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# Understanding Vascular Biology with the Help of Endothelial Progenitor Cells

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## Abstract

Peripheral blood of adults contains low levels of a subtype of progenitor cells that have the capacity to differentiate into mature endothelial cells and hence they are termed as EPCs (Endothelial Progenitor Cells). These cells have the potential to perform neo-vascularization and re-endothelialization of the injured tissue. Bone marrow of adults contains a reservoir of these progenitor cells which can migrate to the peripheral circulation on receiving the appropriate stimulus and thus perform the necessary functions apart from differentiating to mature endothelial cells. Most of the literature states that isolation and selection of EPCs can be performed from different sources by adherence culture and magnetic microbeads. EPCs are normally characterized by the presence of three-antigen combination of CD34, CD133 and VEGFR2. However, when migrating to the circulation i.e. during differentiation, these progenitors start to lose CD133 and begin to express endothelial specific markers like CD31, VE-cadherin and vWF. In context of therapeutic application of these cells, there are few clinical trials which have started to employ EPCs for treating various ischemic diseases though there has been inconsistency in the clinical outcome. Hence, further and deeper understanding in the field of EPCs can bring about a general consensus on their specific identity along with their mechanisms of homing and differentiation which are largely unclear at present. This review summarizes the role of EPCs in some of the major diseases, their isolation, characterization and functionality assays, mechanisms of homing and differentiation along with their circulating levels *in vivo* under certain conditions and factors.

Keywords: Endothelial progenitor cells; Vascular disorders; Vasculogenesis; Angiogenesis; Cell therapy; Regenerative medicine

## Introduction

Endothelium is an inert cellophane-like membrane lining the entire circulatory system which covers a surface area of approximately 1-7 m2 and weighs approximately 1 kg [1]. It consists of more than 1014 cells thus being one of the largest organs of the body. Vascular homeostasis can be maintained only when the endothelium is structurally maintained and functionally active, absence of which leads to thrombosis, hypertension and edema [2]. The endothelium is dynamic and heterogenous with vital secretory, synthetic, metabolic and immunologic functions. EC (Endothelial cell) heterogeneity is absent in arteries and veins which are lined by a single layer of tunica intima comprising of endothelial cells. However, capillary endothelium from different vascular sites appears to be heterogenous thus facilitating different functions performed by them. For example, continuous ECs connected by tight junctions are present in brain and retina thus providing them with an uninterrupted lining allowing only small molecules like water and ions to diffuse thereby maintaining the blood-brain barrier. Discontinuous ECs have fenestrations with large openings thereby facilitating cellular trafficking between intercellular gaps in liver, spleen and bone marrow sinusoids whereas fenestrated ECs with small openings are present in intestinal villi, endocrine glands and kidneys to facilitate selective permeability required for efficient absorption, secretion and filteration [1]. Endothelial progenitor cells (EPCs) serve as progenitors for endothelial cells lining the blood vessels. They are adult multipotent cells like hematopoietic stem cells (HSCs) and they are derived from CD34+ cells [3]. In fact, both EPCs and HSCs seem to arise from a common ancestor, the hemangioblast since most circulating EPCs reside in close association with HSCs and stromal cells within bone marrow. This is also suggested by the fact that many markers like CD34, VEGFR2 and AC133 are shared between HSCs and EPCs [2]. Angiogenesis or vascularization can be stimulated by EPCs in two ways where they can either directly differentiate into mature endothelial cells or they can act via paracrine stimuli releasing various growth factors thereby stimulating the formation and repair of endothelium [3]. Asahara T et al. [4] first reported existence of a bone-marrow derived circulating progenitor for the endothelial cells known as the endothelial progenitor cell (EPC).

EPCs are reported to be capable of regenerating tissues in ischemic areas such as myocardial ischemia, limb ischemia along with remodeling tissues in diabetes mellitus and heart failure [5]. Apart from this, they are believed to have a major role in wound healing [5] and serve as biomarkers to assess the cardiovascular disease risk and progression as well as cancer progression [6]. They can also be expanded *in vitro* for cell therapy in order to facilitate pharmacological screening [3]. It is believed that we could make a suitable coating of these cells for vascular grafts and also use them as vectors for the delivery of angiogenic and anti-angiogenic genes [5].

Thus, it is clear that EPCs have many functions but they have been most widely used as a biomarker or in the form of a cell therapy in various clinical trials to treat patients with heart disease, diabetes, peripheral arterial disease, pulmonary disease and cancer despite the ambiguity in fully characterizing it [7].

EPCs play a major physiological role in maintaining vascular integrity along with homing to the ischemic and tumor tissues and also aid in their revascularization [5]. Their role in various diseases is being understood gradually.

## Cancer

EPCs are like 2 sides of the same coin having both good and bad faces. The good side deals with angiogenesis of wounded tissues and ischemic regions (Controlled vessel formation) whereas the bad side deals with tumor vasculogenesis (Uncontrolled/ Excessive vessel formation). Structurally, blood vessel formation is different in both normal tissues and tumors. Microvessels in tumors are irregular, tortuous, dilated, convoluted and lack functional pericytes. Tumor vessels have errotic growth with transcellular holes and fenetrated endothelium which increases their permeability. Apart from this, they have incomplete basement membrane and both endothelial as well as tumor cells may make up the tumor vessel walls [8]. Cancer cells thrive on tumor vasculature which mostly involves angiogenesis. However, neovascularization also seems to be involved [5] and it has been suggested by accumulating evidence that de novo vessel formation can be initiated by bone marrow derived EPCs in response to oncogenic mediators in solid tumors. Indeed, highly proliferative endothelial-like colonies were formed by EPCs when they were isolated from circulation and exposed to angiogenic factors. However, whether these BM-derived EPCs directly contribute to generation of vessel endothelium by incorporating into tumors and differentiating to endothelial cells (ECs) or indirectly stabilize the tumor vascualture as perivascular cells (PVCs) by localizing around the perivascular regions is still controversial. It is also undetermined whether the tumor type, stage and treatment regulates behaviour of EPCs in direct or indirect manner [9].

Moreover, the numbers of circulating EPCs correlate to the stage, size and type of tumors. In most of the cancers such as lymphomas, leukemia, hepato-cellular carcinoma, colon, breast, ovarian and pancreatic cancer patients, EPCs are used as biomarkers for early tumor detection. Early detection of tumor growth can be traced by tagging EPCs which will help in determining their mobilization and homing to tumor tissues thus serving as a critical determinant of aggressive tumor growth outcome [5]. EPCs are also useful as gene delivery vehicles where these cells can be transduced with a transgene expressing anti-angiogenic factors which can be administered to cancer patients directed at blocking tumor growth [10]. Thus, EPCs could be used as a 'Trojan horse' analogous to liposomes and exosomes for the delivery of chemotherapeutic drugs to tumor tissues. It was seen that blocking EPC mobilization and migration from bone marrow resulted in reduced tumor growth and metastasis which would aid in early detection and surgical intervention of these tumors [11].

## Cardiovascular Disorders

In a healthy individual, vascular homeostasis is maintained by the endothelium which exerts a number of vasoprotective effects such as vasodilation, suppression of smooth muscle cell growth and inhibition of inflammatory responses. The endothelium releases nitric oxide (NO), a potent endogenous vasodilator that not only mediates most of these functions but also prevents oxidation of low density lipoproteins (LDL). Endothelial dysfunction is caused by decreased production or reduced activity of NO resulting in impaired vasodilation [12]. Damage to the endothelial cells is brought about by multiple atherogenic factors or cardiovascular risk factors which lead to penetration of lipids and toxins into the smooth muscle cell layer. This cascade of oxidative and inflammatory events finally culminates in plaque deposition. Plaques, once formed, begin to calcify and become prone to rupture in due course of time which often results in a deadly blood clot. One of the atherogenic factors such as LDL also known as "Bad cholesterol" can penetrate the endothelial wall and contribute to core formation of plaque i.e the foam cells. Also, LDL exposed to free radicals (oxidized LDL cholesterol) aggrevates severe vascular damage by triggering an inflammatory process once they are taken up by the "scavenger pathway" of endothelial cells (http://www.lef.org/protocols/heart-circulatory/coronary-artery-disease-atherosclerosis). People with atherosclerosis, stroke and myocardial infarction have damaged endothelial layer which can further be worsened by numerous pre-disposing factors like age, family history, smoking, diabetes, hyperlipidemia, hypertension, mechanical stress and inflammation. Apart from statin therapy (drugs), EPC transplantation in such patients have also proved beneficial [13]. Tremendous research has been taking place involving the application of EPCs in cardiovascular disorders.

## Diabetes Mellitus

EPCs can also have a potential application in diabetes mellitus. Apart from EPC numbers, certain changes occur in the RBCs of such patients thereby affecting the physiology of endothelium. Persistent hyperglycemic conditions modify the structures on erythrocytes by inducing the formation of advanced glycation end products (AGEs) on them. These then bind to receptor for advanced glycation end products (RAGE) present on endothelium in vivo. Such interaction by diabetic erythrocytes with endothelium initiates oxidant stress which leads to EC activation [1] thereby resulting in loss of anticoagulant properties of endothelium along with gain of pro-coagulant function.

*In vitro* studies of EPC culturing from type 1 diabetic patients showed the presence of an angiogenic inhibitor thus directly providing evidence of reduced angiogenic capacity for *in vitro* tube formation. Another study conducted in type 2 diabetic patients showed an impaired ability of mature endothelial cells to incorporate into tubules. In hyperglycemic conditions, there was reduced number of EPCs. Apart from this, EPCs under hyperglycemic conditions inhibited NO production and matrix metalloproteinase-9 activity as well as displayed impaired migrational and integrative capacities when peripheral blood mononuclear cells (MNCs) were cultivated from healthy donors [13].

Apart from the above mentioned disorders, there are various other less known diseases where EPC numbers and their functions get affected. One such disorder is ankylosing spondylitis (AS) where EPC numbers in patients affected by AS are depleted and have inverse correlation with the disease duration, disease activity and inflammation thereby suggesting inflammation as the main reason in the depletion of EPCs. Such patients also have increased risk of cardiovascular morbidity and mortality and EPCs would probably serve as a therapeutic target for prevention of cardiovascular disorders in AS patients [14]. Acromegaly is a condition in which excess growth hormone is produced by the pituitary gland. Acromegalic patients have reduced circulating EPC levels compared with controls and thus decreased endothelial regenerative capacity possibly due to activation of GH/IGF-1. This increases cardiovascular risk in such patients. However, treatment with somatostatin analogs increased EPC levels by 2-fold in acromegalic patients [15]. Primary Sjogren's syndrome (pSS) is an example of a systemic autoimmune disease which is characterized by chronic endothelial layer damage. It was demonstrated in a study by Bartoloni E et al. [16] that mature EPC levels were higher in pSS patients as compared to healthy controls. Also, there was an inverse correlation between early EPCs and disease duration & diagnosis. Thus, EPCs have a reparative potential in the earliest stages of disease and there is progressive exhaustion of EPC pool during the course of the disease which may eventually lead to defective vascular layer restoration and endothelial dysfunction. Another disorder is COPD (Chronic Obstructive Pulmonary disease) along with PH (Pulmonary Hypertension) where peripheral blood late EPCs are significantly reduced along with a reduction in their proliferation, adhesion and migration capacities. Moreover, increase of pulmonary arterial pressure led to lowered EPC number and function. Also, the severity of pulmonary hypertension negatively correlated with the changes in number and function of EPCs [17].

Thus, apart from the most prevalent diseases, there are a variety of less familiar disorders with low incidence rates where EPCs can be used as biomarkers or as a cell therapy.

## What We Know So Far

### Bone Marrow-Derived Epcs

There is already enough proof to suggest that mesodermal stem cells in the bone marrow of early embryo differentiate to form haemangioblasts, the common precursor of HSCs that forms the inner cluster in blood island from which first embryonic blood cells develop and endothelial-lineage angioblasts (immature but lineage-committed angioblasts) which forms the outer layer of ECs encasing the blood island [1]. These blood islands together with both HSCs and EPCs thus form the primary vascular plexus from which a complex microcirculation arises [18]. This forms a strong basis for supporting MSCs as a good candidate for vasculogenesis.

### Non-Bone Marrow-Derived Epcs

Large numbers of EPCs from non-bone marrow origin such as spleen, intestine, liver, adipose tissue, aortic root of heart as well as adult vascular wall have been reported by Zampetaki A et al. [19] in circulating blood. Spleen is richly supplied with EPCs and mononuclear cells isolated from them demonstrated endothelial cell characteristics and formed tubular-like structures when pre-selected with an endothelial cell medium. Further it was proven that re-endothelialization was sufficiently enhanced and neointima formation was diminished after carotid artery injury by these cells [19].

Irrespective of their source/origin, EPCs have many functions as they could help in vascular therapies such as physiological neovascularization [5] like vasculogenesis, angiogenesis and arteriogenesis [20] to stimulate new blood vessel formation as well as they can also repair damaged endothelial cells thereby bringing about re-endothelialization.

## Vasculogenesis and Angiogenesis

The formation of functional vascular networks takes place in two ways: Vasculogenesis and Angiogenesis. Vasculogenesis takes place where there is no primitive vascular skeleton present. Thus, the first microcirculation arising in embryo from EPCs or angioblasts with the formation of blood islands that comprise EPCs at the periphery & HSCs at the center is known as vasculogenesis. Primary vascular plexus composed of fine capillaries is formed by fusion of these blood islands thus marking the end of vasculogenesis [21]. Since EPCs are involved in vasculogenesis, it was believed that the process of vasculogenesis occurs only during embryogenesis. However, this notion changed when ample evidence showed bone-marrow derived endothelial cells existing on the site of new blood vessel formation in adult life thereby indicating the participation of vasculogenesis in postnatal neovascularization [22].

Angiogenesis is the process where pre-existing mature endothelial cells (ECs) proliferate, migrate & remodel thereby aiding in the further transformations/ branching of the already formed vascular plexus [23]. There are different routes by which angiogenesis occurs as shown from the Figure 1and Figure 2.

Majority of the diseases affecting more than one billion people worldwide are due to defects in angiogenesis. Angiogenesis is an "On and Off" switch which is strictly regulated by both pro-angiogenic and anti-angiogenic factors, imbalance of which may lead to excessive and insufficient angiogenesis. Some of the pro-angiogenic factors include VEGF, bFGF, EGF, TNF-α, Angiogenin, IL-8 and Angiopoietin-1,2 whereas anti-angiogenic factors include IL-12, IFN-α, various statins such as angiostatin, endostatin and vasostatin. Angiogenic therapies designed to "turn on" or "turn off" - are revolutionizing medicine by providing a unified approach for treating various crippling and life-threatening conditions which should hopefully have the same impact in the 21st century that antibiotics had in the 20th century [25], http://www.lymphedemapeople.com/thesite/angiogenesis.htm.

Excessive angiogenesis occurs when abnormal amounts of angiogenic growth factors are produced by diseased cells, overpowering the effects of natural angiogenesis inhibitors. New blood vessels thus formed will feed on the diseased tissues thereby destroying the normal ones. This excessive blood vessel growth favors tumor metastasis in case of cancer.

Cancer, diabetic blindness, age-related macular degeneration, rheumatoid arthritis, psoriasis, and more than 70 other diseases are caused due to excessive angiogenesis. "Anti-angiogenic therapies" with the use of angiogenic inhibitors can be used to curb excessive blood vessel growth. Insufficient angiogenesis occurs when adequate amounts of angiogenic growth factors are not produced by tissues. This condition is also characterized by inadequate blood vessel growth where circulation is not properly restored thereby posing a risk of tissue death. It occurs in coronary artery disease, stroke, delayed wound healing, peripheral vascular disease and tissue-engineered organs. It can be cured by "therapeutic angiogenesis" with the help of growth factors [http://www.lymphedemapeople.com/thesite/angiogenesis.htm].

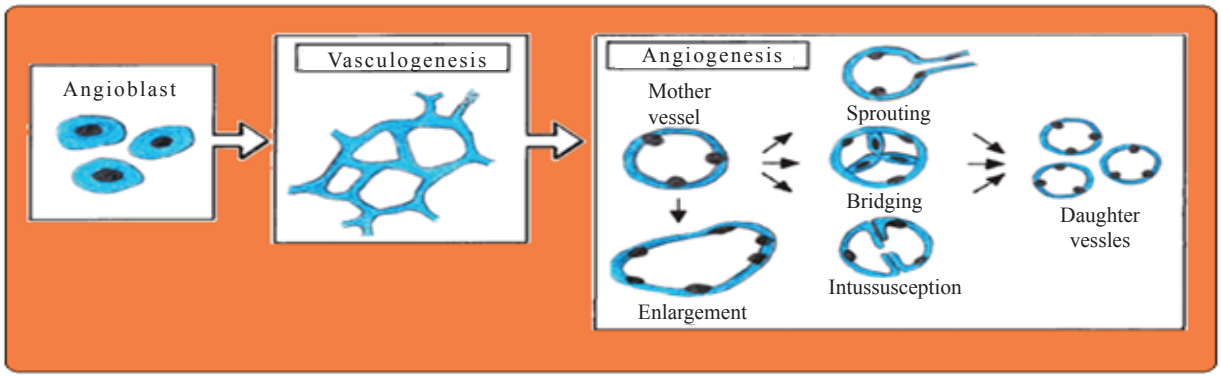


Figure 1: Vasculogenesis and Angiogenesis
Source: Korpisalo P and Yla-Herttuala S; 2010 [24]

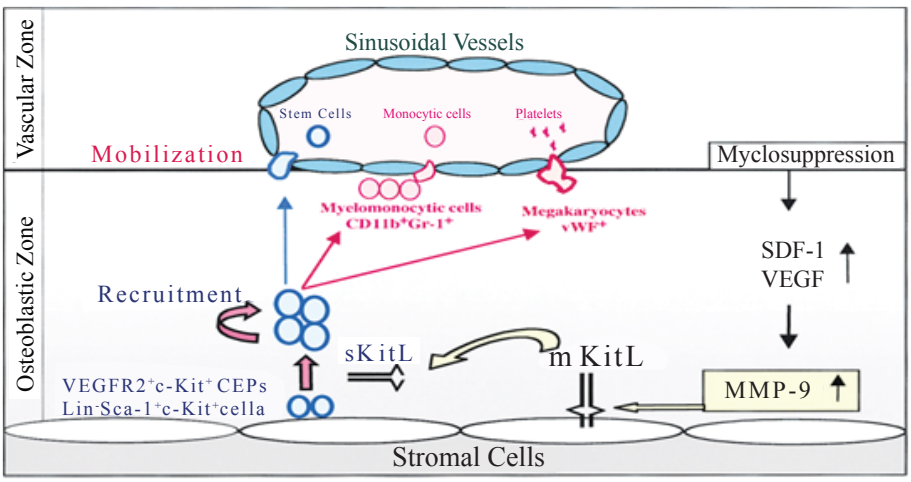


Figure 2: Functional anatomy and recruitment of c-Kit+ stem and progenitor cells is dictated by MMP-9 mediated release of sKitL
Source: Heissig B et al; 2002 [38]

## Isolating Epcs or Methods Used to Define Human EPcs

Isolation and quantification of EPCs can be assisted by a variety of measures which can be grouped into two approaches: Selection based on *in vitro* adhesion, growth, morphology and finally on cell surface markers expressed by them using flow cytometry and immunofluorescence [26]. Since a specific and unique marker or molecular determinant for EPCs is not defined, it limits their isolation. However, putative EPCs have been defined in the human system using three general approaches although all of these have some pitfalls [27].

### First Approach

Low density mononuclear cells (MNCs) are isolated from human peripheral blood or cord blood and plated on tissue culture dishes/ plates coated with fibronectin in a commercially available medium containing endothelial growth factors and fetal calf serum [28-29]. The non-adherent cells are removed after 4-5 days in culture and the adherent cells are functionally examined by their ability to bind acetylated low-density lipoprotein (AcLDL) and Ulex europaeus agglutinin 1 (a plant lectin) [26]. Hence, morphologic appearance, adhesion to fibronectin, cell surface protein expression, AcLDL uptake and lectin binding were the criteria used to define EPCs in this assay. However, this method of short-term MNC adhesion on fibronectin *in vitro* for EPC isolation was flawed by the lack of specificity of the cells obtained [7].

### Second Approach

Although there is no unique protein marker to isolate an EPC [26], this method utilizes monoclonal antibodies and fluorescence activated cell sorting (FACS) analysis to identify particular patterns of cell surface antigen expression on the cells [7,27].

Since both EPCs and HSCs seem to arise from a common mesodermal precursor during embryonic development, Asahara T et al. [4] first described a human EPC by certain cell surface markers that might be expressed by both the hematopoietic and endothelial lineages such as CD34. These authors not only brought forth concepts of circulating EPCs but also suggested that human adult peripheral blood CD34+ cells can function as EPCs in postnatal vascular repair (postnatal vasculogenesis) by migrating and homing to the sites of ischemic and vascular injury in vivo [7,27]. However, Asahara T et al. [4] displayed several limitations such as it not only lacked a protocol to obtain a purified cell population due to insufficient cellular enrichment but also lacked clonal analytical studies and high cellular resolution to prove that new blood vessels were formed in the tissues of mice with induced vascular injury by the infused cells.

Peichev M et al. [30] devised a protocol to separately identify EPCs from circulating mature endothelial cells (CECs) since even mature ECs might circulate at low levels in blood in healthy and diseased humans. Peichev and his colleagues used 3 markers to identify EPCs: CD34, KDR and CD133. CD34, KDR and CD133 are expressed by HSCs but their expression ceases as the HSCs differentiate. However, several labs have independently determined that putative EPCs expressing CD34, CD133, and KDR expressed the CD45 antigen and thus were highly enriched for the hematopoietic activity. Also, these cells did not form endothelial cell lined vessels in vivo nor did they give rise to endothelial cells *in vitro* [31]. Thus, these authors also failed to identify EPCs specifically with their three antigen combination [27].

## Third Approach

This method relies on the colony forming ability of the MNCs plated *in vitro* [7]. Colony forming unit-Hill (CFU-Hill) [29] and endothelial colony forming cell (ECFC) assays [6,32] are the two assays that have been evaluated extensively [27].

## CFU-Hill Assay

Asahara T et al. [4] described EPCs as cluster-forming cells that appeared within 5 days of plating CD34+ cells. This work was expanded on by Ito H et al. [28] who also isolated and plated peripheral blood mononuclear cells (PBMNCs) on dishes coated with fibronectin. The non-adherent cells were removed and replated onto fibronectin-coated dishes one day later, and the number of putative EPCs were indicated by the number of clusters that emerged at 7 days of replating. The EPC cluster assay was further modified by Hill J M et al. [29] by preplating the blood cells for 48 h on fibronectin-coated dishes, then replating the nonadherent cells on dishes coated with fibronectin to quantify the emergence of EPC-derived colonies i.e. EPC colony-forming units (EPC-CFUs). However, when implanted in vivo in collagen gels, these cells did not form blood vessels spontaneously and hence did not display postnatal vasculogenic activity [33].

## ECFCs

Another type of colonies observed are the endothelial colony–forming cells (ECFCs). When blood cells were plated on matrix coated dishes with added growth factors, outgrowth endothelial cells (OECs) were identified within 1–3 wk of culture which possessed clonal endothelial colony–forming cell (ECFC) ability [7]. ECFCs isolated from umbilical cord blood are not only present in higher concentration as compared to adult peripheral blood but also display high telomerase activity (high proliferative capacity) [6] and form human blood vessels in vivo spontaneously [33]. Also, ECFCs do not express CD45, CD14, or CD115 and do not ingest bacteria although they display a host of cell surface antigens normally expressed by primary endothelial cells [26,33].

## Advantage

While the CFU-Hill assay identifies hematopoietic cells, ECFCs display all the properties of an EPC [27].

## Morphology, Phenotypic and Functional Characterization of Epcs

### Morphology of Cells *In Vitro*

EPCs proliferate rapidly and exhibit a heterogenous morphology where the multidimensional colonies formed by them have round clusters associated with adherent spindle shaped cells. On the other hand, endothelial cells (ECs) exhibit "cobblestone morphology" where they grow in monolayers *in vitro* and their growth gets inhibited on reaching confluence [26]. Thus, these two cell types can be easily distinguished *in vitro* by monitoring their morphology.

## Phenotypic Characterization of Epcs

Till date, human or murine EPCs have no specific identifying marker to characterize and quantify them. Thus, many markers should be included which would serve as the best approach to characterize and quantify EPCs. Various hematopoietic stem cell markers such as CD34, CD117 (cKit) and CD133 along with endothelial markers such as KDR (VEGFR2) and VE-cadherin are used to identify EPCs in most cases [34]. It was also demonstrated by various authors that many endothelial lineage markers are coexpressed in circulating EPCs with different intensities apart from VE-cadherin such as vWF, E-selectin on stimulation, CXCR4, CD31, CD146, endothelial NO synthase and CD13 but not the myelomonocytic markers such as CD14 or CD15 [30,35].

## Functional Characterization of Epcs

Certain functional characteristics are highly specific for endothelial cells such as the formation of capillary tubes and production of NO whereas uptake of DiI-Ac-LDL and binding of FITC-UEA-1 lectin are additional general features of isolated EPCs [36]. Some of these functional characteristics are listed below.

## Uptake of Dii-Ac-LDL

Endothelial cells take up acetylated-LDL via the "scavenger cell pathway" of LDL metabolism. Upon incubation of mixed cultures of endothelial and mural cells with 10mg/ml DiI-Ac-LDL (Ac-LDL labeled with a fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) for 4 hours at 37°C, endothelial cells appeared highly fluorescent because of internalization of labeled LDL as visualized by fluorescence microscopy whereas fluorescence was slightly exhibited above background by mural cells. Thus, endothelial cells were identified and isolated from the surrounding mural cells in culture by means of this assay [26].

## Binding of FITC-UEA-1 Lectin

UEA-1 (Ulex europaeus I agglutinin) is a plant lectin specific for some α-L-fructose-containing glycocompounds and when tagged with a fluorescent compound such as FITC, it can be visualized by fluorescence microscopy. Endothelial cells within blood vessel structures of all sizes were found to bind predominantly to UEA-1 as determined by the early studies of human tissues [26].

6.3.3) *In Vitro* Tube Formation: Functionally, endothelial cells are capable of forming tube-like structures *in vitro* when cultured on 3D culture systems composed of collagen or fibrin. This property of tubulogenesis is highly unique to endothelial cells and not shared by hematopoietic cell lineages. Tube formation is a complex process where it recapitulates the morphogenesis events that take place in vivo by undergoing vacuolization, elongation, and coalescence into tube-like structures with the presence of lumina. Therefore, it is important to demonstrate the formation of bona fide tubes present as 3D structures with lumen and not just a 2-dimensional cord of cells when assessing the functional properties of putative EPCs [26].

## Mobilization, Release, Homing and Differentiation of Epcs

### Mobilization and Release of Epcs

Mobilization of EPCs from bone marrow is a complex process which is regulated by a multifaceted interplay between cytokines/chemokines, growth factors, variety of enzymes majorly potent proteases, adhesion molecules and surface receptors [34,36]. Various paracrine signals such as VEGF, GCSF, GM-CSF and SDF-1 are released into the circulation by ischemic and tumor tissues (vascular traumas) which promote the mobilization of EPCs from bone marrow into the circulation, downstream endothelial regeneration and neovascularization [5,37]. Moreover, ischemic foci and tumor tissues have elevated local concentrations of VEGF and SDF-1 and this promotes migration and recruitment of EPCs from circulation to the site of injury [37]. The predominant signal for increase in such mobilizing cytokine levels is hypoxia which is prevalent in both tumor and ischemic tissues thereby bringing about activation of HIF-1 (Hypoxia inducing factor-1) gene ultimately leading to increased synthesis of potent angiogenic factor VEGF. Various other cytokines such as fibroblast growth factor (FGF), SDF-1, osteopontin, CCL2 and CCL5 are also secreted by growing tumors which help in mobilization of EPCs [5].

## Homing of Epcs

The recruitment and incorporation of EPCs to the sites of neovascularization strongly resembles an inflammatory response which requires various multistep adhesive and signaling events to occur in a coordinated sequence such as chemoattraction, adhesion, transmigration and finally differentiation to endothelial cells [39,19].

Various adhesion molecules such as selectins and integrins are involved in the adhesion of EPCs to endothelial cells activated by cytokines and ischemia [39,19]. The initial steps of this process seem to be mediated by P-selectin and E-selectin expressed on endothelial cells. Thus, autocrine and paracrine activation of EPCs takes place by their increased adhesion and interaction to P-selectin and E-selectin on endothelial cells leading to either differentiation or transdifferentiation and proliferation of EPCs as well as vascular growth [5,19].

Various proteases are involved in the process of transmigration of EPCs to the injured tissues by degrading the matrix and thus invading the ischemic tissue. One such protease is cathepsin L which is expressed at high levels in EPCs and impaired recovery was observed following hindlimb ischemia in cathepsin L knockout mice. Another protease detected with a similar property of invasiveness in EPCs is MMP-2. Thus, EPC homing also involves proteases such as cathepsin L and MMP-2 which aid in transendothelial migration [19].

## Differentiation of Epcs Towards Endothelial Lineage

The differentiation process may be initiated when the progenitor cells are on their way to the injured tissue. However, it is difficult to comprehend the exact differentiation cascade of EPCs in vivo though some cytokines like VEGF and SDF-1 as well as mechanical forces such as shear stress generated by blood flow seem to initiate a cascade of events where they not only induce expression of endothelial-specific genes in progenitor cells but also upregulate expression of endothelial cell markers on them. This would make EPCs acquire some phenotypic features of endothelial cells thus increasing the available cell population capable of re-endothelialization of denuded arteries as well as neovascularization of ischemic regions. Apart from this, histone deacetylase (HDAC) activates transcription factors p53 and p21 and hence its activity is essential in the differentiation process [19]. Endothelial gene expression is also regulated by heterochromatin protein 1 alpha (HP1α) which plays an important role in the differentiation and angiogenic function of EPCs [40].

## Conditions and Factors Influencing the Number and Recruitment of Epcs

Various physiological and pathological factors along with drugs, cytokines, chemokines and hormones induce variations in the number, recruitment and function of EPCs both *in vivo and in vitro* (Table 1).

**Table 1: Conditions and factors influencing the number, function and recruitment of EPCs**

LDL = low density lipoprotein; CAD = coronary artery disease; CFU = colony forming units of EPCs; EPC = endothelial progenitor cell; NE = no effect

| **Condition or risk factor** | ***Changes in number/ function of EPCs or CD34+ cells both in vivo and in vitro*** | **Investigators** |
|---|---|---|
| **Physiological:** | | |
| Gender (eg, estrogens) | ↑CD34+/ VEGFR2+ cells | [41] |
| Physical training | ↑EPC number | [42] |
| Age | ↓EPC number | [43] |
| Embryonal development (eg: umbilical cord blood) | ↑ CD133+/ VEGFR2+ cells i.e Circulating EPCs | [21] |
| **Pathological:** | | |
| Coronary artery disease/ number of risk factors<br><br><br>LDL<br>Hypertension<br>Smoking<br>Family history | ↓EPC number and migration<br>↓CD34+/KDR+ cells<br><br>↓CD34+/KDR+ cells, NE on CFUs, ↓Migration<br>↓Migration<br>↓EPCs or CD34+/KDR+ cells, ↓Culture, NE on migration<br>↓EPCs or CD34+/KDR+ cells | [43] |
| Total cholesterol | ↓ In culture<br>↓ Proliferation, migration, adhesion, *in vitro* vasculogenic capacity | [44] |
| Smoking | ↓ Circulating CD45low/ CD34+/ CD133+/ KDR+ cells | [45] |
| Stroke | ↓CFUs | [46] |
| Stable CAD | ↓CFUs<br>↓Migration,<br>↓In vivo vasculogenic capacity | [47] |
| Myocardial infarction | ↑ CD34+ cells<br>↑ CD34+/AC133+/VEGFR2+ cells<br>↑ CFUs | [48] |
| Cerebrovascular atherosclerosis | ↓ CD34+/CD133+ cells in cerebral infarction, no correlation with the degree of atherosclerosis | [49] |
| Vascular injury | ↑ AC133+/ VEGFR2+ cells | [50] |
| Congestive heart failure (Class I-II)<br><br><br><br>Congestive heart failure (Class III-IV) | ↑ CD34+ cells<br>↑ CD34+/AC133+/VEGFR2+ cells<br>↑ CFUs<br><br>↓ CD34+ cells<br>↓ CD34+/AC133+/VEGFR2+ cells<br>↓ CFUs | [51] |
| Diabetes<br>Type 1 diabetes mellitus<br><br><br>Type 2 diabetes mellitus<br><br>Type 2 diabetes mellitus | <br>↓ In culture, ↓*In vitro* vasculogenic capacity<br>↓ In culture, ↓*In vitro* vasculogenic capacity<br><br>↓ Adhesion | [52-54] |
| Growth factors/ Cytokines: | | |
| GCSF | ↑ CD133+/ VEGFR2+ cells | [30] |
| VEGF | ↑ CD133+/ VEGFR2+ cells | [55] |
| **Drugs:** | | |
| Erythropoietin | ↑ Circulating CD34+/ CD45+ cells<br>↑ In culture<br>↑ *In vitro* vasculogenic capacity | [56] |

| | | |
|---|---|---|
| HMG-CoA reductase inhibitors (Simvastatin, Mevastatin, Atorvastatin) | ↑ EPC number | [57] |
| Simvastatin | ↑ Proliferation and migration<br>↑ Adhesion | [58, 59] |
| Atorvastatin, Mevastatin | ↑ Proliferation, ↓ Senescence<br>↑ Migration | [60, 61] |
| **Vardenafil** | ↑ Circulating EPCs | [62] |
| Kallistatin | ↑ Circulating EPCs<br>↑ Proliferation, migration, adhesion, tube formation<br>↓ Apoptosis | [63] |

## Immunological Properties of Epcs

Autologous endothelial progenitor cells (EPCs) can be applied in cardiovascular regeneration but the cell availability in appropriate numbers is a limiting factor. Hence, the other alternative is the use of allogeneic EPCs and therefore the immunogenicity of EPC-derived endothelial cells (EC) is gaining importance [64]. The authors of this paper isolated circulating EPCs from rat and they were differentiated into ECs which were characterized phenotypically and functionally. Major histocompatibility complex (MHC) expression of these cells was determined in response to IFN-γ since cellular allorecognition of ECs is mainly related to their MHC expression and they were compared with rat aortic ECs, and their humoral and cellular allogeneic responses were also analysed *in vitro*. However, for testing the in vivo effects, decellularized aortic grafts were endothelialized with EPC-derived EC *in vitro* and a complete allogeneic mismatch rat aortic interposition model was used for transplantation of this re-endothelialized graft. It was observed that EPC-derived ECs expressed both endothelial-specific markers and low levels of MHC class I with no constitutive expression of MHC class II.

Since the MHC I expression was persistently low, it was unlikely that a natural killer cell response might be initiated. Hence, they were protected against allospecific cytotoxic T lymphocyte activity. Also, the allogeneic stimulation of CD4+ T cells by the EPC-derived ECs was less as compared to the aortic EC. The cells were also protected from complement mediated lysis by preventing effective binding of alloantibodies. Thus, EPC-derived ECs were protected from both cell and humoral mediated immunity *in vitro*. Seeding of EPC-derived ECs into acellular grafts *in vitro* and transplanting them in vivo led to excellent endothelialization and induced only mild inflammation without signs of rejection thus making them useful in therapeutic applications, especially vascular reconstruction [64].

## Future Directions

Successful isolation protocols for EPCs can be established by characterizing specific subpopulation of stem/ bone marrow cells which possesses the beneficial properties for vascular repair [19]. It is highly important that the isolation, cultivation and phenotypic characterization protocols used for EPCs be standardized and improvised for its yield and application [35]. Apart from this, optimal regimens for various aspects of EPCs must be developed such as their activation, stimulation, genetic modification and treatment. A major challenge in the EPC field would be to isolate sufficient numbers of EPCs with their angiogenic potential intact so that they can be used to treat patients with damaged vasculature.

Dealing with clinical trials, there have been instances where reports acquired from two clinical studies employing bone marrow cell transplantation to treat myocardial infarction using similar protocols have displayed conflicting results with one study indicating an improvement of left ventricular ejection fraction and cardiac function whereas the other study found no effect [19]. Apart from human trials, it would be more vital to directly compare the cell populations involved in neovascularization in both humans and rodent models with respect to similar physiological function, state of injury or disease process. It has been observed that EPCs are much more efficient in fostering improvement as well as ameliorating cardiovascular injury after an experimental myocardial infarction in rodent models as compared to their employment in human clinical trials till date [26]. The reasons for such incongruent results can be attributed to both the disease states of either subject examined as well as the cell populations isolated from them.

Moreover, combination cell therapy probably will be the next best approach to regenerate ischemic tissues. Since many ongoing clinical trials utilize MSCs, these can be potentially used in combination with either ECs or EPCs to treat ischemia since MSCs secrete VEGF which would appropriately stimulate cells of the endothelial lineage to induce vascularization and re-endothelialization. However, venture into clinical trials is still far ahead with use of these cells as many questions stem up such as which cells will serve best for vascular regeneration: EPCs or ECs? Next question irrespective of cell type is how to appropriately culture them for clinical trials. Nevertheless, even though specific identity of EPCs is controversial both EPCs and ECs may contribute to cellular therapy for regenerating ischemic tissues due to their inherent vessel-promoting and/ or vascular repair properties [2].

In conclusion, an attempt has been made in this review to give a review of progress in the EPC field, highlighting important findings along with sources of current controversies [26]. Clinical application of EPCs is a new

therapeutic idea venturing into the field of vascular biology and the Ponzo effect is an apt metaphor to describe the status of EPCs for the time being. Whether EPCs will rise up and make a mark in the therapeutic application by cardiologists, neurologists and other physicians trying to treat patients with vascular disease, or will they take sometime in their utilization considering their dark side of adverse effects such as infection, oncogenic transformation, tumor growth, retinopathy, myocardial infarction is just a matter of time [65]. In whichever case, it is essential to further research and understand EPC biology to fully benefit from their regenerative properties which will ultimately lead to their development and utilization as a powerful diagnostic, therapeutic and prognostic tool in a wide array of diseases as well as exploit them as a surrogate marker in clinical or pharmacotherapeutical studies [5,19].

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