granulocyte–colony-stimulating factor (G-CSF) mobilized adult PB, CB, and fetal liver, express AC133, whereas mature ECs do not express AC133. These data indicate that AC133 may be a possible discriminator antigen for undifferentiated EPCs versus differentiated ECs (78). Additionally, these investigators conducted experimental bone marrow transplants with $CD34^+$ cells in patients with left ventricular assist devices to examine antigenic expression profiles of ECs that colonize implanted devices. These studies demonstrated that $AC133^+VEGFR\text{-}2^+$ and $CD34^+VEGFR\text{-}2^+$ cells were detected on the surface of left ventricular assist devices. Thus, these data suggest that *in vivo* angiogenesis may be achieved by the mobilization and recruitment of circulating EPCs to the site of vascular injury (78). Furthermore, these findings suggested to the authors that the circulating $CD34^+$ cells co-expressing AC133 and VEGFR-2 represent a functionally distinct population of EPCs that may play a role in blood vessel formation (78).

Interestingly, despite their original identification and increasing use as a biomarker for vascular disease, $CD34^+AC133^+VEGFR\text{-}2^+$ cells had never been isolated and simultaneously plated in EC and hematopoietic clonogenic assays to ascertain the identity of their clonal progeny. Therefore, our group and others recently challenged the use of the $CD34^+AC133^+VEGFR\text{-}2^+$ surface antigen combination to identify clonally expandable circulating EPCs with a high capacity to acquire an endothelial phenotype (9, 98). By using G-CSF–mobilized PB and CB (enriched sources of both hematopoietic stem cells and EPCs), we isolated and purified $CD34^+AC133^+VEGFR\text{-}2^+$ cells by fluorescence-activated cell sorting and assayed for the presence of clonogenic ECFCs as well as hematopoietic progenitors in the sorted cell population. These studies demonstrated that $CD34^+AC133^+VEGFR\text{-}2^+$ cells are enriched for hematopoietic progenitors, and >98% of these cells express the universal hematopoietic cell surface antigen, CD45. In addition, these studies demonstrated that $CD34^+AC133^+VEGFR\text{-}2^+$ cells do not form ECFCs in culture. Further studies by our group and others suggest that $CD34^+CD45^-$ cells have a higher capacity to acquire an EC phenotype, whereas

$CD34^+AC133^+VEGFR\text{-}2^+$ cells do not differentiate into ECs (9, 98).

To address the need for a more accurate identification strategy for EPCs in the blood, two independent groups recently reported a more rigorous definition by multi-parameter flow cytometry (19, 69). Most recently, Duda and colleagues (19) refined EPC descriptions by devising an alternate set of antigenic expression profiles. Specifically, a standardized flow-cytometric–based method was established for enumerating distinct EPC subpopulations in PB that serve as biomarkers for vascular disease risk and response to antiangiogenic therapies in human cancers (19). In brief, unfractionated MNCs are stained with antibodies directed against endothelial- and progenitor-specific antigens. Viable MNCs are analyzed for $CD34^+AC133^+$ and $CD34^+CD45^-$ cells, which are surrogate markers for CFU-ECs (40) and ECFCs, (9) respectively. Additionally, Duda *et al.* (19) describe a $CD31^+CD45^-CD34^+AC133^-$ population of circulating endothelial cells (CECs, which are hypothesized to be differentiated ECs) that correlate with disease progression in various pathologies (Fig. 3). However, despite the phenotypic identification of CECs, $CD31^+CD45^-CD34^+AC133^-$ cells have never been prospectively isolated and plated into either EC or hematopoietic clonogenic assays to assess the true identity/origin of their clonal progeny. Future studies to address this question are important to enhance our understanding of the biologic implications for changes in circulating levels of these cells.

Regardless of the lack of a consensus definition for circulating EPCs, analysis of EPC concentration in the blood to evaluate for a correlation with disease has been conducted in a variety of clinical settings. As mentioned previously, a common definition for the most primitive EPC precursor in the circulating MNC population is cells that co-express CD34, AC133, and VEGFR-2 (4, 30, 64, 71, 78, 84, 92, 110). Because EPCs are hypothesized to facilitate healing through direct vessel repair or via the formation of new vessels, this EPC definition has formed the basis of many clinical studies designed to determine the role of EPCs in several cardiovascular disorders. Generally, the concentration of circulating $CD34^+$, $VEGFR\text{-}2^+$, or $CD34^+AC133^+VEGFR\text{-}2^+$ cells in the PB correlates with the risk of developing adverse cardiovascular outcomes. Specifically, an inverse correlation with each of these phenotypically defined cell subsets and

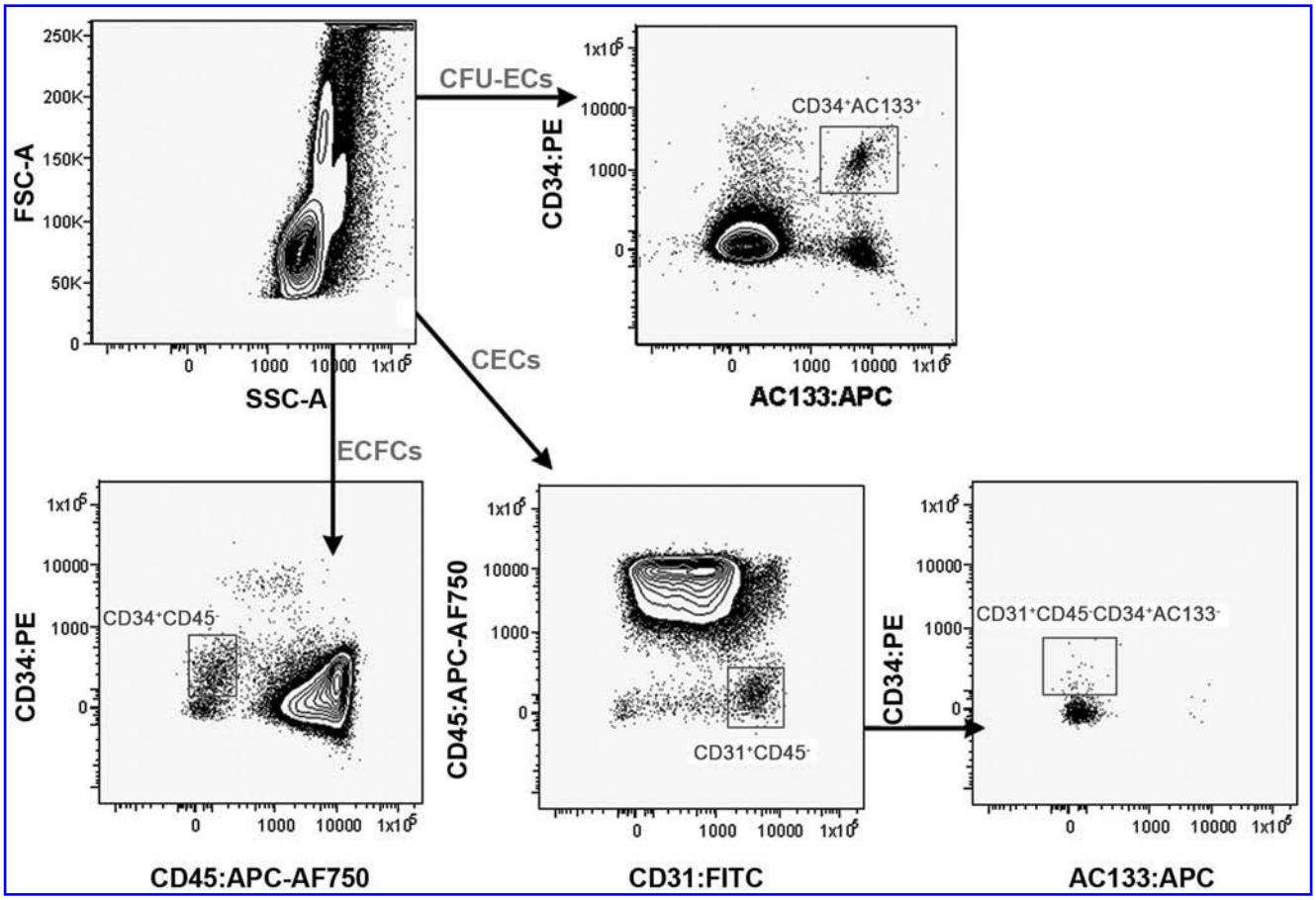


**FIG. 3. Flow-cytometry method to assess accurately the frequency of rare circulating EPC subpopulations in the peripheral blood.** The first fluorescent channel is a "dump channel" used to exclude nonviable cells, platelets, and red blood cells from the analysis. The antigens used are CD34 and CD133 (both stem and progenitor antigens), CD45 (hematopoietic cell antigen), and CD31 (endothelial cell antigen). The gating strategies illustrated depict the three main EPC subpopulations: $CD34^+CD133^+$, $CD34^+CD45^-$, and $CD31^+CD45^-CD34^+CD133^-$, enriched for CFU-ECs, ECFCs, and CECs, respectively.

the highest cardiovascular disease risk category exists (20, 62, 91, 92, 110).

### Interactions Between CFU-ECs and ECFCs

As outlined earlier, adult PB and CB contain subpopulations of EPCs that are identified by *in vitro* culture methods and flow cytometry. Two major subpopulations of EPCs with distinct functional properties have been reported, CFU-ECs and ECFCs. Recent studies are beginning to dissect how CFU-ECs and ECFCs may interact to facilitate blood vessel repair. Our previous studies demonstrate that CFU-ECs are derived from hematopoietic stem cells and exhibit macrophage characteristics, even though they remain classified as EPCs (113). In contrast, ECFCs possess *de novo* vessel-forming ability *in vivo* and are clonally distinct from hematopoietic stem cells (113). Furthermore, it is possible that bone marrow–derived CFU-ECs (*i.e.*, $CD34^+AC133^+$ cells) circulate in the PB to facilitate the angiogenic response of resident and circulating ECFCs (*i.e.*, $CD34^+CD45^-$ cells). In support of this concept, several *in vitro* studies demonstrate that CFU-ECs stimulate ECFC proliferation to form new vessels via endothelial sprouting or to repair sites of endothelial damage (Fig. 4) (48, 49, 113). Recently, Yoon *et al*. (115) tested the hypothesis that the delivery of a mixed population of CFU-ECs and ECFCs would have synergistic angiogenic effects *in vivo* with murine ischemic models. These studies initially demonstrated that *in vitro* coculture of CFU-ECs (or the use of conditioned media from CFU-ECs) stimulated the ability of ECFCs to proliferate and form tubes via secretion of IL-8 and VEGF. When a mixed population of CFU-ECs and ECFCs were injected into a murine model of hindlimb ischemia, an increase in limb salvage and limb perfusion was observed compared with injection of either CFU-ECs or ECFCs alone (115). Furthermore, in mice injected with both CFU-ECs and ECFCs, a marked increase in the number of delivered cells was detected, signifying an enhanced viability or resistance of the mixed cell populations to apoptosis in an ischemic environment. Taken together, these data indicate that CFU-ECs and ECFCs are both operative in vascular repair, which is consistent with their distinct developmental origins. Further studies are now needed to determine the specific molecular interactions between cells that give rise to CFU-ECs and ECFCs in facilitating blood vessel repair.

### Oxidant Stress Response of EPCs

The concept that endogenous reactive oxygen species (ROS) cumulatively damage cells over time emerged as a mechanism for organismal aging and disease >50 years ago. It is now recognized that, in addition to inducing cellular damage, ROS serve as secondary intracellular messengers and affect the overall redox status of a cell (2, 26, 74, 102). Importantly, the intracellular redox environment has a critical role in controlling apoptosis, proliferation, self-renewal, senescence, and differentiation (2, 58, 59, 116). Dysregulation of any one of these phenotypes in EPCs will alter EC function, predisposing to the development of vascular pathology.

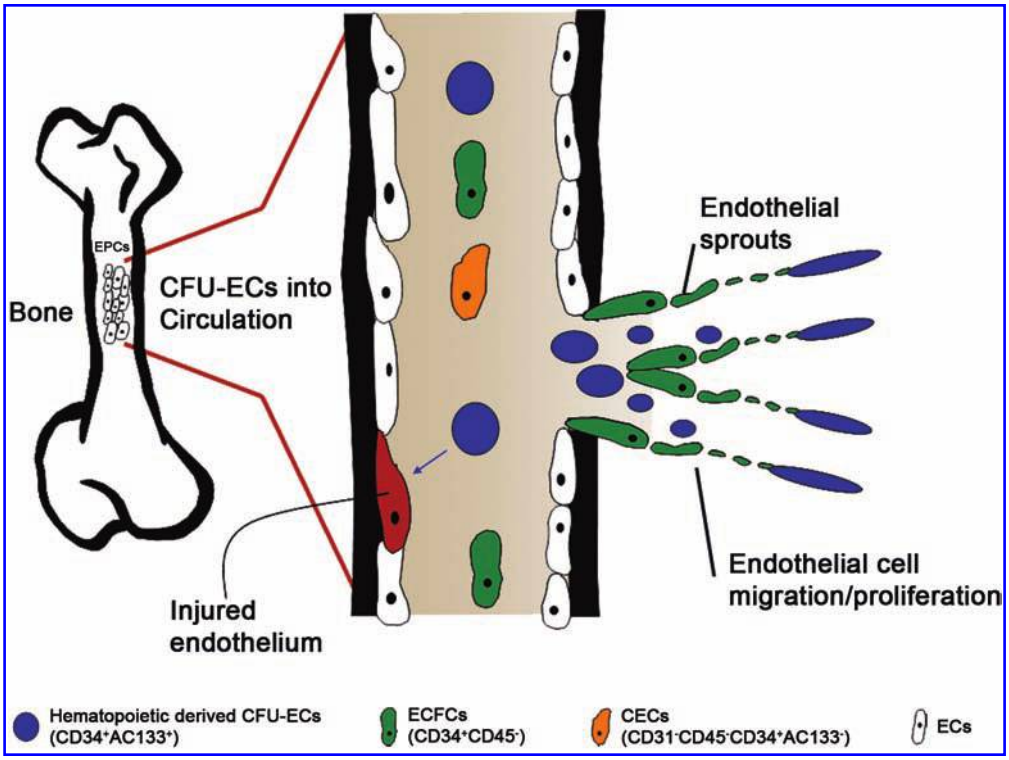


**FIG. 4. Model for the interaction between CFU-ECs, ECFCs, and CECs in angiogenesis and vascular repair.** CFU-ECs ($CD34^+CD133^+$) are derived from hematopoietic stem cells and circulate in the blood, facilitating the angiogenic response of ECFCs ($CD34^+CD45^-$). CFU-ECs and ECFCs are not clonally related. The CFU-ECs stimulate the proliferation of ECFCs to form new vessels via endothelial sprouting or to aid in repairing sites of endothelial damage. The exact function of CECs ($CD31^+CD45^-CD34^+CD133^-$) in sustaining endothelial integrity is less clear.

Numerous disease states enhance oxidant stress *in vivo* before clinically significant vascular disease (13, 67, 97). In addition, acute ischemia is characterized by enhanced ROS production in affected microenvironments. Therefore, it was suggested that EPCs may be resistant to ROS-induced toxicity to facilitate vascular repair and vessel formation in prooxidant tissue microenvironments. To test this premise, Dernbach *et al.* (14) examined oxidant sensitivity of EPCs from healthy adult donors. The culture conditions used to harvest EPCs in these studies isolated an adherent cell population that co-expresses hematopoietic and endothelial antigens (Fig. 1; Method II). Thus, these cells are CACs. Importantly, these studies demonstrated that CACs from healthy adults generate low levels of intracellular ROS and undergo less oxidant-induced apoptosis compared with HUVECs, which were used as differentiated EC controls. Interestingly, CACs had increased expression of the antioxidants manganese superoxide dismutase, glutathione peroxidase, and catalase compared with HUVECs. Based on these findings, the authors speculated that high antioxidant levels in CACs may be the molecular mechanism that promotes survival and vascular regeneration in ROS-rich environments. Additional insight can be gleaned from these important studies after taking into account recent data from our group showing that HUVECs are enriched for ECFCs (Fig. 1; Method III) (48). Therefore, these studies suggest that vessel wall–derived ECFCs, which contain EPCs with *de novo* vessel-forming ability, are highly sensitive to oxidant stress and that CACs, or the angiogenesis-facilitating cells, are equipped with enhanced antioxidant systems to detoxify ROS, resulting in improved resistance to oxidants.

Further evidence that increased antioxidant capacity improves survival of EPCs was shown by He and colleagues (38). In these studies, adult PB-circulating ECFCs were protected against oxidant-induced apoptosis compared with vessel wall–derived ECFCs (*i.e.*, HUVECs and coronary artery endothelial cells) via upregulation of manganese superoxide dismutase (38). However, in our hands, similar levels of $H_2O_2$-induced apoptosis were observed in ECFCs derived from either HUVECs or adult PB (Fig. 5). The apparent discrepancy between these studies may relate to differences in the passage number of ECFCs used, because extended culture enhances EC differentiation, senescence, and risk for cytogenetic alterations. Our studies were conducted on early-passage ECFCs (P<4) in contrast to He *et al.* (48), who used later-passage ECFCs (P4 to 8). In addition, other potential confounding variables include the age of adult PB donors and the health of the pregnancy from which HUVECs were obtained. Nevertheless, given that HUVECs are highly enriched for immature ECFCs compared with adult PB ECFCs, together these data suggest that in the setting of ischemic injury, primitive ECFCs are highly sensitive to oxidant stress, a critical conceptual change within the vascular biology field.

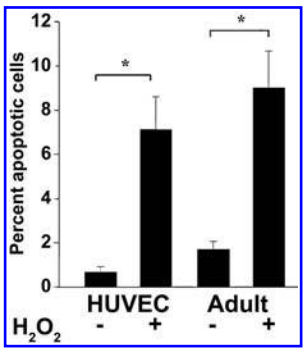


**FIG. 5. HUVECs and ECFCs harvested from adult peripheral blood have similar levels of $H_2O_2$-induced apoptosis.** Low-passage (P<4) HUVECs and ECFCs from adult PB were treated with 200 $\mu M$ $H_2O_2$ before assessing apoptosis by using a TUNEL assay, as previously described (46, 88). The percentage of TUNEL-positive cells is illustrated. Data shown are the mean ± standard error of the mean of a representative experiment conducted in triplicate, $n = 3$ independent experiments with similar results; $^*p < 0.01$ by Student's paired $t$ test.

Formally to test the oxidant sensitivity of circulating ECFCs from different sources of blood, our group examined CB- and adult PB-derived ECFCs for reduced progenitor colony formation and function after oxidant treatment. Single-cell assays evaluated whether primitive ECFCs (*i.e.*, HPP-ECFCs) were differentially sensitive to oxidants compared with more-differentiated ECFCs (*i.e.*, LPP-ECFCs and EC-clusters). Interestingly, CB and adult PB HPP-ECFCs were highly sensitive to oxidant treatment compared with LPP-ECFCs and EC-clusters. Furthermore, matrigel assays and xenograft transplant experiments demonstrated that oxidant treatment severely impairs the vessel-forming ability of ECFCs *in vitro* and *in vivo*, respectively. However, an unexpected finding was that ECFCs from adult PB samples were more sensitive to oxidant stress compared with CB-derived ECFCs. These data are intriguing and support the concept of a developmentally regulated oxidant-stress response in ECFCs, whereby the physiologic process of aging changes responsiveness to oxidant stimuli.

Understanding the molecular pathways contributing to this response may provide important mechanistic insights into the development of endothelial dysfunction and vascular disease.

One component of age-related differences in oxidant responsiveness of ECFCs may be due in part to accumulation of oxidative damage over time from normal metabolic functions, which produce harmful ROS by-products. These internally produced moieties, together with external stressors (*i.e.*, inflammatory cytokines, hyperglycemia, and hypertriglyceridemia), constitute the elements of oxidative stress that ECFCs encounter *in vivo*. Over an individual's lifetime, long-lived cells such as EPCs incur repeated exposures to oxidative stress. Initially, EPCs may compensate by increasing antioxidant responses to counteract the untoward effects of oxidant injury. Over time, oxidant damage likely accumulates in EPCs, diminishing cellular function, and enhancing vascular disease risk. Recent studies from our group support this concept in ECFCs exposed to hyperglycemia *in vitro* or a diabetic intrauterine environment *in vivo* (47).

Emerging epidemiologic data demonstrate that infants born to women with diabetes are at increased risk of developing subsequent vascular dysfunction. However, little is known about the effect of maternal diabetes on fetal EPCs. Therefore, we examined neonatal ECFC function from control and diabetic pregnancies. These studies demonstrated that CB-derived ECFCs from diabetic pregnancies were reduced in number and function compared with controls (47). Specifically, ECFCs exposed to a diabetic intrauterine environment exhibited premature senescence, reduced cytokine-stimulated proliferation, and reduced vessel-forming capac-

ity *in vitro* and *in vivo* compared with neonatal ECFCs harvested from uncomplicated pregnancies. These studies support a model whereby fetal exposure to a maternal diabetic environment results in functional changes in the fetal vasculature consistent with premature aging. Given that oxidant stress is involved in the pathogenesis of diabetic vascular disease in adults (7, 8, 67, 73, 75, 97, 101), together with the premature aging phenotypes identified in ECFCs from diabetic pregnancies, we tested whether ECFCs from diabetic pregnancies had evidence of significant oxidative DNA damage. With a flow cytometry–based quantitation of 8-oxoguanine, a marker of oxidative DNA modification, our recent studies demonstrate that ECFCs from diabetic pregnancies have nearly a threefold increase in oxidative DNA damage (Fig. 6). Future studies to understand further the molecular mechanisms involved in promoting the premature aging phenotypes observed in ECFCs exposed *in utero* to maternal diabetes will be interesting. Furthermore, examining whether CFU-EC or CAC functions are similarly impaired will be crucial to extend current knowledge in vascular disease pathogenesis. Given that the burden of diabetes in pregnancy is increasing (1, 6, 15), identifying the underlying mechanisms responsible for the increased risk of vascular disease in offspring of diabetic pregnancies is paramount to finding potential preventive strategies and to having impact on an escalating health care problem.

In addition to accumulation of oxidative cellular damage, it is possible that the overall redox environment of EPCs may be altered as a consequence of aging. In this case, the cytoplasmic reducing environment of adult ECFCs may be more oxidized, or alternatively, less reduced compared with neonatal ECFCs. Therefore, one might predict activation of redox-sensitive molecules after encountering lower levels of oxidant stress. To determine whether oxidant-induced apoptosis in ECFCs is dependent on a redox-regulated protein, we examined activation of apoptosis signal-regulating kinase 1 (ASK1) in CB-derived ECFCs treated with $H_2O_2$. ASK1 activity is controlled by multiple redox-sensitive proteins including thioredoxin, glutathione-S-transferases, and glutaredoxin (Fig. 7) (11, 18, 31, 43, 66, 89, 94, 95). Oxidation of free sulfhydryl groups within these redox-sensitive proteins results in ASK1 activation, which induces a variety of cellular fates, depending on the stimulus and cell type examined, including apoptosis, senescence, proliferation, and inflammatory cytokine secretion. $H_2O_2$-treated ECFCs had a significant increase in ASK1 activity compared with untreated controls (46). Furthermore, the predisposition of ECFCs to undergo $H_2O_2$-induced apoptosis leading to reduced capillary tube formation in matrigel assays was dependent on intact ASK1 kinase activity. Importantly, these data implicate enhanced ASK1 signaling as a critical molecular mechanism involved in diminished vessel-forming ability of ECFCs after oxidant stress.

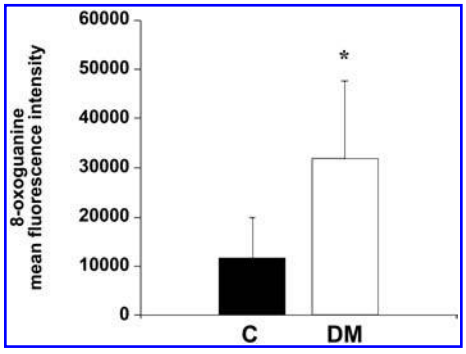


**FIG. 6. ECFCs isolated from the cord blood of diabetic pregnancies exhibit increased oxidative DNA damage compared with controls.** To assess oxidative DNA damage in cord blood ECFCs, ECFCs were fixed and permeabilized, followed by a 60-min incubation with a fluorescein isothiocyanate–labeled Biotrin OxyDNA probe (BD Biosciences), which binds 8-oxoguanine. Labeled ECFCs were then analyzed with flow cytometry, and mean fluorescence intensity was measured. The data shown represent the mean ± standard error of the mean calculated from four different cord blood donors for control (C) and diabetic (DM) pregnancies. *$p < 0.01$ by Student's paired $t$ test. Higher mean fluorescence intensity reflects increased oxidative DNA damage.

## Molecular Mechanisms of Redox Control in Angiogenesis

Redox signaling is an emerging area of investigation in vascular biology. ECs generate ROS such as superoxide and $H_2O_2$, which function as intracellular secondary messengers to regulate many aspects of growth factor–mediated responses, including angiogenesis. A key source of ROS in ECs is through an NADPH oxidase pathway. NADPH oxidase is a major source of ROS in the vasculature (34), with several studies suggesting that $gp91^{phox}$ (also referred to as Nox2) is a critical component of the ROS-generating NADPH oxidase activated by a range of stimulants in ECs (27, 33, 63). Interestingly, Ushio-Fukai *et al.* (106) and Harfouche *et al.* (37) demonstrated that both angiopoietin-1 and VEGF, two important angiogenic cytokines, stimulate EC migration via the activation of a $gp91^{phox}$ containing NADPH oxidase. In addition, $gp91^{phox}$ expression increases in association with the enhanced ROS production observed in pathologic murine models of angiogenesis, including retinopathy and hindlimb ischemia (3, 100). Furthermore, neovascularization in response to VEGF or ischemia is inhibited in $gp91^{phox\,-/-}$ mice, and in wild-type mice treated with NADPH oxidase inhibitors (gp91ds-tat or apocynin), or the antioxidant, ebselen (3, 100, 106). Collectively, these studies support an essential role of $gp91^{phox}$ in angiogenesis.

Another well-known regulator of angiogenesis that is under redox control is the protein ASK1. ASK1 is a member of the mitogen-activated protein kinase kinase kinases and activates the c-Jun $NH_2$-terminal kinase and p38 kinase pathways. Activation of the ASK1 pathway constitutes a pivotal molecular response to numerous types of stress-induced apoptosis (43). ASK1 has an essential role in both oxidative stress– and endoplasmic reticulum stress–induced apoptosis (99). Additionally, ASK1 activation regulates cellular senescence, cytokine production, and proliferation (Fig. 7) (52, 70, 114). Importantly, Yokoi *et al.* (114) recently demonstrated that high glucose induces activation of ASK1 and cellular senescence in ECs and that downregulation of ASK1 activity suppresses EC senescence induced by high glucose. In addition, these investigators demonstrated that ASK1 activation enhances plasminogen activator inhibitor-1 (PAI-1) expression in ECs. PAI-1 is a key protein involved in aging-associated thrombosis and a major inhibitor of fibrinolysis. In diabetic patients, PAI-1 expression in the arterial wall and

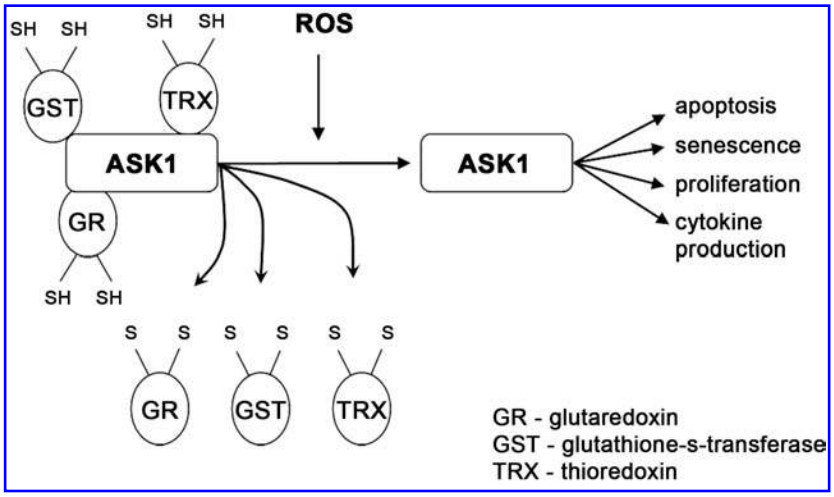


**FIG. 7. ASK1 activity is regulated by multiple redox-sensitive proteins.** Binding of ASK1 to redox-sensitive proteins (GST, GR, and TRX) in a reduced conformation keeps ASK1 inactive. The redox-sensitive proteins "sense" oxidant stress (ROS) by intramolecular disulfide bond formation and dissociate from ASK1. Unbound ASK1 is then activated, which can lead to diverse cellular fates, depending on the cell type and initiating oxidant stress examined.

PAI-1 plasma concentrations are elevated (54, 77). With streptozotocin-induced diabetic mice, an elevation of plasma PAI-1 levels and enhanced senescent ECs in aortas were observed. Interestingly, when ASK1 knockout mice were examined, the changes induced by streptozotocin were attenuated (114). Taken together, these results suggest that hyperglycemia accelerates EC senescence and upregulation of PAI-1 expression via an ASK1-dependent mechanism. Furthermore, in independent studies using ASK1-deficient mice, Yamashita and colleagues (112) implicated ASK1 in the development of endothelial dysfunction and cardiovascular remodeling induced by NO deficiency. Therefore, ASK1 may be a potentially novel therapeutic target in diabetic patients to prevent vascular aging and thrombosis.

Another critical redox protein, glutathione peroxidase type 1 (GPx-1), is involved in maintaining vascular homeostasis. Several studies demonstrate that GPx-1 has a major biologic role in protecting the endothelium from oxidative damage. GPx-1 diminishes oxidant stress by using glutathione to reduce both $H_2O_2$ and lipid peroxides to their corresponding alcohols (80, 105). A correlation between vascular injury and decreased GPx-1 activity has been reported. Lapenna *et al.* (60) demonstrated a decrease in GPx-1 activity in atherosclerotic plaque excised from carotid arteries. Furthermore, Weiss *et al.* (109) showed that overexpression of GPx-1 restored normal endothelial function to cultured ECs exposed to elevated homocysteine concentrations. In addition, Forgionne *et al.* (24,25) showed that mice with a deficiency in GPx-1 not only have endothelial dysfunction, but also have significant structural cardiac and vascular abnormalities. Moreover, loss of GPx-1 expression renders mice susceptible to ischemia/reperfusion injury (24). In contrast to wild-type mice, GPx-1–deficient mice had no increase in circulating EPCs, defined as Ac-LDL$^+$Lectin$^+$VEGFR-2$^+$eNOS$^+$ cells, in response to either exogenous VEGF treatment or ischemic injury. Importantly, it is not clear how this definition of EPCs in mice correlates with the human phenotypes reviewed earlier (Fig. 3) and shown to correlate with

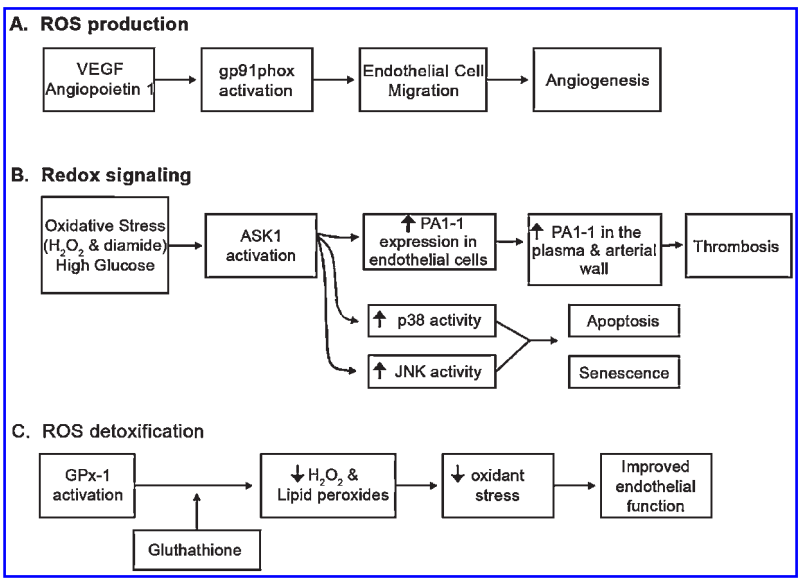


**FIG. 8. Redox molecular mechanisms involved in controlling endothelial function.** The majority of ROS production in ECs is via activation of NADPH oxidase, which stimulates diverse redox signaling pathways that regulate angiogenesis. (**A**) ROS production: gp91$^{phox}$ is activated by a range of stimulants including VEGF and angiopoietin I. This activation leads to the stimulation of EC migration and thus has an essential role in angiogenesis. (**B**) Redox signaling: Oxidative stress and high glucose induce activation of ASK1 and downstream MAP kinases, including p38 MAP kinase and c-Jun $NH_2$-terminal kinase (JNK), which can induce cellular senescence or apoptosis in ECs. In addition, ASK1 activation enhances PAI-1 expression in ECs, which enhances thrombosis. (**C**) ROS detoxification: Glutathione peroxidase type 1 (GPx-1) protects the vascular endothelium from oxidative damage by using glutathione to reduce $H_2O_2$ and lipid peroxides to their corresponding alcohols.

vascular disease risk. Furthermore, Ac-LDL$^+$Lectin$^+$VEGFR-2$^+$eNOS$^+$ cells isolated from GPx-1 knockout mice were functionally deficient in promoting angiogenesis both *in vivo* and *in vitro* and exhibited an increased susceptibility to oxidative stress *in vitro* (28). Collectively, these studies suggest that an imbalance of ROS production, redox signaling, or ROS detoxification contributes to EPC dysfunction and vascular disease (Fig. 8).

### Implications for Human Disease

Evidence of *in vivo* exposure to oxidative stress is observed in several diseases or with risk factors associated with enhanced vascular pathologies, including homocystinemia, diabetes, hypercholesterolemia, and the metabolic syndrome (13, 67, 97). Interestingly, cellular oxidant damage is detected before clinically significant vascular disease. These observations support the concept that increased endogenous oxidant stress promotes the development of vasculopathies (7, 13, 61). Unfortunately, the limited success of antioxidant therapy to treat patients with vascular disease has been disappointing (12). A potential explanation for these findings may be that oxidant injury occurs early in the pathophysiologic process of developing vascular disease. Therefore, by the time symptomatic vascular disease is apparent, irreversible oxidant injury has occurred, making antioxidant therapy unlikely to improve vascular function. Furthermore, antioxidants include a diverse set of compounds that function in a variety of capacities, from direct ROS detoxification to enhancing the function of redox-dependent molecular signaling pathways. Future studies that target specific antioxidant therapy to individuals at high risk of developing vascular disease, before clinical symptoms, will be interesting to determine whether vascular disease can be prevented. Hints that this approach might be useful are the observations that fruit and vegetable intake and moderate red wine consumption, nutritional methods of increasing antioxidant intake, reduce cardiovascular disease risk (21, 32, 53, 111). However, continued mechanistic studies to define how these relatively simple diet-modification strategies protect from vascular disease are warranted and will guide the future development of potential novel antioxidant therapies.

### Summary

Homeostatic regulation of the endothelium is a complex process that requires dynamic interactions between EPCs resident in vessel walls (*i.e.*, ECFCs) and bone marrow–derived EPCs circulating in the PB (*i.e.*, CFU-ECs and CACs) to sustain endothelial integrity and function. Although significant debate remains over the optimal method for defining EPCs (45, 79), numerous studies demonstrate an inverse correlation between circulating EPC numbers and vascular disease risk (10, 22, 23, 40, 44, 68, 76, 104, 107, 108). These observations suggest that prospective enumeration of EPCs may be a useful biomarker for vascular disease risk and a method to evaluate the effectiveness of interventions to reduce vascular morbidities and mortalities. Whether accumulation of oxidant damage over time directly leads to age-related impairments in EPC function is an important unanswered question. However, an irreversible age-related decline in EPC function may explain in part the limited success of clinical trials testing the efficacy of antioxidants in the treatment of patients with vascular disease. Identification of the molecular mechanisms involved in oxidant-induced vascular pathology are beginning to be elucidated, and future studies to examine these pathways in well-defined EPC subpopulations will be important. In addition, future studies that carefully examine the impact of oxidant stress–induced impairments on CFU-EC, CAC, and ECFC function are required to appreciate fully the complexity of vascular disease progression. Analysis of distinct EPC subpopulations together with studies evaluating interactions between oxidant-exposed EPC types will be necessary to more completely understand the role individual EPC subpopulations have in promoting endothelial dysfunction and vascular disease.

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### Abbreviations

acLDL, acetylated low-density lipoprotein; ASK1, apoptosis signal-regulating kinase 1; CACs, circulating angiogenic cells; CECs, circulating endothelial cells; CB, cord blood; CFU-ECs, colony-forming unit–endothelial cells; CFU-Hill, colony-forming unit–Hill; DNA, deoxyribonucleic acid; ECs, endothelial cells; ECFCs, endothelial colony-forming cells; eNOS, endothelial nitric oxide synthase; EPCs, endothelial progenitor cells; FSC, forward scatter; GPx-1, glutathione peroxidase type 1; G-CSF, granulocyte–colony-stimulating factor; HPP-ECFCs, high proliferative potential–endothelial colony-forming cells; HAECs, human aortic endothelial cells; HUVECs, human umbilical vein endothelial cells; JNK, c-Jun $NH_2$-terminal kinase; LPP-ECFCs, low proliferative potential–endothelial colony-forming cells; MNCs, mononuclear cells; PB, peripheral blood; PAI-1, plasminogen activator inhibitor-1; ROS, reactive oxygen species; SSC, side scatter; UEA-1, *Ulex europeus* agglutinin-1; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2.

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