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REVIEW

Human endothelial stem/progenitor cells, angiogenic factors and vascular repair

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Neovascularization or new blood vessel formation is of utmost importance not only for tissue and organ development and for tissue repair and regeneration, but also for pathological processes, such as tumour development. Despite this, the endothelial lineage, its origin, and the regulation of endothelial development and function either intrinsically from stem cells or extrinsically by proangiogenic supporting cells and other elements within local and specific microenvironmental niches are still not fully understood. There can be no doubt that for most tissues and organs, revascularization represents the holy grail for tissue repair, with autologous endothelial stem/progenitor cells, their proangiogenic counterparts and the products of these cells all being attractive targets for therapeutic intervention. Historically, a great deal of controversy has surrounded the identification and origin of cells and factors that contribute to revascularization, the use of such cells or their products as biomarkers to predict and monitor tissue damage and repair or tumour progression and therapeutic responses, and indeed their efficacy in revascularizing and repairing damaged tissues. Here, we will review the role of endothelial progenitor cells and of supporting proangiogenic cells and their products, principally in humans, as diagnostic and therapeutic agents for wound repair and tissue regeneration.

Keywords: endothelial cells; angiogenesis; vasculogenesis; diagnostic biomarkers; tissue engineering; regenerative medicine

1. INTRODUCTION

The human vascular system forms an intricate and dynamic network in the body for the delivery of oxygen and nutrients, for the circulation of cells and their products, and for the removal of carbon dioxide and metabolic by-products. DeWitt (2005) has calculated that blood vessels from an adult would ‘circle the Earth twice’ if placed end to end. The control of blood vessel formation has attracted much attention and significant funding (DeWitt 2005) because blood vessel formation is fundamental to development, its dysregulation can cause or contribute to fatal disease and it is a potential target for therapy. Indeed, Carmeliet estimated in 2005 that over 500 million individuals would reap the benefits of therapeutic developments and intervention in this specialism (Carmeliet 2005), although this could prove to be significantly more.

As early as 1787, John Hunter coined the term angiogenesis after he identified the formation of blood vessels in deer antlers during their yearly regeneration. Since then, it has become clear that haemopoietic stem cells (HSCs) forming blood, and endothelial stem/progenitor cells forming blood vessels, are generated in a highly co-ordinated manner during embryonic development (Watt in press; Watt *et al.* in press). Although there has been considerable debate, there is compelling evidence in mammals to support the origin of HSCs from either a common precursor, the haemangioblast, or, in part, from haemogenic endothelium, possibly by a

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process of endothelial–haemopoietic transition (Tavian *et al.* 1996; Watt *et al.* 2000; Kattman *et al.* 2006; Zambidis *et al.* 2007; Yoshimoto *et al.* 2008; Zovein *et al.* 2008; Dieterlen-Lèvre & Jaffredo 2009; Eilken *et al.* 2009; Lancrin *et al.* 2009; Bertrand *et al.* 2010; Boisset *et al.* 2010; Kissa & Herbomel 2010; Swiers *et al.* 2010 and references therein). However, despite extensive studies on embryonic development of the vascular and haemopoietic lineages, the stages of lineage development from the endothelial stem cell to mature end cells and their sites of origin in humans postnatally have generally remained less well-defined than those for the haemopoietic lineage (Pearson 2009; Yoder 2009). Nevertheless, once formed, the maintenance of the postnatal vascular system requires constant remodelling and dynamic adaptation of vessel and network structures in response to functional needs. These may occur by one of the three processes: (i) angiogenesis, or the formation of new vessels by sprouting from pre-existing vessels, which occurs principally in response to ischaemia; (ii) vasculogenesis, or the de novo differentiation of mature endothelial cells from endothelial progenitor cells (EPCs) derived from bone marrow or originating from precursors within specific regions of the blood vessel wall; and (iii) arteriogenesis, or the process of increasing the size of the lumen of pre-existing arterioles by remodelling and growth to form collateral arteries (Fischer *et al.* 2006).

Pathological neovascularization or excessive blood vessel formation can promote the growth of diseased tissues at the expense of the normal tissue. This can be observed, for instance, in most cancers, in rheumatoid arthritis and in diabetic eye disease (Crawford *et al.* 2009; Hall & Ran 2010; McKeage & Baguley 2010; Szekanecz *et al.* 2010). In contrast, insufficient blood vessel formation after tissue injury can lead to tissue death as is seen, for example, in cardiovascular diseases and with delayed wound healing (Watt & Fox 2005; Martin-Rendon *et al.* 2009; Critser & Yoder 2010). These pathological conditions represent an enormous burden, both to patients and healthcare systems. For example, there are around 6.5 million patients in the United States with chronic wounds and available statistics estimate the cost to health providers of around US$ 25 billion per annum (Sen *et al.* 2009). Similarly in the UK, Posnett and Franks (Posnett & Franks 2008; Posnett *et al.* 2009) estimated in 2008 that around 200 000 individuals suffered from chronic wounds, with NHS (National Health Service) costs of between £2.3 billion to £3.1 billion per annum or around 3 per cent of total estimated expenditure on public health. Comparatively, the estimated costs to the UK health service for treating cardiovascular diseases exceeded £14 billion per annum at this time (www.bhf.org.uk; www.heartstats.org). With an ageing population, expenditure in the treatment of such diseases is likely to increase significantly over the next 20 years.

Therapeutically and diagnostically, there are three potential strategies for the use of EPCs or their products in tissue repair. First, they can be used diagnostically as circulating biomarkers for predicting risk of or response to tissue injury. Secondly, EPCs mobilized into the circulation by growth regulators or obtained from the bone marrow, cord blood or tissues themselves may provide a source of vascular progenitors to facilitate neovascularization. Thirdly, these progenitor cells could be manipulated *in vivo* to enhance their ability for vascular repair.

Despite this, advances in the therapeutic and diagnostic use of these cells and their products have been hindered by the lack of robust standardized or evidence-based methods to define or identify endothelial stem/progenitor cells. Furthermore, molecular controls for new blood vessel formation and vascular remodelling rely on an intricate and regulated cascade of growth factors, inhibitors and signalling molecules, and although there has been a great deal of progress in this area, a full understanding of these processes is fundamental to improved healthcare. Thus, in this review, both basic and preclinical research principally into postnatal human EPCs and their products or regulators will be discussed, as will progress in their clinical use or usefulness for repairing damaged tissues.

2. ENDOTHELIAL PROGENITOR CELLS

2.1. Historical controversies: do postnatal EPCs arise from bone marrow myeloid cells?

A great deal of controversy about EPCs and their role in angiogenesis and vasculogenesis postnatally has arisen because of discrepancies in their identification. This has occurred particularly through the misconception that, for most of those markers currently used, unique endothelial markers exist, even though it has been widely recognized for some time that such biomarkers are not exclusive to the endothelium and are expressed on other cell lineages (Critser & Yoder 2010; Richardson & Yoder 2010). The site of origin of EPCs has also been a matter for debate.

Before the 1990s and based on seminal studies by Folkman (1984), postnatal neovascularization was generally thought to occur only by angiogenesis, despite earlier indications for the repopulation of dacron grafts transplanted into the swine aorta by ‘circulating endothelial cells’ (CECs; Stump *et al.* 1963). In 1997, Asahara and colleagues (1997) ignited interest in postnatal vasculogenesis by demonstrating that peripheral blood mononuclear cells could differentiate into putative ‘endothelial cells’ and become incorporated at sites of neovascularization in animal models of ischaemia. Further studies indicated that putative human ‘EPCs’ could be differentiated into ‘endothelial cells’ *in vitro* and could become incorporated into areas of active angiogenesis in *NOD/SCID* murine models of bone marrow transplantation (Kalka *et al.* 2000). In these studies (Kalka *et al.* 2000), the cultured human cells expressed CD14 together with CD31/PECAM-1 markers, known to be expressed by cells of the monocytic lineage (Watt *et al.* 1993). These cells could also be mobilized into the peripheral blood by cytokines and in response to ischaemia (Takahashi *et al.* 1999). Canine studies confirmed the presence of donor-derived ‘endothelial’ cells in dacron grafts following bone marrow transplantation (Shi *et al.* 1998; Tepper *et al.* 2005).

The postnatal induction of revascularization from bone marrow ‘endothelial precursor cells’ was further supported by using Tie-2 genetically marked murine bone marrow cells in an *in vivo* model of graded vascular ischaemia (Tepper *et al.* 2005). The monocytic lineage thus became a focus for research as the source of either endothelial precursors or of proangiogenic cells, also known as circulating angiogenic cells (CACs).

Hill *et al.* (2003) developed a semi-solid clonogenic colony-forming unit ‘endothelial cell’ (CFU-EC or CFU-Hill) assay and showed that the numbers of CFU-Hill in human peripheral blood correlated inversely with a set of cardiovascular risk factors. These so-called EPCs expressed biomarkers, such as CD31, CD105, CD146, VEGFR-2, CD144, UEA-1 and vWF, which lack specificity for the endothelial lineage. For example, CD144 is also expressed on foetal HSCs in both the mouse and human (Fleming 2005; Kim *et al.* 2005; Ema *et al.* 2006; Oberlin *et al.* 2010) with $CD34^{+}$ $CD45^{+}CD144^{+}$ cells in the human foetal liver generating haemopoietic cells but lacking the potential to generate endothelial cells (Oberlin *et al.* 2010). The EPCs also expressed the more specific haemopoietic markers, CD14 and CD45, and demonstrated AcLDL (acetylated low-density lipoprotein) uptake, a function of both endothelial cells and macrophages (Yoder *et al.* 2007). These human cells do not form secondary/tertiary colonies on replating, do not form chimaeric vessels when transplanted into mice, and are clonally distinct from defined endothelial colony-forming cells (ECFCs) or late-appearing outgrowth endothelial cells as evidenced by JAK-2 mutational analyses of cell subsets (Yoder *et al.* 2007). The CFU-Hill-derived cells have now been shown to be of haemopoietic/monocytic origin and may also contain T cells (Rehman *et al.* 2003; Critser & Yoder 2010; Steinmetz *et al.* 2010).

Elsheikh *et al.* (2005) subsequently identified a subset of human peripheral blood monocytes expressing CD14 and CD309/VEGFR-2 (approx. 2% of $CD14^{+}$ mononuclear cells), which could contribute to revascularization in a model of tissue damage *in vivo*. They concluded that a common monocytic-endothelial progenitor must exist. Next, CD202b/Tie-2^{+} monocytes, representing a similar proportion (approx. 2–7% of mononuclear cells) of the monocytes in peripheral blood to those expressing VEGFR-2, were identified as proangiogenic cells, which were selectively recruited to tumours via angiopoietin-1 produced from activated endothelial cells. These cells enhanced the growth of blood vessels in these tumours but did not differentiate into endothelial cells (De Palma *et al.* 2007; Venneri *et al.* 2007). Interestingly, Tie-2 was also identified on pericyte precursors that are known to support vessel formation and function (De Palma *et al.* 2007) and hence does not possess the endothelial cell specificity implied in other studies (Tepper *et al.* 2005). Following on from these studies, additional experiments (Bailey *et al.* 2006; Zovein & Iruela-Arispe 2006) suggested that, in mice, endothelial cells arose from common myeloid and granulocyte-macrophage progenitors and were components of the bone marrow myeloid progenitor cell lineage, but were not present in the enriched HSC fraction. This novel concept was further studied (Purhonen *et al.* 2008) using VEGFR-2 or Tie-1 promoter transgenic mice together with mobilization of vascular endothelial growth factor (VEGF) and tumour models, but, while bone marrow-derived perivascular cells (possibly proangiogenic monocytes) were recruited to sites of neovascularization, endothelial precursors were not. In more recent studies in mice, local inflammation associated with leishmaniasis was also shown to result in the accumulation of $CD11b^{+}$ monocytic cells at the site of infection (Horst *et al.* 2009; Randi & Bussolati 2009). These cells expressed high levels of ceacam-1, first identified in human cells as a homophilic and heterophilic adhesion molecule (Watt *et al.* 2001 and references therein). Using ceacam-1-deficient mice and bone marrow transplant models, these researchers have further demonstrated that ceacam-$1^{+}CD11b^{+}$ bone marrow-derived monocytic cells promote blood and lymphatic vessel formation in mice, with ceacam-1 potentially interacting homophilically with ceacam-1 on endothelial cells and thereby controlling angiogenesis in inflammation (Horst *et al.* 2009). Analyses of cells populating the Heartmate 1 left ventricular assist device in patients with heart failure have also demonstrated that these are primarily derived from the monocytic lineage and may recruit mesenchymal stem cells (MSCs)/pericytes to the device *in vivo* (Rafii *et al.* 1995). Conversely, the cells populating similar grafts in other species may contain more endothelial lineage cells, thereby demonstrating species differences in the response to implanted devices (see details in Yoder (2010) and references therein).

Taken together, these studies show that bone marrow-derived monocytic cell subsets, which share biomarkers found on and can mimick endothelial cells but express transcriptome and proteome profiles which cluster them more with monocytic cells than endothelial cells, promote neovascularization both *in vitro* and *in vivo* (Watt & Fox 2005; Rohde *et al.* 2006; Purhonen *et al.* 2008; Pearson 2009; Critser & Yoder 2010; Fantin *et al.* 2010; Medina *et al.* 2010 and references therein). However, it is generally accepted that they or their committed myeloid precursors neither give rise to EPCs nor endothelial cells.

2.2. Identifying postnatal EPCs: evidence from culture

Lin *et al.* (2000) demonstrated that, in allogeneic bone marrow-transplanted patients, cells which were $CD14^{-}$ formed late-appearing endothelial cell colonies in culture, the so-called late outgrowth endothelial cells, which were derived from the donor bone marrow. These donor-derived cells declined post-transplant with a switch to the predominance of recipient outgrowth endothelial cells (Lin *et al.* 2000). Lin and colleagues also concluded that circulating EPCs could originate both from existing vessel walls and from the transplanted bone marrow itself, with the latter having much greater proliferative potential *in vitro*.

To more precisely define the postnatal endothelial lineage, Yoder, Ingram and colleagues took advantage of studies on the hierarchical organization of myeloid lineage cells described some years ago before the HSC was clearly defined and where more primitive high proliferative potential colony-forming cells (HPP-CFCs) could be distinguished from more mature low proliferative potential (LPP-)CFCs by colony size, with cell surface markers and by their differential response to growth factors (Bradley & Hodgson 1979; Zhou *et al.* 1988; Bertoncello *et al.* 1991*a*,*b* and references therein). For example, LPP-CFCs formed colonies in response to macrophage colony-stimulating factor (M-CSF), while the HPP-CFCs responded to combinations of growth factors (e.g. interleukin (IL)-3 plus M-CSF or IL-1, IL-3 and M-CSF) and could be purified away from the LPP-CFC by immunomagnetic depletion based on differences in lineage marker expression and by using differential dye efflux technologies, strategies subsequently adapted for enriching HSCs (e.g. Hills *et al.* 2009). Yoder's group therefore analysed the proliferative potential of postnatal human EPCs in defined clonogenic assays *in vitro*, which were reminiscent of these HPP- and LPP-CFC assays (Ingram *et al.* 2004, 2005; Watt & Fox 2005; Yoder *et al.* 2007; Timmermans *et al.* 2009; Yoder 2009, 2010 and references therein; Yoder & Ingram 2009*a*,*b*; Critser & Yoder 2010). In these assays, single EPCs were plated into 96-well collagen 1-coated plates and the number of primary colonies developing over 14 days enumerated. The developing cells showed an endothelial cobblestone morphology and had differing proliferative potentials, with HPP-ECFCs giving rise to LPP-ECFCs which generate endothelial clusters (d14 clusters of 2–50 cells) and finally to mature non-dividing endothelial cells. The LPP-ECFCs formed colonies greater than 50 to less than 2000 cells and did not replate as secondary colonies, while those containing greater than 2000 cells were classified as potential HPP-ECFCs as long as they retained the ability to form at least secondary colonies. Those HPP-ECFCs forming tertiary colonies are thought to possess even higher proliferative potentials. These observations have been confirmed independently by others (Watt & Fox 2005; Melero-Martin *et al.* 2007, 2008, 2010; Kung *et al.* 2008; Melero-Martin & Bischoff 2008; Pearson 2009; Reinisch *et al.* 2009; Zhang *et al.* 2009). These cells and particularly those with high proliferative potential had the ability to form vascular tubules *in vitro* in a stromal-supported co-culture assay (Melero-Martin *et al.* 2008, 2010; Zhang *et al.* 2009). The ECFCs, when selected from human umbilical cord or peripheral blood, were also found to possess relatively high levels of telomerase activity, particularly if they are HPP-ECFCs (Yoder *et al.* 2007) and could be incorporated into the endothelium of vascular structures and inoculate with murine vessels when implanted in a collagen–fibronectin matrix or in matrigel *in vivo* in murine immunodeficient models of vasculogenesis (Yoder *et al.* 2007; Kung *et al.* 2008; Yoder 2009; Reinisch *et al.* 2009; Critser & Yoder 2010; Melero-Martin *et al.* 2010). It was noted by Melero-Martin that the vasculogenic potential *in vivo* decreased with increasing culture periods for the ECFCs (Melero-Martin & Bischoff 2008) and that host myeloid cells (CD11b$^+$ cells) were required for vessel formation *in vivo* (Melero-Martin *et al.* 2008), providing further support for cooperation between EPCs and proangiogenic monocytes. Further studies have demonstrated the stabilization of vessel formation from umbilical cord or peripheral blood ECFCs *in vivo* by supporting stromal/perivascular cells (10T1/2 murine embryonic fibroblasts, human mesenchymal stromal cells derived from bone marrow or adipose tissue; Au *et al.* 2008*a*,*b*; Stratman *et al.* 2009; Traktuev *et al.* 2009; Merfeld-Clauss *et al.* 2010). Key factors involved in pericyte or perivascular-vascular tubule stabilization and vessel guidance include VEGF, angiopoietin-1, transforming growth factor (TGF)-β, Notch and Ephrin signalling pathways and basement membrane proteins (Stratman *et al.* 2009 and references therein).

Thus, the ECFCs represent EPCs with differing proliferative potentials and appear to be synonymous with late outgrowth endothelial cells, but their relationship with endothelial stem cells remains to be defined. The properties of these ECFCs or late outgrowth endothelial cells will be discussed below, and, as appropriate, comparisons will be made with proangiogenic cells.

2.3. Can postnatal ECFCs be distinguished phenotypically?

2.3.1. Are CECs distinct from ECFCs? A great deal of effort has been expended on phenotyping the so-called human 'CECs, CACs or circulating endothelial progenitors (CEPs)' (or collectively as EPCs), which now most likely represent either more mature non-proliferative endothelial cells sloughed-off from vessels in response to tissue damage (CECs) or proangiogenic cells of the myeloid or monocytic lineage (CACs, CEPs, EPCs). Neither are now thought to be true EPCs, with the later most likely deriving from the haemopoietic lineage. These efforts have been described and reviewed in detail elsewhere as have the pitfalls associated with platelet/microparticle contamination of test samples, and the use and limitations of different technologies ranging from automated microscopy to various flow-cytometric techniques (Case *et al.* 2007; Timmermans *et al.* 2007, 2009; Strijbos *et al.* 2008, 2009; Yoder 2009, 2010; Yoder & Ingram 2009*a*,*b*; Critser & Yoder 2010; Estes *et al.* 2010; Mancuso & Bertolini 2010). In brief, it is generally reported that CECs occur in normal adult blood at a frequency of 1 per 1000 to 100 000 cells, although higher concentrations have been cited, and that they are increased in the blood of patients with such conditions as burn injuries and cancers (Duda *et al.* 2007; Fox *et al.* 2008*b*; Lowndes *et al.* 2008; Strijbos *et al.* 2008). The identification of CECs has been surrounded by controversy and is dependent, in part, on the care taken in the enumeration procedure as well as by the selection of biomarkers with which to identify this particular cell subset. Errors can occur, for example, through the venepuncture procedure where vessel damage itself occurs, the lack of Fc receptor blockade or of optimal antibody concentrations during cell labelling and flow cytometry, inaccurate

Table 1. Key biomarkers and their specificity.

CD marker	examples of cell reactivity	comments
CD133	haemopoietic stem cells and some haemopoietic progenitor cells, neural and some other stem cells, epithelial cells, on some tumour-initiating cells, retinal cells	a cholesterol-interacting pentaspan membrane protein, also known as prominin-1
CD34	haemopoietic stem cells, haemopoietic progenitor cells, muscle satellite cells, epidermal precursors, vascular endothelium, hair follicle stem cells, mast cells, eosinophils, fibrocytes	sialomucin belonging to the CD34 family of genes which also includes podocalyxin and endoglycan
CD31	haemopoietic stem cells, haemogenic endothelium, monocytes, platelets, neutrophils, megakaryocytes, NK cells, some T cells, vascular endothelial cells including ECFCs and CECs	immunoreceptor tyrosine-based inhibitory motif containing Ig superfamily molecule, also known as PECAM-1
CD45	leucocytes, not present on erthyrocytes or endothelial cells	protein tyrosine phosphatise existing as multiple isoforms which differ in their cell expression patterns on leucocyte subsets
CD14	monocytes, and trace amounts on neutrophils	phospholoipid-anchored membrane or soluble protein
CD146	endothelial cells including ECFCs and CECs, malignant melanomas and some other cancers, some stromal/perivascular mesenchymal cells, a subset of activated T cells	Ig superfamily member, also known as MUC-18
CD144	endothelial cells, haemopoietic stem cells (at least in foetal liver), perineurial cells	member of the cadherin family of proteins, also known as VE-cadherin
CD105	MSCs, endothelial cells, vascular smooth muscle cells, erythroid precusors, B progenitor cells, circulating $CD34^+$ cells, activated monocytes, macrophages. Follicular dendritic cells, melanocytes, syncytiotrophoblasts, extraglomerular interstitial cells	transmembrane glycoprotein, accessory protein of TGF-β, also known as endoglycan
CD202b	endothelial cells and their progenitors, quiescent haemopoietic stem cells, monocyte subet	type 1 transmembrane protein belonging to the receptor tyrosine kinase family of proteins, also known as Tie-2
CD309	endothelial cells, megakaryocytes, embryonic tissues	type 1 transmembrane glycoprotein belonging to the type III tyrosine kinase receptors, also known as VEGFR-2 or KDR or Flk-1

compensation during multi-colour flow cytometry, in the cell preparation and analysis procedures where platelet aggregates may be mistaken for CECs, in part, by uptake of soluble forms of CD144 and CD146 and lack of a nuclear stain, and, in part, during red cell lysis prior to labelling (Strijbos *et al.* 2008, 2009; Bertolini 2009; Steinmetz *et al.* 2010). Current flow-cytometry protocols include apoptotic Syto16 or live–dead cell stains to define viable cells, and selection for $CD31^+CD146^+$ or $CD34^+CD146^+$ cells within the $CD45^-$ cell subset (Bertolini 2009; Mancuso & Bertolini 2010). Alternatively, $CD146^+$ cells are isolated manually or in automated immunomagnetic CellTracks systems and then detected in the $DAPI^+CD105^+$ $CD45^-$ cell subset (Rowand *et al.* 2007; Erdbruegger *et al.* 2010). While these marker combinations are likely to encompass CEC-containing subsets and while researchers have their preferred methods to enumerate such cells, it must be remembered that CD34, CD31, CD105 and CD146 may be found together on some $CD45^-$ non-endothelial perivascular cells and on ECFCs or their progeny (Timmermans *et al.* 2007; Zhang *et al.* 2009; Watt *et al.* in press), and hence the so-called CEC subset may also include these other cell types. The real distinction for CEC thus comes from the lack of CD45 expression, the non-proliferative status of CECs compared with the other proliferative populations and the identification of these cells as endothelial cells. A list of the CD markers and phenotypes that have been used to identify EPCs, CECs, CACs or proangiogenic haemopoietic cells and ECFCs are listed in tables 1 and 2, respectively, and described further below. The new terminology proposed by Richard and Yoder to replace previous EPC and CAC designations is also cited in table 2 (Richardson & Yoder 2010). This neither distinguishes between CECs and ECFCs nor between proangiogenic haemopoietic cells, which may be myeloid progenitors ($CD14^-$) or committed to the monocytic lineage ($CD14^+$).

2.3.2. Are ECFCs the real EPCs? It was initially postulated that ECFCs or late outgrowth EPCs could be distinguished from CEC on the basis of CD133 expression patterns, but this has now been brought into question. Indeed, although Peichev and colleagues (2000) identified 'endothelial precursors' as $CD34^{+-}$ $CD133^+VEGFR\text{-}2^+$ cells, which were $CD14^-$, they did not include the pan leucocyte CD45 marker, thereby omitting an important marker to distinguish between endothelial and haemopoietic lineage cells. More recently, ECFCs from fresh human umbilical

Table 2. Revised terminology for human endothelial progenitor cells.

revised terminology[a]	biomarkers used in isolation or characterization	published terminology	references
proangiogenic haemopoietic cells	$CD34^+$,$CD31^+CD309^+(VEFGR2^+)$, $CD202b^+(Tie2^+)CD62E^+CD45^+UEA\text{-}1^+$, AcLDL uptake, $CD14^-$	EPC	Asahara *et al.* (1998), Shi *et al.* (1998), Kalka *et al.* (2000) and Takahashi *et al.* (1999)
	$CD133^+CD34^+CD309^+$	CEP	Peichev *et al.* (2000)
	$CD31^+CD309^+CD202b^+$, form myeloid colonies *in vitro*, distinguished from late outgrowth endothelial cells (ECFCs) by lack of expression of BMP2 and BMP4	EPC/CFU-Hill	Hill *et al.* (2003) and Smadja *et al.* (2008)
	$CD45^{dim}CD133^+CD34^+CD144^+$, $CD309^+$	EPC	Fox *et al.* (2008*b*) and Smythe *et al.* (2008)
	$CD34^{bright}CD31^+CD133^+CD45^{dim}CD14^-CD41a^-CD235a^-$	proangiogenic cells	Estes *et al.* (2010)
	key characteristics: $CD31^+CD45^+$ or dim; may be $CD309^+CD202b^+$. More immature progenitors: $CD133^+CD34^+CD14^-$. More mature monocytic lineage cells: $CD133^-CD14^+$		
circulating endothelial cells	key characteristics: $CD146^+CD34^+CD31^+CD45^-DAPI^+CD105^+$ viable, non-proliferative endothelial cells	CEC	Mancuso & Bertolini (2010), Erdbruegger *et al.* (2010), Rowand *et al.* (2007), Strijbos *et al.* (2008, 2009)
endothelial colony-forming cells	key characteristics: $CD34^{bright}CD31^+CD133^-CD45^-CD14^-CD41a^-CD235a^-$viable, proliferative cells forming day 14 endothelial colonies exceeding 50 cells *in vitro*. Also express CD105 and CD146	ECFC	Case *et al.* (2007), Estes *et al.* (2010), Ingram *et al.* (2004), Melero-Martin *et al.* (2010), Reinisch *et al.* (2009), Timmermans *et al.* (2007) and Zhang *et al.* (2009)

[a]Richardson & Yoder (2010) have suggested the terms ECFCs for $CD45^-$ proliferative endothelial progenitor cells and proangiogenic haemopoietic cells for the $CD45^+$ haemopoietic subsets that support vessel formation and that express either the more primitive stem cell marker CD133 or the monocytic lineage marker CD14. CECs as defined by Bertolini (2009) possess a similar phenotype to ECFCs but are non-proliferative mature viable endothelial cells.

cord blood or bone marrow have been enriched by immunomagnetic or flow-sorting based on CD34 positivity and CD45 negativity (Case *et al.* 2007; Timmermans *et al.* 2007). These cells have also been shown to be CD133$^-$ but to express VEGFR-2 (Case *et al.* 2007; Timmermans *et al.* 2007; Zhang *et al.* 2009; Estes *et al.* 2010). They also express CD31 (Estes *et al.* 2010). Cells expressing CD34, CD45 and CD133, as well as CD31, were shown to belong to the haemopoietic lineage (Case *et al.* 2007; Timmermans *et al.* 2007, 2009; Estes *et al.* 2010) and most likely include cells previously classified as CACs. As indicated in table 1, human CD34 is an activation marker and specific CD34-glycosylated epitopes are expressed, though not exclusively, by human haemopoietic stem/progenitor cells, some stromal/pericyte cell populations and most endothelium with the expression of CD34 decreasing on endothelial cells during culture (Sutherland *et al.* 1988; Hernandez *et al.* 2009; Strilic *et al.* 2009; Zhang *et al.* 2009; Campagnolo *et al.* 2010; Civin 2010). Human CD133, AC133 or prominin-1 is also found on haemopoietic stem/progenitor cells but again is not specific for this lineage (Miraglia *et al.* 1997; Corbeil *et al.* 2000; Mizrak *et al.* 2008). CD45, a protein tyrosine phosphatase consisting of a number of isoforms differentially expressed by leucocyte subsets, is neither found on cells outside the haemopoietic lineage nor on erythrocytes (Saunders & Johnson 2010). Finally, CD31, also known as PECAM-1, is present on subsets of haemopoietic cells (e.g. HSCs and monocytes) as well as on endothelial cells and does not have the endothelial specificity often cited (Watt *et al.* 1993, 1995).

Using these markers and based on previous assays, Estes *et al.* (2010) have refined flow-cytometric approaches to describe a polychromatic flow-cytometric assay for enumerating a population of human peripheral blood ECFCs, together with two distinct populations of haemopoietic progenitor cells, one proangiogenic and one non-angiogenic. They define the methodology used not only in labelling cells with multiple fluorescently tagged antibodies, but also detailed specification for the flow-cytometric analyses (see also discussions on this subject in Yoder 2010). Of note, all three subsets were CD31$^+$ (expressed by both endothelial cells and haemopoietic subsets) and expressed CD34 brightly. In this protocol, ECFCs were defined as having a phenotype of CD31$^+$CD34^{bright}CD45$^-$CD133$^-$CD14$^-$CD41a$^-$CD235a$^-$, while the proangiogenic cells were CD31$^+$CD34^{bright}CD45^{dim}CD133$^+$CD14$^-$CD41a$^-$CD235a$^-$ and the non-angiogenic cells CD31$^+$CD34^{bright}CD45^{dim}CD133$^-$CD14$^-$CD41a$^-$CD235a$^-$ (table 2). Live–dead cell staining was used in all cases as previously described (Smythe *et al.* 2008; Estes *et al.* 2010). Of further note was the exclusion of CD14$^+$ cells and the VEGFR-2 surface marker in these studies. Exclusion of CD14$^+$ cells would select for more immature CD133$^+$ myeloid or multi-potent haemopoietic progenitors, while removing a proportion of autofluorescent haemopoietic cells (Watt *et al.* 1980; Estes *et al.* 2010). For the VEGFR-2 biomarker, Estes and colleagues (2010) were unable to find a reliable anti-VEGFR-2 antibody for flow cytometry. Thus, the putative CD14$^+$VEGFR-2$^+$ subset of proangiogenic cells was not measured in this assay nor was the ability of the proangiogenic fraction to support vessel formation. These researchers report a tight distribution of between 1.2 and 1.8 in the ratio of proangiogenic to non-angiogenic progenitors in healthy blood donors, with values less than 1 indicating vascular disease and those greater than 2.2 occurring in those individuals with the specified cancers (Estes *et al.* 2010). Interestingly, the ECFC-containing subset was below the detection limit in healthy donors, but was reported as being detectable in individuals undergoing treatment for cancers and those with vascular disease.

2.4. Content and proliferative potential of ECFCs in the normal circulation

As indicated from phenotypic studies combined with *in vitro* culture assays, the levels of ECFCs in the normal human adult circulation are low (Watt *et al.* 1980; Estes *et al.* 2010). From our own unpublished studies using Ingram and Yoder's *in vitro* ECFC clonogenic assay, we have found significantly more (on average 27-fold) ECFCs in the human umbilical cord blood at birth than in adult peripheral blood from normal blood donors. Estimates for ECFC levels in normal human adult peripheral blood are cited as around 2 ECFCs per 10^8 mononuclear cells (some analyses suggest these may be as high as about 30 ECFCs per 10^8 mononuclear cells) or 0.05–0.2 ECFCs per millilitre of blood, although this may vary with age (Yoder *et al.* 2007; Estes *et al.* 2010). This compares with around 400 proangiogenic cells per millilitre of normal human adult peripheral blood (Fox *et al.* 2008*b*; Lowndes *et al.* 2008; Smythe *et al.* 2008) and would explain the difficulty in detecting ECFCs based on phenotype and flow-cytometric analyses described above. The higher levels of ECFCs in human umbilical cord blood compared with adult peripheral blood is consistent with studies from Ingram's group (Ingram *et al.* 2004; Javed *et al.* 2008). The ECFC-derived progeny expressed CD31, CD144, CD146, CD73 and CD105, but lacked CD133, CD14 and CD45 expression (Zhang *et al.* 2009). The CD34 surface phenotype varied, with around 25 and 50 per cent of those derived from adult and umbilical cord blood, respectively, after three to five passages expressing this molecule (G. Tsaknakis & S. M. Watt, unpublished data). The umbilical cord blood ECFCs also had a much greater proliferative ability (approx. 100-fold more cells generated after 14 days in culture) than adult peripheral blood ECFCs and formed vascular tubules *in vitro* (Ingram *et al.* 2004). Additionally about threefold more ECFCs were found in the umbilical cord blood at term (37–40 weeks of gestation) than at 24–31 weeks of gestation (Javed *et al.* 2008).

Although ECFC levels are low in human peripheral blood, they can also be found for example in the blood of canine, swine and non-human primate species (Hu *et al.* 2002; Huang *et al.* 2007; Milbauer *et al.* 2009), but are almost non-existent in the circulation of normal C57BL/6J adult mice greater than 8 weeks of age. They are, however, more prevalent in younger mice and following VEGF priming of mice

(Somani *et al.* 2007). Differences have been noted in the numbers of HSCs and their progeny among different strains of inbred mice. Whether the low ECFC numbers in older C57BL/6J mice is specific to this mouse strain or is a common feature of all murine strains is unknown.

2.5. *Do endothelial stem/progenitor cells reside in specific niches postnatally: vessel walls versus bone marrow?*

The actual postnatal site of residence of endothelial stem/progenitor cells has been debated for some time and it has been unclear if these cells normally reside in the bone marrow after birth or are associated with specific regions of the vascular wall, either in the bone marrow and in other tissues, from whence they can exit or be mobilized into the peripheral blood (Tilki *et al.* 2009; Klein *et al.* 2010; Yoder 2010). Although it has been proposed that postnatal organs including the intestine and liver can also contribute to the CECs pool (Aicher & Heeschen 2007; Aicher *et al.* 2007), and that 'proangiogenic cells' can be found in adipose tissue, muscle and dermis (Grenier *et al.* 2007; Saif Heeschen & Archer 2009), the robust demonstration that these cells are ECFCs or their precursors rather than myeloid or mesenchymal-derived proangiogenic cells has not always been conclusively made. In contrast, the human umbilical cord vein and aorta have been shown to contain significant numbers of ECFCs at birth, with a proportion having high proliferative potential (Ingram *et al.* 2005; Zhang *et al.* 2009). It is possible that these cells or their foetal bone marrow counterparts are the source of ECFCs that enter and circulate in the umbilical cord blood before birth. Using chimaeric and genetically modified murine transplant models, it has been suggested the bone marrow contribution to the endothelium is about 1 per cent or at vessel branch points around 3–4% (Crosby *et al.* 2000; Foteinos *et al.* 2008; Zacchigna *et al.* 2008), although the latter studies did not clearly distinguish between endothelial and haemopoietic-derived cells. In other studies in rats (Reidy & Schwartz 1981; Prescott & Muller 1983), it appeared that endothelium contributing to vascular repair mainly originated from the endothelium neighbouring the wound injury and hence from the vessels themselves. Thus, whether ECFCs mobilized into the circulation from around the site of the wound or from the bone marrow contribute to the initial stages of vessel repair, as has been suggested for the formation on new vessels in tumours (Yoder & Ingram 2009*a*,*b*) or whether ECFCs originate principally from the vessels themselves remains unclear and again species differences may exist in the contribution of such cells to wound repair (see also Pearson 2009; Yoder 2010 for further discussions). Evidence for the potential location of vasculogenic niches within vessel walls after birth is discussed below.

The walls of large and medium-sized vessels (arteries and veins) are composed of three concentrically arranged layers. These are the tunica intima (lumen/blood facing region), the tunica media and the outer tunica adventitia, with endothelial cells, smooth muscle cells and fibroblasts or adventitial stromal cells being the predominant cell types associated with these three respective regions. The adventitia also contains nerves, which control vessel wall contractility, and microvessels penetrating the media and intima are formed in the adventitia (Tilki *et al.* 2009; Klein *et al.* 2010). Endothelial cells within the intima of large vessels, such as the aorta, were reported to be mostly quiescent in the adult under normal homeostatic conditions (Wright 1968; Schwartz & Benditt 1976, 1977; Joyce *et al.* 2009; Pearson 2009; Tilki *et al.* 2009; Yoder 2010). For example, Schwartz and Benditt (1976) reported that, while the overall daily replicative rate of the aortic endothelium of the normal rat at birth was maximally 13 per cent, it dropped to around 0.1–0.3% at five to six months of age. However, proliferative clusters or 'niches' with up to 60 per cent of the cells proliferating were observed to be unevenly distributed within the aortic intima when the analyses were done in three-month-old female rats (Schwartz & Benditt 1976). These findings supported earlier studies in guinea pigs which suggested the presence of replicative endothelial 'niches' in aortic branches (Wright 1968). The postnatal replicative capacity of endothelial cells in vessels other than the major vascular conduits can also vary and this may also differ among species. For example, in the adult human cornea, endothelial cells do not generally divide *in vivo*, but do not lose the capacity to do so *in vitro* or in response to injury, while bovine corneal endothelium more closely resembles the bovine aorta in its content of ECFCs, including HPP-ECFCs (Joyce *et al.* 2009; Huang *et al.* 2010; Yoder 2010). Another example is the rat pulmonary microvascular endothelium, which has been shown to have a higher proliferative rate and more HPP-ECFCs than the pulmonary artery endothelium in postnatal life (Alvarez *et al.* 2008; Yoder 2010). Thus, a variety of factors (e.g. hypertension, age, injury, species differences, location, etc.) may influence the endothelial replicative rate and hence their capacity for wound repair (Schwartz & Benditt 1976, 1977; Tilki *et al.* 2009; Klein *et al.* 2010; Yoder 2010).

Recent studies suggest that, at least in large vessels, EPCs are located in 'vascular wall stem cell niches' in both the subendothelial zone of the intima or in the 'vasculogenic zone' in the adventitia close to the medial layer, with both regions also containing MSCs (Zengin *et al.* 2006; Ergun *et al.* 2007; Crisan *et al.* 2008; Passman *et al.* 2008; Campagnolo *et al.* 2010; Klein *et al.* 2010; Watt *et al.* in press). The 'vasculogenic zone' has also been reported to harbour myeloid cells or their haemopoietic progenitors (HSC/HPC), and progenitors for smooth muscle cells and fibroblasts (Ergun *et al.* 2007; Klein *et al.* 2010), the latter potentially originating from MSCs. Thus, the key questions are (i) whether the bone marrow or vascular wall-associated stem/progenitor cells (perhaps in the bone marrow, other tissues or the large conduit vessels) make a major contribution to new blood vessel formation, or whether both have significant but distinct roles, and (ii) whether the cells required for revascularization can be mobilized into the peripheral blood and

provide a source of cells for therapeutic or diagnostic use. It should be noted in this respect that some studies suggest that the bone marrow does not contribute substantially to vascular endothelium (Göthert *et al.* 2004; De Palma *et al.* 2005). Using transgenic and parabiotic mouse models, Purhonen and colleagues (2008) were subsequently unable to demonstrate a contribution of bone marrow cells to vessel formation in tumours. As Yoder (2010) surmises, the endothelial cells within vessel walls could migrate, be mobilized or shift position in response to injury, and hence the contribution from bone marrow cells may be minimal. Clearly, the mechanisms associated with wound repair and revascularization of tumours by endothelial cells and their progenitors and proangiogenic counterparts need to be determined to optimize therapeutic benefits.

2.6. *Can EPCs be mobilized into the circulation by angiogenic factors and contribute to neovascularization in humans?*

It is known that the normal human adult blood contains a minor proportion of circulating HSCs and more HPCs, the latter being principally committed erythroid progenitors (Ho *et al.* 1998; Cheung *et al.* 2007). One explanation for this circulation of haemopoietic progenitor cells is that this is a remnant of the circulating stem/progenitor cell pool during foetal development. Another explanation is that these circulating progenitors may have the potential to rapidly enter the bone marrow or tissues in response to haematological stress, thereby allowing their rapid maturation and maximizing, for example in the case of erythroid cells, the delivery of oxygen to tissues when most needed. As indicated above, other non-haemopoietic stem/progenitor cells, such as ECFCs and MSCs, circulate in the normal adult peripheral blood in low or even negligible numbers under homeostatic conditions (He *et al.* 2007; Salem & Thiemermann 2010; Yoder 2010). It is also evident postnatally that ECFCs, MSCs and HSC/HPCs reside in the bone marrow where they may be associated with the vascular niche, and that they may also exist in niches within the walls of other vessels (Ergun *et al.* 2007; Watt & Forde 2008; Tilki *et al.* 2009; Watt *et al.* 2009, in press; Campagnolo *et al.* 2010; Klein *et al.* 2010; Lymperi *et al.* 2010). It has been demonstrated that these cells can be released into the circulation in response to injury (e.g. ischaemia) or to mobilizing or angiogenic factors, although, with the lack of specific biomarkers, the identity of the cells mobilized and the site of mobilization have been fraught with controversy. Attempts will be made to distinguish between the cell types and to highlight the molecules known to play a key role in this process and which include growth factors, cytokines, hormones and pharmacological substances. In particular, the studies will be focused on burn injuries, cardiovascular disease and the mobilizing factors used to treat blood disorders.

2.6.1. Angiogenic factors and burn injuries. VEGF is one of the most widely studied angiogenic factors. In 2001, Gill *et al.* (2001) demonstrated that VEGF levels were elevated in the plasma of patients 6–12 h after burn injuries or coronary artery bypass grafting and that this correlated with the rapid (approx. 50-fold) but transient mobilization of proangiogenic cells into the bloodstream, returning to basal levels by 48–72 h post-injury. They also described increased levels of circulating late endothelial outgrowth cells in blood collected 12 h after the injury in both groups of patients. We extended these studies to demonstrate that, in normal adult blood donors, circulating levels of factors, such as VEGF and CXCL-12 (also known as SDF-1), the ligand for the chemokine receptors CXCR-4 and CXCR-7 (Thelen & Thelen 2008; Watt & Forde 2008), correlated with the numbers of circulating proangiogenic cells (Smythe *et al.* 2008). We further demonstrated that levels of such factors in the adult human peripheral blood strongly correlated with the severity or total surface area of the burn injury and that these factors peaked rapidly within 24 h of the thermal injury and then declined over several days to weeks (Fox *et al.* 2008*a*,*b*). The rapid release of VEGF also correlated with increased circulating cell-free mRNA levels of endothelial-specific genes, such as *ECSM2*, in the plasma of burn patients (Fox *et al.* 2008*a*), although these mRNA levels remained elevated after VEGF levels had declined. The acute elevation of ECSM2 mRNA levels also correlated with the burn severity in terms of per cent total body surface area burnt in these human subjects (Fox *et al.* 2008*a*). Interestingly, *ECSM2*, also known as endothelial cell-specific chemotaxis receptor (ECSCR), is an endothelial-restricted cell surface receptor that appears to enhance the sensitivity of VEGF in angioblasts during vasculogenesis (Verma *et al.* 2010). Taken together, these results suggest that in response to burn injury and increased circulating VEGF and CXCL-12, both proangiogenic and endothelial cells (including late endothelial outgrowth cells) are rapidly mobilized into the peripheral blood. Other factors have also been shown to be elevated after burn injury. These include pro-inflammatory factors, such as IFN-γ, IL-10, IL-17, IL-4, IL-6, IL-8, eotaxin, granulocyte colony-stimulating factor (G-CSF), IL-13, IL-15, IP-10, MCP-1 and MIP-1α, which are differentially elevated in the peripheral blood of adults and children with burns (Finnerty *et al.* 2008). Finnerty *et al.* (2008) have suggested that such differences may provide insight into the higher rates of morbidity and into the development of potentially differential therapeutic treatments in adults compared with children suffering from major burn injuries. The relationship between the levels of such factors and the levels of proangiogenic cells or ECFCs in the circulation was not determined in this latter study, although the effects of some of these factors on cell mobilization into the peripheral blood have been studied separately.

2.6.2. Angiogenic factors and cardiovascular diseases. Leone and co-workers (2009) have recently reviewed in detail the studies on mobilizing factors and heart disease, although many of the initial studies did not

clearly distinguish between proangiogenic cells, circulating mature endothelial cells and ECFC/late endothelial outgrowth cells, and the mobilized cells are globally referred to as EPCs. Some of these studies are exemplified here and attempts are made to relate published findings to current definitions of ECFCs, CECs and proangiogenic cells of the monocytic lineage. First, it has been established that patients at risk of developing cardiovascular diseases have reduced numbers of proangiogenic cells (CFU-Hill) in their circulation with the number of such cells being inversely related to the Framingham cardiovascular risk score (Hill *et al.* 2003). There are, however, conflicting data on the numbers of such cells in patients with coronary artery disease (Leone *et al.* 2009). As with severe burn injuries, acute myocardial infarction (AMI) can induce a rapid mobilization of these proangiogenic cells into the circulation, a response that correlates with increased levels of circulating angiogenic factors. Shintani *et al.* (2001), for example, demonstrated enhanced VEGF levels in the circulation of patients 3–28 days post-AMI. These correlated with elevated levels of cells, which phenotypically could encompass proangiogenic cells, CECs or ECFCs. There was no significant elevation in bFGF, G-CSF, GM-CSF, IL-6 or IL-3 levels measured over 28 days post-AMI in these patients. The studies of Massa *et al.* (2005) initially demonstrated markedly increased numbers of circulating cells with a proangiogenic phenotype at the early stage after AMI. These reached a peak within 7 days post-AMI and then declined to basal levels after 60 days. The increased 'proangiogenic progenitor cell' levels strongly correlated with increased VEGF levels (but not SCF or CXCL-12) in the patients' plasma (Massa *et al.* 2005). Others have shown increases in VEGF, G-CSF and CXCL12 in the circulation in such patients (Leone *et al.* 2009), although it has also been reported that VEGF presents two waves of release during AMI, the first one in the acute phase (24–48 h) and the second in the subacute phase (7 days) (Pannitteri *et al.* 2006). In heart failure, cells with a proangiogenic phenotype and those forming CFU-Hill were found to be increased along with VEGF and CXCL-12 (but not G-CSF) at the early phase (NYHA I and II) and were reduced in late phases (NYHA III and IV) of heart failure (Valgimigli *et al.* 2004). Leone and colleagues (2009) have suggested that increased $CD34^+$ cell mobilization and the conflicting coronary artery disease data on cell mobilization may be related to the myocardial ischaemic burden in these patients. More recently, Massa has demonstrated increased ECFCs in the circulation of patients following AMI (Massa *et al.* 2009). The numbers of ECFCs were increased from 0–3 (median 0) per 10^7 MNCs in normal blood donors to 0–53 (median 19) per 10^7 MNCs in AMI patients when measured within a median time of 3 h from the onset of symptoms. In the latter case, ECFCs correlated with the percentage of $CD34^+CD45^-$ and $CD34^+VEGFR\text{-}2^+$ cells, but not of $CD133^+CD34^+$ cells in the peripheral blood. Thus, it would appear that both proangiogenic and ECFCs are mobilized in response to acute cardiovascular injury.

2.7. Mobilizing factors in animal models

The effects of the factors described above and of such factors or drugs as G-CSF and erythropoietin (Epo) and the CXCR-4 antagonist AMD-3100 or Plerixafor, which have been used therapeutically to treat haematological disorders (Watt & Forde 2008), on the mobilization of proangiogenic cells and ECFCs have been analysed in animal models to varying extents. Some of these studies will be described here.

Asahara *et al.* (1997) showed that *in vivo* administration of VEGF in animal models resulted in high numbers of bone marrow-derived 'proangiogenic' cells being released into the circulation and promoting neovascularization. Subsequent studies demonstrated that VEGF gene transfer led to sufficient mobilization of such cells from the bone marrow to contribute to postnatal angiogenesis and vasculogenesis (Kalka *et al.* 2000; Hattori *et al.* 2001). Fibroblast growth factor (FGF) has also been shown to have an intermediate but crucial role by regulating the oestrogen-induced mobilization of a bone marrow cell subset, most likely of haemopoietic origin. In FGF-2-deficient mice, this cell subset failed to mobilize following oestrogen treatment, whereas implantation of wild-type bone marrow completely restored the circulating levels of these cells (Fontaine *et al.* 2006). Epo is another cytokine reported to have a critical regulatory effect on cell trafficking. Although it has a major role in erythropoiesis, Heeschen and co-workers (2003) demonstrated in experimental animal models that it has a beneficial role in augmenting postnatal vascularization via $VEGFR\text{-}2^+$ cell mobilization, again most likely proangiogenic cells. Endogenous Epo levels have been directly correlated with proangiogenic cells of the haemopoietic lineage in the peripheral blood of patients suffering from ischaemic coronary artery disease and it has been suggested that Epo has the equivalent angiogenic potency as VEGF. In humans, a pilot study (Ferrario *et al.* 2009) has shown poor $CD34^+CD45^+$ HPC mobilization but an increase in $CD34^+CD45^-$ cell mobilization by Epo in AMI patients. Whether the latter represent mature CEC or also contain ECFCs is unknown. Although initially used to lower circulating levels of cholesterol, cholesterol-lowering 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors or statins have also been shown, in animal models, to augment mobilization of bone marrow HSC/HPCs and proangiogenic cells, which facilitate vascular repair (Dimmeler *et al.* 2001; Llevadot *et al.* 2001; Tousoulis *et al.* 2008). Increased CXCL12 levels achieved by gene delivery using adenoviral vectors have additionally led to enhanced $VEGFR\text{-}2^+$ cell levels in murine models, while the CXCR4 antagonist, AMD3100, favoured $VEGFR\text{-}2^+$ cell translocation into the bloodstream (Yin *et al.* 2007). It seems most likely given present knowledge that, in most of the experiments described above, these cells represented proangiogenic cells of the haemopoietic lineage or mature endothelial cells.

These studies contrast with others (Shepherd *et al.* 2006), where both proangiogenic cell and late outgrowth endothelial cells (ECFCs) mobilization were

analysed and cell subsets clearly distinguished. The latter research revealed that, in healthy human subjects, AMD3100 administration induced an approximate tenfold increase in proangiogenic cell levels in the circulation accompanied by an increase in HPP-ECFCs, when compared with control subjects. The former contributed to vascular repair in an ischaemic animal model, but there were insufficient HPP-ECFCs obtained to allow their analysis *in vivo*. Since G-CSF is widely used in the clinical setting of bone marrow transplantation as a mobilizing agent of HSC/HPC into the peripheral blood (Watt & Forde 2008; Dimmeler 2010), Shepherd *et al.* (2006) also compared AMD-3100 and G-CSF in human volunteers and demonstrated that both mobilized proangiogenic cells and HPP-ECFCs, with G-CSF generating more effective proangiogenic cells as assessed in *in vivo* animal models of vascular repair and slightly higher numbers of HPP-ECFCs. Administration of G-CSF in rat models of balloon-injured carotid arteries resulted in high numbers of mobilized cells, which contributed to endothelial regeneration, but, based on phenotype, these are more likely to be of haemopoietic than the endothelial lineage (Takamiya *et al.* 2006). More recent studies (Pitchford *et al.* 2009) have compared the effects of VEGF, G-CSF and AMD3100 on the mobilization of cells in mice, in part, confirming Shepherd *et al.*'s findings in the human. They used a novel *in situ* hind limb perfusion assay to collect mobilized cells and analysed these for late outgrowth endothelial cells, MSCs or stromal progenitor cells and haemopoietic progenitor cells (Jones *et al.* 2009; Pitchford *et al.* 2009, 2010). G-CSF mobilization of late outgrowth endothelial cells was modest, and its effect was mostly on the mobilization of HSC/HPC. When comparing the treatment of mice with AMD3100 or with a combination of G-CSF and AMD3100, no significant differences were observed in mobilized late outgrowth endothelial cells numbers, thus suggesting that there is no synergistic effect between G-CSF and CXCR4 antagonist in this species. However, treatment of mice with VEGF followed by AMD3100 administration led to substantially greater levels of mobilized late outgrowth endothelial cells (Pitchford *et al.* 2009), suggesting a potential superior angiogenic role for VEGF compared with G-CSF or AMD3100 alone. These findings may also highlight the importance of increased levels of VEGF and CXCL-12 in patients following burn injury for late outgrowth endothelial cells and ECFC mobilization (Fox *et al.* 2008*a*,*b*). Whether the mouse exactly recapitulates the human in their angiogenic responses is unclear given the species differences in ECFCs and CEC cited earlier. Finally, the list of mobilizing factors described above is not exhaustive. Other factors such as the angiopoietins, PDGF and IGF-1 as well as soluble cell surface receptor isoforms (e.g. CD146, VEGFR-2, N-cadherin, Notch, EphB4, etc.) regulate endothelial cell proliferation or other functions and may also directly or indirectly control their mobilization or that of proangiogenic cells into the circulation (Al Haj & Madeddu 2009; Leone *et al.* 2009; Li & Harris 2009; Azam *et al.* 2010; Harhouri *et al.* 2010; Kebir *et al.* 2010; Padfield *et al.* 2010; Saharinen *et al.* 2010; Sheldon *et al.* 2010 and references therein).

These studies nevertheless suggest that, in response to injury, angiogenic factors are rapidly released into the circulation, thereby encouraging both human proangiogenic and endothelial progenitor cells (ECFCs) to escape their tissue-specific niches and enter the bloodstream. This would allow them to rapidly reach sites of injury where they could contribute to the inflammatory response or promote new blood vessel formation and restore vascular perfusion, thereby supplying the nutrients necessary for wound healing. The observations described above and many more not cited have led to the rapid progression of this basic research into the clinic. However, it must be stressed that the robust definition and efficacy of the cell populations and their products responsible for revascularization at sites of injury are of paramount importance in optimizing treatments, which will benefit patient care.

3. WHAT IS THE DIAGNOSTIC AND THERAPEUTIC POTENTIAL OF EPCs OR THEIR PRODUCTS IN WOUND AND TISSUE REPAIR AND CANCERS?

3.1. Diagnostic disease and therapeutic biomarkers

Wound healing following tissue damage is classically divided into four temporally overlapping phases—haemostasis (the early phase with provisional extracellular matrix deposition), inflammation, proliferation and remodelling (the cellular phase with collagen deposition), with new vessel formation occurring during the proliferative phase (Nguyen *et al.* 2009). Proangiogenic cells such as fibroblasts, monocyte/macrophages and smooth muscle cells are also involved in the cellular phase. Many studies have demonstrated a correlation between the levels of mobilizing factors, circulating proangiogenic cells (particularly from the monocytic lineage), disease progression and clinical outcomes. More recently and as noted earlier using a set of defined biomarkers (for proangiogenic cells $CD31^{+}CD34^{bright}CD45^{dim}CD133^{+}CD14^{-}CD41a^{-}CD235a^{-}$; for non-angiogenic cells $CD31^{+}CD34^{bright}CD45^{dim}CD133^{-}CD14^{-}CD41a^{-}CD235a^{-}$) with specified flow-cytometric techniques, Estes and co-workers (2010) suggested, for example, that ratios of circulating proangiogenic to non-angiogenic cells of less than 1 are predictive of vascular disease in the human. In contrast, the circulating endothelial precursors, ECFC/late outgrowth endothelial cells, generally occur in normal human blood at low levels and are not as easily detected as their proangiogenic and non-angiogenic counterparts. Indeed, very few studies have actually analysed these more recently defined cell subsets or indeed cell-specific biomarkers in the blood of patients with vascular disease or injury. Their usefulness as diagnostic or prognostic biomarkers to predict vascular disease or damage or to follow-up therapies therefore requires more detailed studies in different patient groups.

Within the limitations of the assays described, studies from various groups have assessed the levels of CECs and/or proangiogenic haemopoietic cells (CEPs) in patients with various cancers and the changes in the levels of these cells in response to chemotherapy. These have been reviewed recently (Strijbos *et al.* 2008; Bertolini 2009 and references therein). Of note were the initial observations that

— CEC and proangiogenic haemopoietic cell levels in the peripheral blood may act as a prognostic model, being increased in patients with certain advanced cancers and returning to normal levels with cancer remission,
— higher CEC baseline levels in the peripheral blood of breast cancer patients undergoing chemotherapy predicted disease-free and overall survival at least over approximately 2 years of follow-up,
— breast cancer patients who responded to chemotherapy plus the anti-angiogenic compound bevacizumab showed higher baseline levels of viable CECs, and
— CEC levels might be used as a surrogate marker for optimizing anti-angiogenic drug treatments or drug escalation, and for defining vascular toxicity (e.g. cardiovascular side effects) long term after anti-cancer treatments.

Thus, with clearer and more robust definitions of CECs, proangiogenic haemopoietic cells and ECFCs and additional randomized clinical trials in treating cancer patients where these cells are assessed, it should be possible to determine the efficacy of these cells as surrogate markers for monitoring prognosis and therapeutic effects of existing and newly developed cancer therapeutics.

3.2. Cell therapies and tissue engineering for vascular repair

3.2.1. Early clinical trials and cardiovascular repair. A number of early studies in animal models indicated that the bone marrow or blood-derived ‘EPC’ could enhance vascular repair. This led to the rapid translation of this research into the clinic. Many of the studies have concentrated on treating cardiovascular disease using the mononuclear cells or $CD34^+/CD133^+$ cells selected from mobilized peripheral blood or bone marrow, or cultured MSCs. We have recently carried out systematic reviews for 21 randomized clinical cellular therapy trials for treating AMI and ischaemic heart disease (Brunskill *et al.* 2009; Martin-Rendon *et al.* 2008*a*,*b*, 2009 and references therein). These randomized trials were restricted to intracoronary or intramyocardial infusions of autologous mononuclear cells derived from bone marrow or G-CSF-mobilized peripheral blood. Sample sizes varied among the trials (1000 patients treated in total) and follow-up times were limited, but the procedures reviewed appeared safe. A small improvement (e.g. in left ventricular ejection fraction (LVEF)) was observed particularly when larger cell numbers (greater than or equal to 10^8) were directly infused into the heart, although the persistence of positive effects long term requires further assessment (Martin-Rendon *et al.* 2008*a*,*b*, 2009; Brunskill *et al.* 2009; Assmus *et al.* 2010; Chavakis *et al.* 2010). Another systematic review of intracoronary cell therapy following percutaneous coronary intervention for AMI also showed clinical benefit (Lipinski *et al.* 2007). In non-randomized studies reported earlier, some adverse events have been observed. The MAGIC trial and those trials reported by Mansour and colleagues demonstrated high rates of in-stent restenosis or de novo stenosis following AMI and intracoronary infusion of G-CSF-mobilized blood cells or enriched $CD133^+$ bone marrow cell progenitors, suggesting a higher risk of artherosclerosis progression (Bartunek *et al.* 2005; Mansour *et al.* 2006). Further systematic analyses of randomized studies are necessary to assess the efficacy of these cellular therapies and further define any adverse events.

Peripheral artery occlusive disease (PAOD), often the consequence of artherosclerosis which manifests itself in the lower extremities, has also been treated with cellular therapies, mostly in non-randomized clinical trials using bone marrow or mobilized peripheral blood mononuclear cells delivered directly to the affected site (Martin-Rendon *et al.* 2009). There are reports of beneficial effects for more than a year, particularly in terms of pain relief. Some adverse events have been reported. Randomized trials and better-defined cell populations are therefore required to prove the safety and efficacy of these PAOD therapies.

All these cellular therapies have limitations. Studies in animal models of AMI suggest that at least some cell types derived from bone marrow (e.g. MSCs) are not retained in the heart and that the positive effects of cell therapies may be of a paracrine or hormonal nature, which limit inflammation and scar formation, affect remodelling, increase revascularization and perhaps enhance the survival or proliferation of endogenous cardiac stem/progenitor cells (Stuckey *et al.* 2006; Carr *et al.* 2008; Martin-Rendon *et al.* 2008*c*; Quevedo *et al.* 2009; Sato *et al.* 2010 and references therein). More recent studies have however compared the contribution of late outgrowth endothelial cells to MSC for cardiac revascularization in a swine model of AMI and found the former cells superior (Dubois *et al.* 2010). Other approaches have analysed the functional effectiveness of induced pluripotent stem (iPS) and ES-derived cells in scaffolds or in scaffold-free grafts or have taken a drug-discovery route, for example by assessing the effects of factors, e.g. thymosin, on cardiac revascularization and vascular repair (Stevens *et al.* 2009; Jujo *et al.* 2010; Kong *et al.* 2010; Maltais *et al.* 2010; Qian & Srivastava 2010; Shrivastava *et al.* 2010 and references therein, our unpublished studies).

3.2.2. Strategies for clinical use of ECFCs. There are three current strategies for the use of ECFC/late outgrowth endothelial cells. First, they can be used for cellular therapy as agents of neovascularization, for example to rescue critical ischaemia in patients and promote healing as described above, or as vehicles for the delivery of angiogenic factors. Secondly, the capacity of ECFC/late outgrowth endothelial cells to

differentiate into endothelial cells can be used for re-endothelialization of damaged vessels and maintenance of the integrity of the endothelium. Thirdly, ECFC/late outgrowth endothelial cells may be used as a source of endothelium for tissue engineering applications to create a microvasculature.

3.2.2.1. Delivering angiogenic factors. Initial attempts to promote angiogenesis *in vivo* consisted of intracoronary infusions of VEGF and bFGF. Although improved flow was reported, the growth factors were only transiently present and patients suffered significant hypotension (Al Haj & Madeddu 2009 and references therein). Gene therapy techniques have also been employed to deliver angiogenic factors. Adenoviral vectors encoding VEGF and injected directly into ischaemic tissue elevated the expression of VEGF and showed only a mild increase in systemic VEGF. Gene transfer of naked DNA encoding for VEGF (phVEGF) for the treatment of critical limb ischaemia produced improvement in the ankle-brachial index, angiographic evidence of newly visible collateral blood vessels and the demonstration by magnetic resonance angiography of improved lower extremity blood flow (Isner & Asahara 1999). Conversely, similar approaches with anti-angiogenic molecular therapies (e.g. angiostatic proteins, siRNAs to VEGF and its receptors, miRNAs which downregulate angiogenesis) are being researched to suppress excess endothelial proliferation as may occur in some ocular diseases (Anderson *et al.* 2010; Rajappa *et al.* 2010). Cells can also function as vehicles of gene delivery. Retrovirally transduced skeletal myoblasts produced VEGF causing a 30-fold increase in capillary density within one week. The cells that are involved in microvascular regeneration, such as proangiogenic mesenchymal/perivascular cells may prove the most suitable vehicle for the controlled delivery of biologically active factors (Padfield *et al.* 2010).

3.2.2.2. ECFCs for re-endothelialization and vascular engineering. Stents or prosthetic vascular grafts coated with endothelial cells have been cited as a way to reduce the incidence of neointimal formation and consequent premature graft failure, although drug-eluting stents have been, in part, effective in reducing in-stent restenosis (Padfield *et al.* 2010). The implantation and population with cells of left ventricular assist devices and vascular grafts in the human and in animal models have also been reviewed recently (Yoder 2010). As described, it is thought that proangiogenic cells mobilized from the bone marrow enhance ECFC mobilization to, and their proliferation on and re-endothelization of stents after percutaneous coronary intervention. While citing their limitations and describing known adverse events with these approaches, Padfield further suggested that this re-endothelization can or could be enhanced pharmacologically with proangiogenic mobilizing factors (e.g. statins, G-CSF, Epo, peroxisome proliferation activator antagonists), cellular therapies (e.g. infusion of proangiogenic cells and ECFCs) and stent-based therapies (e.g. VEGF-2 gene-eluting stents, CD34 cell capture stents; Padfield *et al.* 2010). Further research and analysis of outcomes of clinical trials adopting some of these approaches are awaited.

3.2.2.3. Engineering the microcirculation. The shortage of transplantable organs and tissues (e.g. in severe burn injuries) drives the need to create an organ or tissue substitute suitable for human transplantation. To engineer a three-dimensional tissue construct for transplantation, a suitable scaffold, specific precursor cells and a vascular supply to the developing tissue are generally required. *In vivo* for survival of three-dimensional constructs, delivery of oxygen and nutrients and removal of metabolic by-products are crucial; hence the need for creating microcirculatory networks *in vitro*.

The early attempts at forming vascular networks *in vitro* relied on culturing endothelial cells on plastic and in collagen gels, which spontaneously self-assembled into tubular structures. When co-cultured, endothelial cells recruited pericytes to their abluminal surface stabilizing the microvessels. Since endothelial cells in isolation were unstable within collagen gels, human umbilical vein endothelial cells (HUVECs) were transfected with bcl2 and then seeded into fibrinogen/collagen 1 gels to achieve longer survival *in vitro* and *in vivo* with the formation of perfused HUVEC-lined capillary-like microvessels (Zheng *et al.* 2004). Black and colleagues (1998) co-cultured keratinocytes, dermal fibroblasts and HUVECs. The resulting graft contained spontaneously formed capillaries with a basement membrane. Levenberg *et al.* (2005) produced skeletal muscle constructs *in vitro* by co-culturing mouse myoblasts, embryonic fibroblasts and HUVECs in a porous scaffold. The skeletal muscle construct was successfully implanted *in vivo* and the *in vitro*-developed vasculature was maintained. Several studies have demonstrated that *in vitro* or *in vivo* assembly of a microcirculatory network can follow *in vitro* co-culture cell seeding. Koike *et al.* (2004) seeded HUVECs and 10T1/2 mesenchymal precursors in a three-dimensional fibronectin type I collagen gel and then implanted these into SCID mice. The newly formed vessels spontaneously connected to the systemic circulation and survived *in vivo* for 1 year. Au *et al.* (2008*a*,*b*) evaluated the use of human bone marrow-derived MSCs (hMSCs) and EPCs for use as vascular progenitor cells. hMSCs efficiently stabilized nascent blood vessels *in vivo* by functioning as perivascular precursor cells. The engineered blood vessels also remained stable and functional for more than 130 days *in vivo*. More recently, Hendrickx *et al.* (2010) and our laboratory have demonstrated the ability of blood-derived late outgrowth endothelial cells/ECFCs and human dermal fibroblasts to form microvessels in dermal substitutes *in vitro* (A. Athanassopoulos *et al.*, unpublished studies). Hendrichx extended these studies to an *in vivo* model of wound healing (Hendrickx *et al.* 2010). Finally, Reinisch has produced animal serum-free cultures of hMSCs and ECFCs *in vitro* and these form a microvasculature in immunodeficient mice

(Reinisch *et al.* 2009). Such approaches open the way for the therapeutic use of such cells in tissue repair.

3.2.3. Limitations and challenges in the use of EPCs in tissue repair. Many of the revascularization studies for tissue repair have been conducted in animals or have used cell populations in human clinical trials, which very likely contain only minor numbers of ECFCs (Kirton & Xu 2010 and references therein). This is particularly pertinent in cell therapies for cardiovascular repair where blood or bone marrow mononuclear cells, $CD34^+$ cells or $CD133^+$ cells enriched in haemopoietic cells have most often been used and where only small improvements in LVEF have been observed (Martin-Rendon *et al.* 2009). Additionally, the origin of the ECFCs that might promote tissue repair is not understood. For example in the adult, it is unclear if revascularization proceeds from ECFCs positioned in the vessel wall and adjacent to the damaged vasculature or if ECFCs are mobilized from the bone marrow and tissues into the circulation in response to tissue injury from whence they home into and attempt to repair the damaged tissues.

One of the simplest approaches would be to source and use autologous ECFCs and their supporting proangiogenic cells with or without an appropriate scaffold to promote tissue revascularization and repair as this would overcome human leucocyte antigen system incompatibility issues faced with allogeneic transplants. As ECFCs occur in low numbers postnatally, the challenges here would be to generate sufficient normal proliferating ECFCs *in vitro* in a timely fashion. Indeed, Reinisch and colleagues (2009) have expanded human ECFCs in animal protein-free conditions and these cells appear to retain phenotypic and functional characteristics as well as genomic stability. Finally, we now are developing the technologies to generate endothelial cells from iPS cells (Taura *et al.* 2009). These cells or the factors that regulate endothelial production and revascularization may form the basis of future autologous cell therapies or in drug discovery, if efficacy and safety issues can be addressed. Despite these limitations, it is encouraging that the grafting of a human donor trachea, precolonized with autologous epithelial and chondrogenic cells, into a patient suffering from TB, resulted in a fully functional trachea which was revascularized by the patients own cells post-transplantation (Hollander *et al.* 2009).

4. CONCLUSIONS

Proangiogenic cells, endothelial progenitor cells (ECFCs) or their products have the potential to facilitate vascular repair. ECFCs generally lack the unique markers, which make their identification difficult. They also represent a small fraction of all circulating blood or bone marrow cells. However, they might, for example, be mobilized into the blood after growth factor administration or injury or collected from tissues (e.g. umbilical cord, placenta) or cord blood at birth, be expanded *ex vivo* and stored in a manner reminiscent of haemopoietic stem/progenitor cell strategies, or be reprogrammed to provide a continuous supply of endothelial cells. Their subsequent transplantation, particularly if incorporated into engineered grafts, or the use of revascularization promoting compounds identified through drug-discovery routes could then be used to enhance neovascularization of the appropriate damaged tissues. This has clear therapeutic potential, for example, in the delayed wound healing and ulceration that accompanies peripheral vascular disease and in diabetes, in patients with major burns which can be life-threatening and in ischaemic cardiovascular disease, where the consequences of acute or chronic injury are myocardial cell death and loss of cardiac function. Clinical trials are already investigating the diagnostic potential of cells and their products and their therapeutic capacity to promote revascularization. However, a better understanding is needed of endothelial lineage differentiation, the feasibility of clinical expansion of such cells and the vasculogenic capacity of expanded or reprogrammed cells in the context of vascular tissue engineering applications.

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