Endothelial progenitor cells: novel biomarker and promising cell therapy for cardiovascular disease

Shaundeep SEN∗†‡, Stephen P. McDONALD∗†, P. Toby H. COATES∗†§ and Claudine S. BONDER†‡§
∗Central Northern Adelaide Renal and Transplantation Service, Royal Adelaide Hospital, North Terrace, Adelaide, SA 5000, Australia, †Department of Medicine, University of Adelaide, Frome Road, Adelaide, SA 5000, Australia, ‡Human Immunology, Centre for Cancer Biology, SA Pathology, Frome Road, Adelaide, SA 5000, Australia, and §Centre for Stem Cell Research, University of Adelaide, Frome Road, Adelaide, SA 5000, Australia

ABSTRACT

Bone-marrow-derived EPCs (endothelial progenitor cells) play an integral role in the regulation and protection of the endothelium, as well as new vessel formation. Peripheral circulating EPC number and function are robust biomarkers of vascular risk for a multitude of diseases, particularly CVD (cardiovascular disease). Importantly, using EPCs as a biomarker is independent of both traditional and non-traditional risk factors (e.g. hypertension, hypercholesterolaemia and C-reactive protein), with infused ex vivo-expanded EPCs showing potential for improved endothelial function and either reducing the risk of events or enhancing recovery from ischaemia. However, as the number of existing cardiovascular risk factors is variable between patients, simple EPC counts do not adequately describe vascular disease risk in all clinical conditions and, as such, the risk of CVD remains. It is likely that this limitation is attributable to variation in the definition of EPCs, as well as a difference in the interaction between EPCs and other cells involved in vascular control such as pericytes, smooth muscle cells and macrophages. For EPCs to be used regularly in clinical practice, agreement on definitions of EPC subtypes is needed, and recognition that function of EPCs (rather than number) may be a better marker of vascular risk in certain CVD risk states. The present review focuses on the identification of measures to improve individual risk stratification and, further, to potentially individualize patient care to address specific EPC functional abnormalities. Herein, we describe that future therapeutic use of EPCs will probably rely on a combination of strategies, including optimization of the function of adjunct cell types to prime tissues for the effect of EPCs.

Key words: biological marker, biological therapy, cardiovascular disease, endothelial dysfunction, endothelial progenitor cell, flow cytometry.

Abbreviations: ACEi, angiotensin-converting enzyme inhibitor; AGE, advanced glycation end-product; AMI, acute myocardial infarction; AngII, angiotensin II; ATRA, angiotensin receptor antagonist; bFGF, basic fibroblast growth factor; BM, bone marrow; BMC, BM cell; CAD, coronary artery disease; CPC, circulating progenitor cell; CRP, C-reactive protein; CVD, cardiovascular disease; CXCL, CXC chemokine ligand; CXCR, CXC chemokine receptor; DPP IV, dipeptidylpeptidase IV; EC, endothelial cell; ED, endothelial dysfunction; EPC, endothelial progenitor cell; EPO, erythropoietin; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; HIF-1α, hypoxia-inducible factor-1α; HMGBl, high-mobility box group 1; ICAM, intercellular adhesion molecule; IGF, insulin-like growth factor; LDL, low-density lipoprotein; LV, left ventricular; LVEF, LV ejection fraction; MMP, matrix metalloproteinase; MNC, mononuclear cell; NO, NO synthase; eNOS, endothelial NOS; oxLDL, oxidized LDL; PECAM-1, platelet EC adhesion molecule-1; PI3K, phosphoinositide 3-kinase; PPARγ, peroxisome proliferator-activated receptor γ; PVD, peripheral vascular disease; SDF-1, stromal cell-derived factor-1; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; vWF, von Willibrand factor.

Correspondence: Dr Claudine Bonder (email claudine.bonder@health.sa.gov.au).
INTRODUCTION

Diseases of the vascular system, including cardiac, peripheral and cerebrovascular disease, pose a major health burden. Ischaemic heart disease and cerebrovascular disease are the leading causes of death in both developed and developing economies [1]. Current therapies for CVD (cardiovascular disease), including lifestyle and pharmacological control of risk factors and surgical revascularization, do not completely reverse the pathophysiology [2]. New approaches to the management of CVD based on EPCs (endothelial progenitor cells) may offer novel avenues to reduce this burden of disease.

As discussed below, the current literature describes two specific EPC groups: (i) directly identified peripheral circulation EPCs, and (ii) cultured EPCs from blood, BM (bone marrow) and other sources, which are expanded ex vivo, and may mature and attain other cellular characteristics. The potential differences between cell identification in these two settings must be recognized and considered when examining the current EPC literature.

The inner lining of the blood vasculature, formed by ECs (endothelial cells), is the major site for the pathological processes that contribute to CVD. Normal operation of ECs requires a balance of a number of diverse functions (Figure 1A), including regulation and buffering of normal blood flow, facilitation of transfer of gases, solutes and cells to underlying tissue, and aiding in cellular repair [3]. BM-derived EPCs contribute directly to vascular homeostasis through both direct cell-to-cell contact, as well as autocrine and paracrine effects [4]. The inherent heterogeneity of ECs at differing anatomical sites provides specialized EC actions throughout the body (reviewed in detail elsewhere [5,6]). These differences in ECs reflect blood vessel diversity, from the tightly associated ECs of the major conduit arteries, where vessel integrity is paramount, to fenestrated ECs that populate end organs, such as the kidney and liver. Atherosclerosis and arteriosclerosis are the two major causes of occlusive vascular disease. Figures 1(B) and 1(C) show the stepwise progression in ED (endothelial dysfunction) during these diseases that occur as a consequence of exposure to risk factors, including smoking and lipids. Importantly, vascular ECs are integral to cardiac function. Regenerative medicine with EPCs provides a new frontier as a single-cell system with both diagnostic and therapeutic potential.

EPCs: REGULATORS OF VASCULAR HEALTH

The immature status of EPCs provides an attractive opportunity to target multiple sites throughout the body which exhibit heterogeneity in their endothelium. Indeed, EPCs have been shown to overcome these variations in the vasculature by exhibiting functional effects in arteries, capillaries and veins. They were first identified by Asahara and co-workers in 1997 [7] and have been shown to be a reliable biomarker for this disease, as well as a therapeutic potential in ischaemic syndromes. As EPCs constitute only 1–5 % of the total BMC (BM cell) population and less than 0.0001–0.01 % of peripheral circulating MNCs (mononuclear cells) [8], current research races to identify means of expanding this population of cells.

EPC characterization

There is no single cell-surface or other marker available for identification of an EPC. The most widely accepted current definition is co-expression of the surface markers CD133, CD34 and VEGFR2 (VEGF (vascular endothelial growth factor) receptor 2) [9]. Although these markers are not unique to EPCs, their combination characterizes a specific progenitor cell at a specific maturation stage (Figure 2).

The CD34 marker was the first to be used to define EPCs [7]. Its expression defines EPC ‘stage specificity’, as it decreases in intensity as EPCs differentiate [10,11]. The function of CD34 is predominantly for cell-to-cell adhesion, as a ligand for E-selectin, with an ability to regulate the tightness of EC adherence to adjoining cells [12]. Notably, CD34 has also been used since the 1980s for identification of BM-derived haemopoietic progenitor and stem cells [13].

CD133 is a five-transmembrane protein [14] found on 20–60 % of CD34bright cells in BM and blood, but not on mature ECs [15]. Although a function has been ascribed to CD133 as an ‘organizer’ of membrane topology by regulating lipid composition within the plasma membrane (reviewed elsewhere [16]), it is likely to have additional affects that have yet to be determined.

VEGFR2, otherwise known as KDR (kinase insert domain-containing receptor) or Flk1, is one of three VEGFR family members, including VEGFR1 (Flt1) and VEGFR3 (Flt4) [17]. It was initially identified on cells involved in vasculogenesis and, unlike VEGFR1, was present on cells with potential to differentiate into mature ECs [18]. VEGFR2 is a receptor tyrosine kinase for VEGF, which is critical for EC functions, including maturation and migration [17].

Other cell-surface markers used for EPC identification, such as vWF (von Willibrand factor) [19], CD31 [20] and CD144 [21] are in fact mature EC markers, whereas CXCR4 (CXC chemokine receptor 4) is found on numerous cell types (reviewed elsewhere [22]).

This identification difficulty has lead to the use of functional assays to delineate EPCs from other cell types. These functional assessments also augment the use of EPCs as a biomarker by defining further the vascular insult caused by a specific risk factor.
Figure 1  Role of ECs in health and disease

Under normal conditions (A), ECs maintain (1) blood vessel integrity, (2) balance anti-coagulation and (3) clotting, (4) modulate the immune system by regulating leucocyte trafficking, and (5) maintain vascular tone along with pericytes and SMCs. In contrast, during disease (B and C), senescent ECs with decreased repair capacity cause (6) vessel denuding, (7) plaque formation, and (8) rupture and thrombosis, with vessel occlusion, as well as (9) vasoconstriction and (10) arterial stiffening.

Activation, release and homing of EPCs from BM

The functional characterization of EPCs is based on the process by which these cells are activated, homed to sites of need and exert influence. EPCs reside within a stem cell niche in the BM characterized by low oxygen tension [23] and high levels of SDF-1 (stromal cell-derived factor-1)/CXCL12 (CXC chemokine ligand 12), a potent chemoattractant for EPCs [24] which binds via the receptor CXCR4 [25]. EPCs mobilize out of the BM in response to peripheral tissue hypoxia and trauma (Figure 3A), which cause the production and release of EPC-activation factors, such as HIF-1α (hypoxia-inducible factor-1α) [26], VEGF, EPO (erythropoietin),
oestrogen or CXCL12 to a concentration greater than that in the BM [27–29]. These agents act via the PI3K (phosphoinositide 3-kinase)/Akt pathway to activate eNOS [endothelial NOS (NO synthase)] by phosphorylation of Ser1177 and result in the increased production of NO from L-arginine [30–32]. The physical release of EPCs from the BM is also dependent on the production of NO and the local activity of MMPs (matrix metalloproteinases) (e.g. MMP-9). MMP-9 causes the release of soluble kit ligand from EPCs in the BM, allowing the cells to move out to the peripheral circulation [33]. A reduction in circulating NO results in reduced MMP-9 levels and mobilization of EPCs, and decreased in vivo capillary formation [34], even in the face of elevated VEGF blood concentrations [35]. Following release from the BM, EPCs follow cytokine gradients to activated tissues, then act in one of three ways: paracrine, integration or new-vessel formation (Figure 3B).

Diurnal variation in peripheral EPC number is seen with lowest levels at the start of the day, little change by 15.00 hours, and then a significant increase (approx. 25%) by 22.00 hours [36]. This may be due to parallel diurnal changes in the hormones GM-CSF (granulocyte/macrophage colony-stimulating factor) [37] and G-CSF (granulocyte colony-stimulating factor) [38]. In addition, a reduction in stromal cell production of SDF-1 occurs in response to noradrenaline release by sympathetic nerve fibres embedded in the BM. Noradrenaline release occurs during daylight hours and, as SDF-1 levels decrease in the BM niche, increased numbers of haemopoietic stem cells are released into the peripheral circulation [39]. Subsequent EPC attachment requires direct cell–cell contact via adhesion molecules. For example, EPC expression of P-selectin, E-selectin, ICAM-1 (intercellular adhesion molecule-1) and PECAM-1 (platelet EC adhesion molecule-1), as well as integrins α4, β1, β2, β3 and β5, facilitate EPC binding to the vascular endothelium [40]. β1 and β2 integrin levels are increased further on EPCs by HMGB1 (high-mobility box group 1), a nuclear protein that is released from damaged cells. HMGB1 acts via RAGE [receptor for AGEs (advanced glycation end-products)] on EPCs to significantly increase adhesion to EC-associated fibronectin and ICAM-1 [41].

**Regulation of ECs and the vasculature by EPCs**

ECs require ongoing autocrine and paracrine survival factors for the maintenance of normal function, in part through the bias of cell survival over senescence or
apoptosis. These pro-survival factors, in the form of VEGF, IGF-1 (insulin-like growth factor-1) and SDF-1, can originate from the myocardium [42] and skeletal muscle [43], as well as EPCs themselves [4]. Activated EPCs are a potent source of NO, an essential mediator of normal vessel relaxation, thrombosis and repair [44–47], as well as improving arterial compliance through augmented large artery distensibility [48]. Low levels of NO are associated with ED [49,50], and low EPC numbers are associated with reduced NO-dependent small vessel function [51]. In a murine model, infusion of EPCs increased NO production and improved endothelial function [52].

**EPCs contribute to new blood vessel formation**

Under the influence of VEGF [53], SDF-1 [24] and MCP-1 (monocyte chemotactic protein-1)/CCL2 [54], EPCs home to sites distal to pre-existing vasculature and form new vessels, or aid in the propagation of new vessels from pre-existing ones [55]. Studies also suggest that EPCs
may endothelialize vascular grafts both ex vivo [56] and in vivo [57], as well as denuded large arteries in a rabbit model [58]. In situ maturation of EPCs is augmented by the presence of platelets and fibrin clots, which act as intermediaries in cell tethering [59]. Proteomic studies implicate thymidine phosphorylase as being critical to EPC defence against oxidation-related apoptosis and also directly influencing ECs in a paracrine fashion to improved migration and tube formation [60]. The vasculature associated with malignancies is fast growing and often initially distant to pre-existing normal vessels, and EPC incorporation is seen in these vessels [61]. It is hypothesized that these newly recruited EPCs produce and release VEGF which causes the internalization of VE-cadherin on resident ECs releasing them from their surroundings for contribution to angiogenesis [62]. Importantly, our current understanding of EPCs is limited by the identification and ex vivo-expansion factors with the two most widely described EPC populations, i.e. early and late outgrowth EPCs differing substantially in phenotype and function (Figure 2).

**EPCs AS A CVD BIOMARKER**

Damage to ECs from such factors as oxidative and shear stress predates atherosclerotic changes [63], and ED is an early and integral factor in the progression of atherosclerotic plaques and arteriosclerotic stiffening [64]. Both processes lead to disruption of normal blood flow and subsequent tissue ischaemia [65,66]. For example, the presence of ED in coronary [67], conduit [68] and resistance [69] arteries is an independent risk factor for both cardiovascular and all-cause mortality [67,70,71]. Cardiovascular risk factors which affect endothelial function, such as cholesterol [72], diabetes [73], smoking [74] and age [75], in parallel affect EPC function and number (summarized in Table 1).

As listed in Table 1, vascular trauma [76], AMI (acute myocardial infarction) [77–79], acute coronary syndrome [80] and surgery or burns [76] cause acute hypoxia and vascular injury and increase EPC number. Guven et al. [81] found an increased expansion capacity of EPCs collected from patients experiencing acute coronary ischaemic events, with the greatest growth capacity in EPCs from patients with the worst vascular disease. In comparison with acute events, as shown in Table 1, many chronic disease states correlate with deficiencies in the number or function of EPCs. Lower circulating EPC numbers are an independent predictor of coronary artery ED [82,83].

Further vascular insult, such as thrombosis with vascular occlusion, which occurs in acute coronary artery syndromes, induces local elevations of VEGF, which recruits EPCs to the site of injury [77]. The pro-survival effect of early EPCs on resident EC viability and replacement of dying ECs [42,84] contributes to enhanced neovascularization and improved LV (left ventricular) function. However, with ongoing hypoxia or injury comes a fall in VEGF/SDF-1 production from target tissue and an inability of the BM to maintain adequate EPC production [85].

Changes in EPC number and function during CVD allow their use as a biomarker [86]. In clinical studies, low circulating numbers and reduced functional capacity of EPCs is currently being used to predict future cardiovascular events, independent of other cardiovascular risk factors, and has been validated in high-risk [87,88], as well as low-risk [89], population groups. A reduction in EPC migratory capacity correlates with increased atherosclerotic load in humans (measured by carotid intima-media thickness) [90]. In a recent study by Fadini et al. [91] of pooled data from four studies of 1057 high-risk patients, the lowest tertile of CPC (circulating progenitor cell; defined as CD34+ ±KDR+) number was able to independently predict future major cardiac events, to an average of 1.7 years [91]. The benefit of CPC count to predict events was above that of classic risk factors and was enhanced by a concurrent elevation in human serum CRP (C-reactive protein).

Secondly, in humans, restoration of EPC number or augmentation of function is possible through pharmacological and other means, and is associated with an improved risk profile [92–94]. Furthermore, infusion of EPCs in a rabbit model of atherosclerosis was associated with the reversal of atherosclerotic plaques [58].

EPCs provide a clinical advantage over the use of other biomarkers. Measurement of circulating number or function of EPCs correlate directly with endothelial function, whereas other biomarkers only correlate with end-tissue damage or stress {CK-MB (creatine kinase-MB) [95], troponin [96], NT-pro-BNP (N-terminal pro-brain natriuretic peptide) [97–100]}, or the agents which cause the insult {oxLDL (oxidized LDL (low-density lipoprotein))] [101] and CRP [102]).

The current use of EPC number alone, however, may not fully explain vascular risk in certain settings, including in patients taking agents that alter EPC activity. Rupp et al. [103] investigated the effect of atorvastatin prescription to patients with and without CAD (coronary artery disease) with regard to ex vivo EPC ability to differentiate into ‘cardiomyogenic’ cells in the presence of rat myocytes. They found that EPCs from treated individuals had a greater differentiative capacity, which did not relate to actual peripheral EPC numbers from the patient donor [103]. Numaguchi et al. [104] have demonstrated in a post-AMI cohort treated with stenting that the ability of a patient’s EPCs to more efficiently differentiate to a mature EC phenotype and attach (in vitro) was associated with better LV function at 6 months. As shown in Table 1, a number of different
Table 1  Human disease states associated with altered circulating EPC number and function

<table>
<thead>
<tr>
<th>Disease</th>
<th>EPC number</th>
<th>EPC function</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD</td>
<td>↓</td>
<td>↓</td>
<td>In vivo, decreased numbers of EPCs have been associated with reduced coronary endothelial reactivity [82]. Impaired in vitro migration capacity of EPCs is associated with increased carotid intima-media thickening [90], a marker of atherosclerosis and future cardiac risk. EPCs from IHD subjects have reduced or impaired CXCR4 function [175]. Decreased migratory capacity to SDF-1 [176]. Numbers increased early, then decreased in the latter stages of chronic heart failure [85].</td>
</tr>
<tr>
<td>Ischaemic cardiomyopathy</td>
<td>↑ Early in disease, then ↓ later</td>
<td>↓</td>
<td>For both Type 1 [177] and Type 2 [178] diabetes. In rodent models, association with impaired sympathetic release of noradrenaline into BM, resulting in increased BM SDF-1 levels and a reduction in EPC release into the peripheral circulation [179], as well as microangiopathy causing EPC apoptosis [180]. Elevated HbA1c is also associated with decreased EPC and increased macrophage release from the BM [181]. In humans, increased numbers of adipocytes at the expense of other cell types are found in the BM, which may lead to decreased hemangioblasts [182]. Brunner et al. [183] observed decreased circulating EPC numbers in non-proliferative diabetic retinopathy, but markedly elevated levels of mature EPCs in patients with proliferative lesions. Other recent studies in humans suggest the roles of oxidative stress, [184] and AGEs [185] causing decreased EPC function due to cell injury.</td>
</tr>
<tr>
<td>Diabetes</td>
<td>↓</td>
<td>↓</td>
<td>For both Type 1 [177] and Type 2 [178] diabetes. In rodent models, association with impaired sympathetic release of noradrenaline into BM, resulting in increased BM SDF-1 levels and a reduction in EPC release into the peripheral circulation [179], as well as microangiopathy causing EPC apoptosis [180]. Elevated HbA1c is also associated with decreased EPC and increased macrophage release from the BM [181]. In humans, increased numbers of adipocytes at the expense of other cell types are found in the BM, which may lead to decreased hemangioblasts [182]. Brunner et al. [183] observed decreased circulating EPC numbers in non-proliferative diabetic retinopathy, but markedly elevated levels of mature EPCs in patients with proliferative lesions. Other recent studies in humans suggest the roles of oxidative stress, [184] and AGEs [185] causing decreased EPC function due to cell injury.</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>↓</td>
<td>↓</td>
<td>Reduced numbers and impaired general functional capacity with increasing total cholesterol and LDL ([186], but see [186a]). EPC senescence is secondary to oxLDL [187], with in vitro studies showing dephosphorylation of Akt by oxLDL in the presence of VEGF, leading to decreased EPC differentiation [188]. Atorvastatin reverses the effect in both rat [112] and human [55,188] models, including humans with stable CAD [111]. For isolated hypercholesterolaemia, dietary change + exercise [189] significantly increased EPC numbers.</td>
</tr>
<tr>
<td>Hypertension</td>
<td>↓</td>
<td>↓</td>
<td>Decreased numbers seen in hypertensive patients with or without diabetes or IHD [190]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>In vitro migratory capacity of human EPCs is specifically reduced by hypertension [105]. Hypertension is associated more with arteriosclerosis than atherosclerosis, and it may be postulated that this specific effect on EPCs explains some of this difference. Increased senescence of EPCs in humans with pre-hypertension and hypertension has been shown [191,192], but the aetiology is unclear. Reduced release of EPCs from BM is known to be associated with elevated levels of complement C3a, and elevated C3a with reduced circulating EPCs has been found in humans with resistant hypertension [193]. Furthermore, in patients with resistant hypertension, low circulating EPC numbers independently correlate with endothelium-dependent vasodilation [194].</td>
</tr>
<tr>
<td>Smoking</td>
<td>↓</td>
<td>↓</td>
<td>Decreased number with ongoing use [105], but cessation is associated with an increase in number [195]. Functions, including proliferation, migration, adhesion and tube formation, are reduced in healthy smokers [196].</td>
</tr>
</tbody>
</table>
Table 1 (Continued)

<table>
<thead>
<tr>
<th>Disease</th>
<th>EPC number</th>
<th>EPC function</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aging</td>
<td>↓</td>
<td>↓</td>
<td>Decreased number with increasing age [197], and association with endothelial dysfunction [198].</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>In mice, older EPCs have been shown to be less effective than younger EPCs at preventing atherosclerotic lesions in a hypercholesterolaemic state [199].</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chang et al. [200] have suggested a decreased response to hypoxia by reduced stabilization of HIF-1α in peripheral tissues of the elderly causes reduced recruitment rather than a primary EPC problem.</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>↓</td>
<td>↓</td>
<td>Reduced number [201], as well as function, correlating with endothelial dysfunction [202].</td>
</tr>
<tr>
<td>Inflammation</td>
<td>↓</td>
<td>↓</td>
<td>CRP: direct reduction in function through blockade of eNOS [203], and a reduction in CRP is also associated with an improvement in EPC number [80].</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TNFα: reduced number, which is possibly related to myelosuppression [85].</td>
</tr>
<tr>
<td>Chronic kidney disease</td>
<td>↓</td>
<td>↓</td>
<td>Decreased number and function in both chronic and end-stage kidney disease [204,205].</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Haemodialysis is associated with increased EPC number, but reduced migratory function [206].</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kidney transplantation improves the migratory capacity and adhesion in vitro of EPCs, but overall cell numbers are decreased [207].</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Improved EPC numbers are found with better functioning grafts [208].</td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>↓</td>
<td>↓</td>
<td>Reduced function, in particular disabling the ability of transplanted cells in a mouse model to respond to an ischaemic stimulus [209].</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A human pilot study of EPC therapy for pulmonary hypertension showed a significant improvement in a 6-min-walk test 12 weeks after a single bolus of EPCs. There was a concurrent improvement in pulmonary vasculature and right heart pressures [210].</td>
</tr>
</tbody>
</table>

Disease states alter specific EPC functions, such as hypertension and reduced EPC migratory capacity [105]. Given these factors, although EPCs may more accurately reflect endothelial health compared with other biomarkers, care must be taken in their use in a clinical setting. Disease activity and co-morbid conditions should be taken into consideration, along with identification issues. Furthermore, EPC functional assessment may also be required and may in fact provide a pathway to tailor CVD therapy in individuals.

**Increase in the number or function of endogenous EPCs by pharmacological or lifestyle intervention**

This has been attempted pharmacologically in both acute and chronic settings. Intervention studies have shown associated improvement in EPC number or function with the agents outlined below.

**G-CSF**

In patients with known CAD, circulating EPC numbers increased, as well as the ability of those cells to express CXCR4 and mature into outgrowth EPCs in vitro in response to a 5 day course of G-CSF at 10 μg/kg of body weight [106]. The randomized double-blind placebo-controlled G-CSF-STEMI (G-CSF ST-Segment Elevation Myocardial Infarction) trial prescribed G-CSF or placebo to late-presentation AMI patients. At 3 months, the treatment arm showed improved neovascularization of the infarct area, but no improvement in LV function [107].
### Figure 4  Strategies for increasing EPC numbers and function at target sites

This specifically includes increasing the number of EPCs delivered to the target site and improving function once there.

<table>
<thead>
<tr>
<th><strong>Increase Bone Marrow EPC Mobilisation</strong></th>
<th><img src="https://via.placeholder.com/150" alt="Image" /></th>
</tr>
</thead>
<tbody>
<tr>
<td>- Lifestyle – Diet / Weight Loss / Exercise / Smoking Cessation</td>
<td></td>
</tr>
<tr>
<td>- Immediate Pharmacological – EPO / G-CSF</td>
<td></td>
</tr>
<tr>
<td>- Long-Term Pharmacological – Statins / PPARγ agonists / ACEi / ATRA</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Ex vivo EPC Expansion and Reinfusion</strong></th>
<th><img src="https://via.placeholder.com/150" alt="Image" /></th>
</tr>
</thead>
<tbody>
<tr>
<td>- Autograft – Bone marrow or peripheral blood source +/- pharmacological stimulation</td>
<td></td>
</tr>
<tr>
<td>- Allograft – ability to build bank of cells for rapid infusion from peripheral, umbilical cord blood or bone marrow</td>
<td></td>
</tr>
<tr>
<td>- Zenograft</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Reverse Engineer EPC from Differentiated Cell Sources</strong></th>
<th><img src="https://via.placeholder.com/150" alt="Image" /></th>
</tr>
</thead>
<tbody>
<tr>
<td>- Cell options including mature ECs, mononuclear cells</td>
<td></td>
</tr>
<tr>
<td>- Combination of agents likely to be required to drive dedifferentiation</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Increase EPC Homing/Action at Target Site</strong></th>
<th><img src="https://via.placeholder.com/150" alt="Image" /></th>
</tr>
</thead>
<tbody>
<tr>
<td>- Direct Injection of EPCs into target tissue</td>
<td></td>
</tr>
<tr>
<td>- Direct injection of chemoattractants into target tissue</td>
<td></td>
</tr>
<tr>
<td>- Increase target tissue production of chemoattractants (VEGF, SDF-1) through upregulation of RNA production</td>
<td></td>
</tr>
</tbody>
</table>

**ACEis (angiotensin-converting enzyme inhibitors) and ATRAs (angiotensin receptor antagonists)**

ACEis [87] and ATRAs [108] both may increase EPC number. There are several mechanisms of action, including a reduction in oxidative stress by blockade of the effect of AngII (angiotensin II) on its receptor [AT1R (AngII type 1 receptor)] and increasing VEGF release (reviewed elsewhere [109]).

**Oestrogen**

In animal models, short-term oestrogen replacement improved EPC survival via the up-regulation of eNOS [29] and MMP-9 activity in BM, increasing EPC release [110].

**Statins**

Atorvastatin up-regulates NO production by activating the PI3K/Akt/NOS pathway [45,87,111], leading to increased EPC activation in humans [45] and peripheral differentiation (demonstrated in a mouse model) [112]. This has been shown in humans for both atorvastatin [111] and rosuvastatin [92].

**PPARγ (peroxisome-proliferator-activated receptor γ’) agonists**

Studies have shown the ability of PPARγ agonists to reduce EPC senescence, improve adhesion and increase EPC number in humans [113–115].
EPO analogues
Lipsic et al. [116] injected 300 μg of darbepoetin α pre-angioplasty following AMI. They showed a 2.8 times increase in circulating EPC number by 72 h, but no difference in cardiac outcome and no adverse side effects [116].

Lifestyle modifications
Increasing exercise time also positively influences EPC number in both low-risk [117] and stable CAD [118] patient groups. Concurrent elevations in VEGF and IL-6 (interleukin-6) seen with strenuous activity precedes an increase in peripheral EPC numbers [119]. Conversely, ‘detraining’ is associated with decreases in EPC numbers, with the latter proportional to a worsening in brachial artery flow-mediated dilation [120]. As noted, many of these studies were animal-based or utilize surrogate markers of CVD risk, rather reporting long-term outcomes.

Infusion of autologous in vitro-expanded EPCs for acute therapy of tissue ischaemia
Following infusion of EPCs in mice, target tissues have supraphysiological levels of VEGF [121], SDF-1, IGF-1, bFGF (basic fibroblast growth factor) and Ang-1 (angiopoietin-1). However, recruitment of endogenous BM EPCs potentiates the effect of the initial exogenous EPC infusion, further improving clinical outcomes [42]. Cell-infusion therapy relies on appropriate EPC isolation and phenotypic stability, and subsequent infusion ensuring delivery of cells to target tissues for therapeutic effect. Most recent EPC studies include those collected from peripheral blood, but also from liver [122], spleen [123], umbilical cord blood [124], adipose tissue [125] and BM [55]. An alternative strategy for human EPC collection includes G-CSF administration to mobilize CD34+ cells into the peripheral circulation, where they are collected [126]. However, Honold et al. [127] have demonstrated cleaving and inactivation of CXCR4 on human EPCs collected from peripheral blood following G-CSF mobilization. This effect caused a decrease in EPC migration in vitro, as well as reducing new vessel formation in a mouse model of hindlimb ischaemia [127]. How this has an impact upon human clinical trials requires further investigation.

In cardiac studies, non-targeted peripheral intravenous infusion of human EPCs into rats has shown a poor efficiency of delivery of cells to the heart (approx. 1% of infused EPCs). This proportion increases to 2% if active ischaemia is present, and is greater than 4% with direct LV injection [128]. Although directly injecting EPCs into infarcted tissue may improve clinical outcomes, including up to a 30% reduction in LV end-diastolic pressure in a rodent model [129], this is unlikely to be possible in all human clinical settings. An alternative is to inject pro-angiogenic cytokines into target tissue to augment EPC migration. The combination of VEGF and SDF-1 appears most effective in human in vitro studies [130]. A novel approach based on this is to pharmacologically improve the half-life of pro-angiogenic cytokines in vivo. The cell-surface peptidase CD26/DPP IV (dipeptidylpeptidase IV) is found on a subpopulation of CD34+ cells, as well as multiple cell types in the periphery. Its action is to cleave and inactivate SDF-1 (at its position two proline), which in turn inhibits CD34+ migration [131]. Increased activity of DPP IV on CD34+ cells can be found in some, but not all, diabetic patients. It is associated with decreased cell migration and tube formation in vitro [132]. Sitagliptin is a DPP IV inhibitor and a novel treatment for diabetes by increasing incretin hormone levels (reviewed elsewhere [133]). It is postulated that agents such as sitagliptin could be used in conjunction with other factors to augment EPC homing by increasing SDF-1 half-life.

Clinical intervention studies
There are now a multitude of clinical trials involving interventions with EPCs or similar cells taking place, investigating diseases such as myocardial infarction, diabetic neuropathy and pulmonary hypertension (see www.clinicaltrials.gov). Published studies arising from these trials are listed in Table 2.

CAD
A meta-analysis of cell-infusion trials for cardiac disease by Lipinski et al. [134] included both unsorted and sorted BM and peripheral blood MNCs. Cells were infused within 14 days of myocardial injury and follow-up was for a minimum of 3 months. A decreased risk of heart failure and death up to 2 years post-cell infusion was found, despite only a 3% improvement in LVEF (LV ejection fraction). The authors suggested this modest change in LVEF was similar to other interventions, such as angioplasty, and that other factors, including improved survival of hibernating myocardium, may have been involved [134]. The optimal timing of EPC infusion post-tissue infarction remains unclear; cell delivery at 2 days had no benefit [135], whereas delivery at 3–7 days was efficacious [136]. Awad et al. [54] have postulated that CD14+ leucocytes are the first cell type which influence endothelial repair at ischaemic sites: it is possible that the CD14+ cells, with decreased angiogenic capability themselves, prime the environment for EPC activity. The currently recruiting JUVENTAS (reJUVenating ENdothelial progenitor cells via Transcutaneous intra-Arterial Supplementation) trial will attempt to circumvent timing problems by repeated autologous BM-derived EPC intra-arterial infusion for critical PVD (peripheral vascular disease) [137].
Table 2  Comparison of the completed EPC studies in humans
A number of different cell types and cell collection techniques were used. There are limited long-term data available; however, there appeared to be no significant risk associated with any of the studies at up to 24 (Flores-Ramirez et al. [215]) and 49 (Pasquet et al. [212]) months. PB, peripheral blood; S/E, side effects TBPI, toe brachial pressure index.

<table>
<thead>
<tr>
<th>Author</th>
<th>Clinical condition</th>
<th>Intervention</th>
<th>Cell definition</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kalka et al. [139]</td>
<td>Critical leg ischaemia</td>
<td>Intramuscular injection of naked plasmid DNA encoding VEGF</td>
<td>KDR+/VE-cadherin+/CD34+/E-selectin</td>
<td>30 Times increase in the number of circulating ‘EPCs’ following increased tissue expression of VEGF. Clinical outcome not reported</td>
</tr>
<tr>
<td>Dobert et al. [211]</td>
<td>AMI</td>
<td>Intra-coronary injection of autologous BM or peripherally derived EPCs 4±2 days post-AMI</td>
<td>CD34+/CD45−</td>
<td>Improved LVEF from 53–58 %, with no difference in residual tissue perfusion by PET and SPECT for either cell type.</td>
</tr>
<tr>
<td>Lipis et al. [116]</td>
<td>AMI</td>
<td>Single bolus of 300 μg of darbepoietin α</td>
<td>CD34+/CD45−</td>
<td>8 Times increase in EPCs at day 3, but no significant increase in LVEF at 4 months.</td>
</tr>
<tr>
<td>Wang et al. [210]</td>
<td>Pulmonary hypertension</td>
<td>Autologous EPC infusion</td>
<td></td>
<td>Significant improvement in 6-min-walk test, with no side effects</td>
</tr>
<tr>
<td>Pasquet et al. [212]</td>
<td>AMI</td>
<td>Intra-coronary injection of G-CSF-mobilized PB MNCs</td>
<td>CD34+</td>
<td>Significant improvement in LVEF at 49 months; however, with evidence of endothelial and myocardial differentiation of CD34+ cells</td>
</tr>
<tr>
<td>Kawamoto et al. [213]</td>
<td>Critical limb ischaemia from PVD or Bergers disease</td>
<td>Autologous PB CD34+ MNCs collected following G-CSF mobilization</td>
<td>CD34+</td>
<td>Significant improvement in symptoms, TBPI and oxygen pressure, without major S/E related to the infusion at 12 weeks. Moderate S/E reported from G-CSF.</td>
</tr>
<tr>
<td>Yang et al. [214]</td>
<td>Severe CAD</td>
<td>Intra-coronary stent-adenovector virus coding the human hepatocyte growth factor gene</td>
<td>CD34+/CD117+</td>
<td>Increased CD34+ cells in PB, as well as increased levels of MCP-1, IL10</td>
</tr>
<tr>
<td>Flores-Ramirez et al. [215]</td>
<td>Patients on the heart transplant list due to congestive heart failure with LVEF &lt;35%</td>
<td>Intra-coronary infusion of autologous EPCs collected after G-CSF mobilization</td>
<td>CD133+</td>
<td>Significant improvement in LVEF at 24 months, with no side effects related to infusion</td>
</tr>
</tbody>
</table>

**PVD**
Fadini and co-workers [138] have recently published a meta-analysis of cell therapy trials for severe peripheral arterial disease. They demonstrated two important points: (i) that BM-MNCs are superior to peripheral circulating MNCs, and (ii) direct intramuscular injection of cells gave greater benefit than intra-arterial routes. Both routes of administration groups had improvement in subjective and objective outcomes compared with traditional conservative management, with the safety of therapy also being confirmed [138]. These results may be related to greater EPC percentages found in the BM compared with the periphery and also the advantage of direct cell infiltration, rather than reliance on cell homing that may be limited by reduced production of cytokines by chronically ischaemic tissues.

**Gene transfer to endogenous EPCs to enhance survival and longevity of phenotype or to augment target tissue production of activating cytokines**
Targets for action include increasing VEGF expression from ischaemic tissue to promote endogenous EPC release [139] to increasing the activity of SK (sphingosine kinase), a lipid enzyme recently identified as an EPC...
control mechanism with increased enzymatic activity retaining BM-derived EPCs in a progenitor phenotype [140]. The current ENACT-AMI (Enhanced Angiogenic Cell Therapy—AMI) trial will attempt to up-regulate eNOS activity in autologous EPCs by transfection with a human eNOS-pVAX plasmid before reinfusion as a means of augmenting EPC function [141]. Others have transduced human EPCs to increase VEGF expression for improved revascularization of skin flaps in nude mice [142]. An alternative strategy is to manipulate pro-EPC factors. Feng et al. [143] increased HDL (high-density lipoprotein) production in mice by transfer of the human APOA1 (apolipoprotein A1) gene, resulting in increased EPC release from BM, incorporation into ischaemic tissue and improved revascularization. Balancing these paracrine factors is essential for optimal EPC function, and this is depicted graphically in Figure 4.

**Addition of supporting cell lines to augment EPC function**

Although the endothelial lining is critical to vessel function, the support structure within the vessel wall remains as crucial. As shown in Figure 1, dysfunction of vessels relates to problems in both vessel zones. SMCs (smooth muscle cells) are integral in maintaining vessel tone in response to chemokines released by the endothelium, as well as maintaining the physical structure and shape of the vessel. Conversely, an *in vitro* study of human EPCs has demonstrated that, under exposure from shear stress, SMCs promoted differentiation of EPCs into mature ECs via the up-regulation of Akt [144]. Murine studies have suggested the role of Ang-1 originating from SMCs activating Tie-2-positive EPCs to improved survival and tube formation [145]. An *in vitro* human study has also demonstrated augmented tube formation with the combination of late outgrowth EPCs and SMCs in a scaffold [146]. Other pre-clinical studies included human mesenchymal progenitors [147] and adipose stromal cells [148], which were injected into mice along with EPCs. Both additional cell types augmented vessel formation. Pericytes are another ubiquitous vascular cell that may augment EPC function further. Their complex relationship with the endothelium is reviewed elsewhere, but involves physical support, as well as releasing VEGF [149]. To date, there are no completed human clinical trials of EPCs with additional cell types. These findings do, however, explain in part the results of whole BMC infusions.

Despite their promise, these therapeutic options have not changed clinical practice yet. A number of substantial issues including EPC classification, as well as inadequate optimization of cytokine dosing and timing, remain unresolved. Furthermore, unlike unsorted BM-MNC trials, there are limited long-term data on symptoms, survival or side effects (BM trials show variable therapeutic responses to 2 [150], 3 [151] and 4 [152] years, but with no side effects). Concern has been raised for *de novo* malignancy post-EPC infusion, on the basis of the potential role of EPCs in vessel growth in neoplasms (reviewed elsewhere [153,154]). An autopsy report on a patient directly injected with unsorted autologous BM-MNCs 11 months prior to death showed enhanced vasculogenesis and transformation of pericytes towards a myocyte phenotype, with no evidence of abnormal cell activity [155]. There have been no reports of malignancy in EPC studies thus far. Pro-arrhythmic effects of infused cells in regenerating myocardium have also been raised, but no evidence of this has been found with EPCs [156] or unsorted BM-MNCs [157]. The use of G-CSF for peripheral release of BM-MNCs is associated with a number of well-known side effects, but with no reported cases of increased malignancy risk in otherwise normal individuals (http://pi.amgen.com/united_states/neupogen/neupogen_pi_bcp_english.pdf).

**ISSUES IN EPC IDENTIFICATION AND CHARACTERIZATION**

The inconsistency seen in clinical studies of treatment of ischaemic tissue with EPCs is probably due to an inability to isolate a pure population of EPCs and, hence, differing functional capabilities. This reflects a lack of consensus on identifying a ‘true’ EPC. As a result, a number of different terms have been used to describe cells that have some EPC characteristics or functions in common. These include early colony-forming ECs, progenitor-derived mature ECs, and haemopoietic- and myeloid-derived progenitors, all of which have been termed EPCs [158–160]. It is becoming increasingly evident that the definition of EPCs has been loosely applied and now encompasses a variety of cells including CD45 haemopoietic stem and progenitor cells (CD45 is found on early EPCs, but only at very low expression levels and disappears rapidly [8]), CD116+ myeloid cells as well as CD14+ monocytes and macrophages [161–163]. Of greater concern is that this ‘EPC’ signature may also be found on cancer cells [164]. Specifically, the protein AC133-CD133/1, used as a surface-marker target, is differentially expressed on some colon cancer cells [165]. Clearly, a novel cell-surface marker specific for EPCs is required to unequivocally distinguish these cells from closely related cells. In the interim, we and others advocate the use of co-expression of CD133, CD34 and VEGFR2 as being indicative of an early EPC phenotype [166].

The ambiguity of the ‘EPC’ markers in EPC identification presents two major issues. The first is EPC maturation. EPCs may either maintain their earlier phenotype and function, or progress to become mature ECs (see Figure 4). The loss of CD133 and reduced CD34
Table 3 Functional assays used for identifying EPCs

<table>
<thead>
<tr>
<th>Assay</th>
<th>Test/basis for testing</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrigel tube-forming capacity</td>
<td>Ability of cells to elongate and align in culture, followed by tube formation.</td>
<td>Tubes defined by the presence of a lumen. All endothelial lineage cells can perform this function, with mature ECs being the most adept.</td>
</tr>
<tr>
<td>Migration</td>
<td>Cell movement across a cell layer or synthetic membrane, mimicking in vivo function.</td>
<td>Inherent function of many cell lines; differentiation improved with the use of chemotactic agents including SDF-1 and VEGF.</td>
</tr>
<tr>
<td>eNOS activity</td>
<td>Measured by PCR/Western blotting; integral part of cellular activity.</td>
<td>Present in other cell types (mature ECs and CD14+ cells).</td>
</tr>
<tr>
<td>In vivo vasculogenesis</td>
<td>Mouse model of hindlimb ischaemia post-femoral artery occlusion; cells infused; vascular growth and activity measured by Doppler assessment of tissue perfusion and cytokine levels.</td>
<td>Mature ECs have no ability to improve blood flow [216]. EPCs alone or in combination with CD14+ cells promote vessel growth.</td>
</tr>
<tr>
<td>Lectin Ulex europaeus-1 binding</td>
<td>Binds to L-fructose residues on cell surface.</td>
<td>Marker of mature ECs [217] and also present on monocytes.</td>
</tr>
<tr>
<td>Labelled acetylated LDL</td>
<td>LDL-receptor-mediated endocytosis.</td>
<td>Maximum uptake seen in mature ECs [218], as well as macrophages and SMCs [219]. No evidence of uptake by immature EPCs.</td>
</tr>
<tr>
<td>Production of cytokines (VEGF, SDF-1 and IGF)</td>
<td>Measurement by expression of mRNA as well as production in vitro and in vivo [4].</td>
<td>Paracrine function of EPCs, underlying their ability to influence endothelium and tissue resident progenitors. Not unique.</td>
</tr>
<tr>
<td>Differentiation capability</td>
<td>Role of EPCs to provide new ECs to vessel wall.</td>
<td>Increased EC growth from EPCs correlates with improved in vivo endothelial function in healthy human controls [220].</td>
</tr>
</tbody>
</table>

Expression occurs with maturation, as well as increased expression of EC markers (e.g. vWF, VE-cadherin and PECAM-1). The second is the ability of other cell lines to act as pseudo-EPCs or differentiate into mature ECs. Haemopoietic [167] and multi-potential stem cells may do this, as well as more mature monocyte and macrophage-derived cells [168]. It is generally agreed that EPCs differentiate from an immature form that expresses CD133+/VEGFR2 [169]. Confusion arises with myeloid cells expressing CD14+/CD34− and endothelial markers, which are functionally able to form tube-like structures in vitro [169]. Reyes et al. [170] have described a MAPC (multipotent adult progenitor cell), which is CD34+/CD133+/VEGFR2+. These cells, under the influence of VEGF, differentiate into CD34+/VEGFR2+ and functional mature ECs [170]. Technically, this poses a problem if cultured EPCs progress from early to late stage or can be contaminated by other cell lines with similar actions [171], including tissue resident progenitors. Recent research also highlights the possibility of cultured MNCs picking up debris or microparticles from damaged platelets. These particles express vWF and PECAM-1, which are taken up and expressed by the MNCs, in particular by monocytes. The presence of these surface markers also increases the angiogenic potential of the MNCs as measured by tube formation in matrigel, as well as increasing colony-forming units [172]. These cells are obviously not EPCs, but have gained mature EC markers and function. This phenomenon has not yet been studied in vivo. An alternative strategy to characterize EPCs is to assess functional capacity, with a number of in vitro assays being developed. Table 3 summarizes some of the most commonly used in vitro assays including those detecting EPC migration, tube formation, and cytokines and NO production. These characteristics may be used in conjunction with cell-surface markers to more specifically define and describe EPCs.

As important as the maturation process, there is no consensus on EPC isolation or culture techniques. Currently, MNCs are isolated from whole blood by gradient density separation prior to seeding on to fibronectin, vitronectin or rat-tail collagen for 3–72 h. As EPCs are unable to bind to fibronectin [173], adherent cells are then discarded and the non-adherent fraction cultured. Pre-culture EPC selection enables improved cell purity and may be achieved using FACS or magnetic bead separation. Notably, both processes rely on antibody binding, usually one or more of CD34, CD133 and VEGFR2, which are not unique to EPCs. Over time, culture media have also evolved from basic M199 to proprietary media such as endothelial growth medium with added cytokines and supplements [ascorbic acid, steroids, VEGF, bFGF, IGF and endothelial growth...
factor] (most commonly obtained from Lonza). The aim of the culture environment is to promote persistence of an early EPC phenotype at the expense of other cell lines. No one culture medium, proprietary or otherwise, is advertised specifically for EPCs. Importantly VEGF, while improving EPC survival and proliferation, also promotes cell maturation. Clearly, standardized plating and culture techniques remain an ongoing objective for EPCs.

FUTURE TARGETS FOR THE USE OF EPCS IN CVD AND CONCLUSIONS

Progress in the use of EPCs as a therapy remains limited by identification and ex vivo-expansion factors and, as a result, variable functional attributes. These matters are the focus of ongoing research, especially the search for a unique EPC marker. Nevertheless, EPCs are a robust biomarker of CVD (based on their direct interaction and influence on endothelial function, and the unique ability to monitor their peripheral number or function as a marker of response to therapy). In the research setting, continued understanding of EPC function improves insight into vasculogenesis and the pathology of vascular disease.

The future use of EPCs, beyond that of a biomarker, relates to cellular regenerative therapies. Up until now, only modest clinical responses have been observed with the use of EPCs alone. However, the understanding that EPCs act in concert with supporting cells will lead to the co-infusion of EPCs with pericyte progenitors or SMCs [174], or the addition of chemotactants for supporting cells. This would enable EPCs to be used both for repair and maintenance of existing vasculature within the body, as well as having the potential to endothelialize implantable devices, such as stents and cardiac valves, to improve their function. Long-term ex vivo growth of vessels and organs will require the use of EPCs in conjunction with other stem and progenitor cell types to reproduce the specialized vasculature.

REFERENCES

278 S. Sen and others


© The Authors Journal compilation © 2011 Biochemical Society


Received 24 August 2010/12 October 2010; accepted 26 October 2010
Published on the Internet 13 December 2010. doi:10.1042/CS20100429