Endothelial Colony Forming Cells (ECFCs) for tissue regeneration: in vitro characterization

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Endothelial Colony Forming Cells (ECFCs) for tissue regeneration: in vitro characterization

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CHAPTER 1

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1. ANGIOGENESIS, ARTERIOGENESIS AND DE-NOVO BLOOD VESSEL FORMATION AS PHYSIOLOGICAL CONCEPTS FOR PROSPECTIVE TISSUE REGENERATION AND REPAIR IN CLINICAL SETTINGS

Various pathological conditions that affect the cardiovascular system can lead to tissue ischemia. Destruction of the vascular network results in functional impairment and reduction of reparative capacity of ischemic tissue. The lack of oxygen and nutrients hampers the regenerative ability of tissue-resident stem and progenitor cells, while the influx of circulating immune and immature cells which also are necessary for tissue repair is reduced. Therefore, establishment of an adequate and functionally competent vasculature is of utmost importance for initiation and completion of tissue regeneration.

The neovascularization of ischemic tissue encompasses angiogenesis, arteriogenesis and de-novo vessel formation. The common environmental factor that initiates and consecutively regulates each of the three neovascularization mechanisms is the reduced oxygen availability in the ischemic area. The lower the oxygen tension is, the stronger the signal to start neovascularization will be. These processes of neovascularization represent the armory of how the human body fights ischemia which is a characteristic feature of certain pathological conditions such as myocardial infarction or peripheral arterial disease. The prospects of manipulating one of these physiological reparative mechanisms for therapeutically assisted tissue regeneration opens new venues of research in the field of regenerative medicine.

1.1 Hypoxia as driving process of neovascularization in post-natal life.

Oxygen concentrations in the human body differ depending of the vascularization of the tissue. The highest O_2 concentration exists in the lungs (13.5%), while in the circulation it varies from 5-12.5% to reach between 0.6%-5% in bone marrow and tissues. Therefore, hypoxia exists and the tissue is ischemic whenever the oxygen concentration is below the minimal physiological O_2 tension that ensures proper function of that tissue. Once present, the cells can sense low O_2 levels and adapt their functions by altering gene expression. This cellular response to hypoxia is mainly regulated by a family of transcription factors known as hypoxia-inducible factors (HIFs), which consists of HIF-1α, HIF-2α, and HIF-3α. The regulation of HIFs is oxygen dependent and regulated by a family of prolyl hydroxylase domain proteins (PHDs) which hydroxylates the alpha subunits of HIFs. Proline hydroxylation of HIF-1α and HIF-2α results in proteasomal degradation subsequent to binding Von Hippel Lindau (pVHL) protein. During hypoxia, HIF-1α and -2α predominantly regulate the expression of genes which possess hypoxia-
responsive elements and are crucially involved in the adaptation of cell metabolism during low O_2 levels, cell survival, proliferation, differentiation, and neovascularization and tissue repair. The prenatal knockdown of HIF-1α in mice is incompatible with life due to an excess of inadequate vascularization and vascular defects which further underpins the crucial role of HIFs in vascular formation. However, mice lacking HIF2α died at mid-gestation with bradycardia and reduced levels of noradrenaline, suggesting that HIF-2α regulates embryonic catecholamine synthesis in response to physiologic hypoxia. The difference in HIF1α and HIF-2α was further underscored by the finding that HIF-1α via VEGF-A primarily enhanced sprouting angiogenesis, while HIF-2α was also involved in the stabilization of microvessels.

Under hypoxic conditions, as a result of activation of HIFs, the cells release a plethora of trophic factors that play crucial roles in activation and regulation of the signaling pathways important for neovascularization and tissue repair. The presence of growth factors, cytokines and chemokines such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), tumor necrosis factor-alpha (TNF-α), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), stromal cell-derived factor 1 (SDF-1, CXCL12) triggers a concrete and finely tuned interaction between immune, endothelial, mural, and tissue resident stem and progenitor cells which results in formation of new vasculature within tissue affected by an ischemic insult.

1.2 Angiogenesis

Angiogenesis represents formation of new blood vessels from the pre-existing ones. At present, sprouting angiogenesis is acknowledged as a main physiological mechanism that ensures formation of new blood vessels in post-natal life. Intussusceptive angiogenesis is another mechanism of vessel formation that consists of splitting of a pre-existing one in two new blood vessels. Given the fact that intussusceptive angiogenesis mainly represents remodeling of pre-existing vascular networks and is a flow dependent mechanism, it is likely that sprouting angiogenesis is responsible for the major part of neovascularization. Sprouting angiogenesis is mainly a hypoxia-initiated and VEGF-mediated process. During angiogenesis different cells types such as myeloid cells, endothelial cells and endothelial progenitor cells as well as mural cells such as pericytes participate in the formation of new vasculature in the ischemic area. As a result of the effect of VEGF, certain endothelial cells of pre-existing vessels within the ischemic tissue acquire a so-called tip-cell phenotype. These endothelial tip cells are responsible for the initial formation of budding sprouts which represents the first step of angiogenesis. The sprouting consists of processes of degradation of basal membrane and surrounding extracellular matrix (ECM) by matrix metalloproteinase enzymes.
General introduction

(MMPs)\textsuperscript{19} and the uPA/uPAR fibrinolytic system\textsuperscript{20,21}; cell migration and subsequent proliferation; and formation of immature tube-like structures. During sprouting the tip-cells are guiding the invasion into the ECM, while the adjacent proliferating endothelial cells – called stalk cells – support further sprout elongation\textsuperscript{22}. These cells create a lumen and are aligning in such a way that they form the wall of the future blood vessel. Over time, the tip cells anastomose with the other tip cells of nearby sprouts forming vascular loops\textsuperscript{23}. Myeloid cells such as monocytes/macrophages positioned in the gap between two tip-cells of two neighboring sprouts assist the interconnection of two sprouts and formation of vascular loops\textsuperscript{24}. Once formed and perfused, these vascular loops are further stabilized by formation of a basal membrane and incorporation of supporting mural cells such as pericytes\textsuperscript{17}. Depending on the needs of local microenvironment a newly formed vascular network is further remodeled by anastomosing of the sprouts into bigger vessels or by pruning\textsuperscript{25} of a part of the new vessels. This ensures appropriate delivery of oxygen and nutrients.

The guidance, formation and maturation of the sprouts depends mainly on the concentration gradients of VEGF, SDF-1, PDGF, and angiopoietins (Ang-1/2) present in ischemic tissue, in which VEGF plays a most crucial role\textsuperscript{18,26}. A finely tuned interplay between VEGF and Notch/DLL signaling pathways regulates the switch between tip and stalk cell phenotype of endothelial cells and the further elongation, branching and fusion of the sprouts\textsuperscript{26}. The presence of PDGF-\(\beta\beta\) ensures attraction and incorporation of mural cells to newly formed blood vessels\textsuperscript{27}. Angiopoietins regulate the final vessel maturation and stabilization\textsuperscript{28}. An excess or lack of these growth factors leads to impaired angiogenesis characterized by an unstable vascular network with abnormal structure and function. When the angiogenic signals dissipate, the sprouting process ceases down and the endothelial cells acquire a quiescent phenotype, which enables them to perform their normal physiological functions optimally.

The vast amount of acquired knowledge about angiogenesis offers the prospect that on the one hand manipulation of this process may represent new opportunity for treatment of ischemic diseases. On the other hand, by restricting angiogenesis, a new modalities to fight cancer and specific eye disease (adult macular degeneration) can be developed.

1.3 Arteriogenesis

Although enlisted as a mechanism that participate in neovascularization of the ischemic tissue, arteriogenesis is not a process that directly contributes to alleviation of tissue ischemia by generating new vessels. Arteriogenesis is a process that increases the input of oxygen and nutrients by remodeling of the pre-existing collateral arteries within the
tissue. It takes place in the vascular tree distal of the occlusion of blood vessel, and is a flow mediated hypoxia-independent mechanism. As a result of increased flow, the endothelial cells are activated and start secreting trophic factors such as TNF-α, MCP-1, eNOS, GM-CSF that attract myeloid cells as well as upregulating receptors such as ICAM-1 and VCAM-1 which are important for EC-myeloid cell interaction. Monocytes represent the major myeloid cell type and, once differentiated into macrophages, are the crucial protagonist in the arteriogenic process. These cells secrete a plethora of chemokines, growth factors and proteases involved in vascular growth. As a result of this paracrine and ECM-remodeling effect of macrophages, the endothelial cells as well as smooth muscle cells proliferate leading to the widening of existing collaterals. This widening leads to a marked increase in blood flow, as according to the law of Poiseuille the amount of fluid flow is determined by the fourth order of the radius of the vessel. Increased blood flow through remodeled collaterals ensures improvement of delivery of oxygen and nutrients into the tissue. Pre-clinical animal studies have offered a solid base for the suggestion that manipulation of arteriogenesis represents a promising and feasible tool for tissue regeneration and repair. Stimulation of the mechanisms that regulate arteriogenesis, by application of growth factors and different cell types emerged as a new concept to improve blood flow to ischemic tissue.

1.4 Vasculogenesis or de-novo vessel formation
Vasculogenesis refers to the process of de novo formation of a primitive vascular network by differentiation of hemangioblasts into endothelial cells. Until the discovery of endothelial progenitor cells, it has been accepted that vasculogenesis occurs only during the embryonic and fetal development. In the last two decades it has become clear that vasculogenesis also can in adult life. Postnatal vasculogenesis is composed of several steps involving mobilization, recruitment, differentiation, and finally incorporation of endothelial progenitor cells (EPCs) into neovascularization sites. Under influence of VEGF, SDF-1, GM-CSF or tissue hypoxia, EPCs are mobilized into circulation from the bone marrow or other tissues. Once recruited into ischemic sites, these cells differentiate under influence of pro-angiogenic factors into mature endothelial cells that incorporate into the existing vascular endothelial lining or initiate assembly of new vascular structures. At present, the scientific literature recognizes two distinct types of EPCs each one of them participating during vascularization in a different manner. Depending on the time that they appear during in vitro culture after isolation from peripheral blood, the EPCs are clustered as early and late outgrowth EPCs. The so-called early EPCs belong to myeloid lineage and participate in neovascularization (including angiogenesis) in a paracrine fashion by releasing pro-angiogenic factors. On the other hand, the late outgrowth EPCs also referred to as blood outgrowth endothelial cells,
endothelial outgrowth cells or endothelial colony forming cells (ECFCs) do not belong to hematopoietic cell lineage. They exhibit a pronounced vascularization ability in vivo by physically incorporating into newly formed blood vessels\textsuperscript{40}.

Adequate regeneration of the tissue affected by an ischemic insult relies on efficient angiogenesis that can be facilitated by tissue engineering or cell-based therapeutics involving endothelial cells (ECs). In vivo studies pointed out that ECFCs possess all biological features to be considered as a cell source of preference for de novo vascularization either directly as source of endothelial cells in tissue engineering or for cell based therapies (see also Chapter 2).

### 1.5 Prospect for therapeutic neovascularization

Development of tissue ischemia results in irreversible tissue damage mainly mediated through necrosis and apoptosis. The body’s first reaction to tissue injury is hemostasis which represents platelet aggregation and the formation of a fibrin clot. The hemostatic plug usually further lowers the local oxygen level, a lowering that is further aggravated by invading phagocytes. Hypoxia - usually accompanied by sterile inflammation - sends strong prosurvival signals to the surrounded tissue as well as to more distant tissues via the systemic circulation. Local signals as well as the signals distributed via the systemic circulation attract and recruit immune and stem/progenitor cells from the local environment or from more distant tissues such as bone marrow. Activation of a complex network of signaling pathways initiates the intrinsic tissue regenerative process which results in differentiation of tissue-resident stem/progenitor cells in tissue-specific somatic cells and reconstitution of an adequate surrounding ECM. However, a crucial step in tissue regeneration and repair is the formation of new vasculature or the expansion of the existing one in order to provide an adequate supply of oxygen and nutrients. Vasculogenesis, angiogenesis and arteriogenesis are the three physiological mechanisms that enable re-establishment of adequate blood flow in injured area. Pre-clinical studies have shown that by intervening/interfering with some of the crucial components that govern these processes it is possible to accomplish faster and more efficient tissue repair. At present, angiogenesis, arteriogenesis and vasculogenesis attract the interest of the scientific world as promising tools for regenerative medicine. Accumulated knowledge about neovascularization mechanisms have been translated into pre-clinical and clinical concepts for tissue regeneration and repair.

Depending on the pathological background of the event that resulted in tissue ischemia it is possible to stimulate one of these neovascularization processes in order to initiate or facilitate efficient tissue repair or to preserve its function. Therapeutic arteriogenesis might be used to improve the symptoms of myocardial ischemia in...
patients with coronary ischemic disease by facilitating the recruitment of collateral vessels. Application of MCP-1\textsuperscript{41} or GM-CSF\textsuperscript{42} resulted in an increased influx of monocytes which correlated with an increased collateral blood flow after femoral artery occlusion in animals. Moreover, intracardiac injection of bone marrow cells has been reported to have a positive influence on left ventricular ejection fraction and in coronary flow reserve in patients post-acute myocardial infarction\textsuperscript{43}. Injection of EPCs in ischemic tissue or into the systemic circulation results in tissue regeneration and repair mediated mainly via angiogenesis and to lesser extent by de-novo vascularization. These cells not only formed new fully functional vascular networks that were interconnected with the host vasculature, but - by secreting different trophic factors - they also enabled generation of a permissive environment for differentiation of tissue resident stem/progenitor cells into tissue specific somatic cells. Combined with other stem cells and embedded in biocompatible scaffolds EPCs have been successfully used in tissue engineering proof-of-concept research in animal models of bone, skin, and peripheral ischemia. At present, EPCs represented through ECFCs are considered a most promising cell type for regenerative neovascularization.

2. IDENTIFICATION AND CHARACTERIZATION OF ECFCs

The identification of EPCs shifted the paradigm of how post-natal vascularization occurs. The emergence of EPCs offered an distinct and unique insight into the mechanisms that govern post-natal neovascularization. Until their identification it was widely accepted that angiogenesis is the main vascularization process in post-natal life and that it is mainly driven by mature endothelial cells that line the already existing vasculature. The discovery of EPCs offered proof that the post-natal neovascularization is not only restricted to angiogenesis and arteriogenesis but also that - as a result of EPC recruitment - de novo blood vessel formation takes place in the adult organism. The concept of EPCs as a driving force of post-natal neovascularization was first introduced by the seminal work of Asahara\textsuperscript{44} who identified a subpopulation of circulating mononuclear cells from peripheral blood (PB) that exhibited pro-angiogenic features in vivo. To that end, the PB mononuclear cell (MNC) fraction was enriched by magnetic beads for CD34\textsuperscript{-}-cells and plated in endothelial differentiation medium. After a period of 24h, the non-adherent fraction was removed and clusters composed of spindle-shaped cells emerged in cell culture after 3-7 days. Phenotypical and functional characterization revealed that these cells - besides expressing endothelial cell surface markers (CD31, VEGFR-1, Tie-2) - also exhibit markers exclusively associated with hematopoietic cell lineage (CD45). These
cells differentiated from CD34+ PB-MNCs and named as EPCs actively participated in neovascularization after implantation in mice and rabbit models of unilateral hindlimb ischemia.

This discovery was translated into a Colony-Forming Units-Hill assay (CFU-Hill assay) which in short time became a gold standard for enumeration of circulating EPCs from peripheral blood, whose number of in vitro formed colonies correlates with the susceptibility for cardiovascular disease as well as with other diseases. The existence of EPCs also initiated a widespread enthusiasm of using these cells for regenerative neovascularization. In a short term plenty of studies emerged reporting a beneficial effect of implantation of EPCs in different animal models of vascular or myocardial ischemia. Later research identified that these EPCs (henceforth CFU-Hill cells) belong to the hematopoietic cell lineage and participate in neovascularization only in a paracrine manner which might explain their poor performance in treatment of acute myocardial infarction in human subjects. On the other hand, inoculation of PB-MNCs in endothelial differentiation medium for 4 days yields cells with endothelial-myeloid phenotypical characteristics and pro-angiogenic function in vitro and in vivo. These cells, obtained from PB-MNCs by this method and regarded at the time of identification also as EPCs, differ from CFU-Hill cells. These circulating angiogenic cells (CACs) exhibit the same spindle-shaped morphology as CFU-Hill cells but grow in a wide spread pattern in cell culture instead of being grouped in small dense cell clusters.

Extensive research of late EPCs phenotypical- and in vitro and in vivo functional characteristics identified that these cells are a distinct type of EPCs different from the CFU-Hill cells (early EPCs) and CACs. The late outgrowth EPCs, also referred to as endothelial colony-forming cells (ECFCs) due to their unique ability to form secondary and tertiary...
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colonies upon re-plating, can be obtained from cord or peripheral blood, white adipose tissue\textsuperscript{54} or lung vasculature\textsuperscript{55} from humans. These cells, in contrast to CFU-Hill cells and CACs, are characterized by robust proliferative and clonal capacity; by pronounced in vivo vascularization ability; by physically incorporating into newly formed blood vessels; and by being neither derived from nor belonging to the myelo-monocytic lineage. Moreover, ECFCs are phenotypically indistinguishable from other cultured endothelial cells derived from other vascular beds such as human umbilical vein endothelial cell (HUVEC) or human microvascular endothelial cell (HMVEC)\textsuperscript{56,57,58}. The inability of CFU-Hill cells to actively incorporate into newly-formed vasculature in vivo and to acquire a truly endothelial phenotype indicates that their pro-endothelial phenotype (expression of some endothelial markers) might be mere an in vitro phenomenon. In addition, CFU-Hill as well as CACs possess neither clonal ability nor proliferative potential that matches ECFCs. Therefore, based on the differences in expression of phenotypic markers, the in vitro and in vivo neovascularization potency as well as differences in clonal ability, it indicates that the late outgrowth endothelial progenitor cells or ECFCs, but not the CFU-Hill cells (early EPCs) or CACs can be considered as putative true endothelial progenitor cells.

2.2 Differences between CB-ECFCs and PB-ECFCs

The mononuclear cell fraction obtained from cord or peripheral blood is the most used source to isolate ECFCs. These cells can also be isolated from lung\textsuperscript{59} and white adipose tissue\textsuperscript{54} of human as well as other tissues/organs as already reported for different animal models\textsuperscript{60,61,62}. However, research related to the comparison of biological features and regenerative ability of ECFCs is performed almost exclusively with cells isolated from umbilical cord blood (CB) or peripheral blood (PB) samples. As one can expect, there are important differences between the ECFCs isolated from CB and PB due to the different physiological conditions that exist during fetal and adult life. The circulating EPCs that give rise to ECFCs are significantly more enriched in CB than in PB making the isolation of these cells from CB much easier than from PB\textsuperscript{57}. Once isolated in vitro CB-isolated ECFCs exhibit a proliferation ability that enables generation of sufficient number of cells for clinical application in shorter period than PB-ECFCs\textsuperscript{57}. In addition, PB-ECFCs exhibit hallmarks of senescence at earlier time point during in vitro expansion\textsuperscript{40} and are more sensitive to oxidant stress\textsuperscript{43} than CB-isolated ECFCs. Moreover, the CB-derived ECFCs form vascular networks that last longer than the ones built by PB-ECFCs\textsuperscript{64,65,66}. These important in vitro and in vivo differences suggest that CB-ECFCs might represent a more appropriate cell type for regenerative treatments than PB-ECFCs. However, it was reported that the CB-derived but not the PB-isolated ECFCs demonstrate signs of a significant degree of karyotype abnormalities during long-term expansion\textsuperscript{67}. This
finding indicates that CB cells cannot be considered free of risk in clinical application. Comparative gene profiling of CB- and PB-isolated ECFCs revealed that CB-derived cells have higher mRNA levels of ICAM-1 and vWF and lower levels of pro-inflammatory genes such as IL-1β and TNF-α. Higher mRNA levels of the proteolytic enzymes MMP1 and MMP9 were detected in PB-ECFCs, which indicates that these cells are better equipped for remodeling of the ECM than CB-ECFCs. With respect to the ability to trigger an immune response toward autologous immune cells, the CB cells were less immunogenic than the cells isolated from adult blood. Comparative proteomic analysis of ECFCs derived from cord- or peripheral blood identified 34 differentially regulated proteins. Most of the upregulated proteins in CB-ECFCs were involved in cell proliferation and protection against oxidative stress, which likely underlie the increased survival of CB-isolated cells after in vivo application as well as their better vascularization of ischemic tissue compared to PB-ECFCs. There is a strong likelihood that the differences in cellular composition, levels of trophic factors and oxygen concentration between cord and peripheral blood are most likely responsible for the observed differences between CB- and PB-ECFCs. Cohen et al. have reported that treatment of adult T-lymphocytes with heat-inactivated serum derived from peripheral blood elicited a strong proliferation response which was absent when cord blood-derived serum was used. Therefore, the circulating EPCs that give rise to ECFCs from cord and peripheral blood are residing in different environments. Being exposed to different conditions and factors, they differ in their in vivo and in vitro response to stimuli.

Whatever the underlying processes or mechanisms are that contribute to differences in the neovascularization ability between CB- and PB-ECFCs, it should be noted that at present peripheral blood represents a more feasible cell source for isolation of ECFCs that can be used for treatment of ischemic diseases. Use of PB for isolation of ECFCs from patients grants application of these cells in an autologous manner, thus reducing the risk of unwanted immunological reactions. Unavailability of autologous CB-derived ECFCs for treatment of the present patient population that might be in a need of these cells restricts CB to be considered as a viable source of ECFCs in the near future.

2.3 Origin of ECFCs

It has been more than 25 years since the first isolation of ECFCs, yet the question about the origin of these cells remains still unanswered. These cells can be isolated from peripheral and cord blood by inoculation of freshly isolated MNCs with endothelial differentiation medium on a rat-tail collagen I, fibronectin or gelatin. Several research groups have also reported derivation of ECFCs from other human tissues such as white adipose tissue and intima of large blood vessels, lung as well as from mouse.
rat lungs, and from porcine, rabbit and rat bone marrow. One of the drawbacks of identification and isolation of EPCs is the heterogeneity of the primary inoculum of MNCs, wherein the precursor cells that give rise to ECFCs reside. However, based on the cell surface expression of antigens associated with stem/progenitor and endothelial differentiation phenotypes it is possible to select a specific cell population that generates to ECFCs. By this approach it is also possible to identify an accessory cell population that is involved in outgrowth of ECFCs in vitro.

The circulating immature cells that develop as ECFCs reside in the cell fraction positive for CD34. Removal of CD34+ MNCs diminishes the outgrowth of ECFCs. It is interesting that a two-compartment co-culture of CD34+ MNCs with the cells positive for CD14 or CD11b but negative for CD34 generates more ECFCs colonies than the CD34+ MNCs fraction cultured alone. This implicates that CD34+/CD14+ or CD34+/CD11b- cells which originate from the hemato-myeloid cell lineage have an important role during in vitro endothelial differentiation of circulating CD34+ endothelial precursors. These studies have also identified that this paracrine effect of CD14+ or CD11b+ MNCs is mediated via angiogenin-1 or SDF-1, respectively. Interestingly, removal of the cells positive for CD45 from a CD34+ MNCs population yields ECFCs colonies suggesting that CD45- MNCs cells (mainly T-lymphocytes) are dispensable for outgrowth of ECFCs. By using of 4-color flow cytometry of purified CD34+ cells, Delorme et al, have confined the surface antigen phenotype of circulating EPCs that give rise to ECFCs to a cell population that is characterized by expression of CD34 and CD146 and absence of CD45, CD133, or CD117. These CD34+/CD146+/CD45-/CD133-/CD117- fulfilled the typical in vitro and in vivo features of late outgrowth EPCs. Further research performed with implementing more strict investigational criteria and techniques such as polychromatic flow cytometry (PFC), colony assays, immunomagnetic selection, and electron microscopy have confirmed the report of Delorme et al. At present, the phenotypical characterization of ECFCs includes that the cells must be positive at least for the endothelial cell lineage markers CD31, CD34, CD146, CD309, CD144 and ought to be negative for the hematopoietic surface antigens CD14, CD45 as well as for mesenchymal stem cell markers CD70 and α-smooth muscle actin.

The proposed set of markers for identification of circulating endothelial precursor cells in CB and PB partially overlaps with the set of markers usually associated with hematopoietic stem cell lineage which sparked a debate about the hematopoietic origin of EPCs. Early research on EPCs biology suggested that EPCs might originate from hematopoietic stem cell pool. Comparison of the cell surface markers and genomic profiling, and assessment of clonal and in vitro and in vivo neovascularization potency clearly delineated that ECFCs and the myeloid cells characterized by the
General introduction

CFU-Hill assay are two different cell subsets that in different ways participate in the formation of new blood vessels. Clonal analysis of ECFCs and CFU-Hill cells derived from patients with myelodysplastic syndrome or from patients with polycythemia vera or with chronic myeloproliferative disorders bearing either JAK2 V617F or BCR-ABL mutations further underpinned that ECFCs as true EPCs are not hematopoietic in origin. At present, the question about the origin of the circulating progenitor cells that differentiate into ECFCs remains still unanswered.

Lineage tracing studies identified that primitive multilineage HSCs/progenitor cells responsible for the generation of all blood cell types during definitive hematopoiesis arise from hemogenic endothelium. During embryonic development, the primordial stem cells give rise to hemangioblasts from which all mesodermal precursors originate. It appears that the hemogenic endothelium is a one step further in the hematopoietic commitment of hemangioblasts. While the HSCs, endothelium and MCSs originate from the hemangioblasts, the hematogenic endothelium is unable to produce cells that belong to the mesenchymal stem cell pool. It remains unknown whether the hematogenic endothelium might also give rise to angioblasts or EPCs that reside in different tissues and are responsible for neovascularization and vascular repair in postnatal life.

Endothelial precursors that give rise to ECFCs reside in different tissue compartments. Bone marrow is still considered as a primary source of endothelial precursors although recent data indicate that rather the subendothelial space of vessel wall is the predominant space from where ECFCs precursors are released in the circulation. Isolation of ECFCs from white adipose tissue, lung and placenta implies that tissue-resident, dormant ECFCs precursors reside in these as well as other tissue components. Normal physiological and pathological signals mobilize these precursors to participate either in maintenance of vascular homeostasis or in tissue repair and regeneration.
Figure 1. Origin of ECFCs

*massive ischemia that overcomes the potential of tissue-resident endothelial precursors to re-establish vasculature

Bone Marrow

Vascular Endothelium

Tissue-resident EPCs

PMID:
25669621
17620112
20590667

PMID:
22530103
15585655

PMID:
adipose tissue: 24982574
lung: 24710033, 21882012
placenta: 24106336, 27004134

*maintenance of vascular integrity and repair of local denudation

umbilical vein endothelial cells (HUVECs)
aortic endothelial cells (HAECs)
pulmonary artery endothelial cells (PAECs)

*maintenance of vascular homeostasis and angiogenesis within a tissue
Nevertheless, collective evidence supports the hypothesis that EPCs can be found except in blood also in the vascular compartment of different tissues. The identification of a complete hierarchy of EPCs in cell cultures of HUVECs, human aortic endothelial cells (HAECs)\textsuperscript{70} and human pulmonary arterial endothelial cells (HPAEC)\textsuperscript{83} derived from the vessel wall indicates that the intimal space of blood vessels might act as a reservoir of cells with potent vascularization and reparative potential. Isolation of ECFCs from solid tissues of humans and animals by employing different cell culture methods or magnetic pre-selection for CD31 antigen strongly supports the hypothesis that the vasculature harnesses precursor cells able to differentiate into ECFCs. Although the bone marrow (BM) usually is associated as a place where the stem and progenitor cells reside, it appears that EPCs that give rise of ECFCs are not present in BM. Tura et al\textsuperscript{76} reported that an application of GM-CSF, a known factor that triggers the migration stem and progenitor cells from BM into circulation, did not mobilize EPCs that give rise of ECFCs. In addition, existence of cells with multilineage potential able to differentiate into endothelial cells within vasa vasorum of tunica adventitia of large blood vessels\textsuperscript{87,88} further supports the consensus that the vasculature is enriched with ECFC-generating EPCs. Whatever is the location where these EPCs reside, these cells can be released into the circulation accessing the sites of neovascularization and vascular repair where they differentiate into mature endothelial cells.

In vitro studies underlined several growth factors and signaling networks that govern the differentiation of circulating EPCs into ECFCs. Angiogenin-1 and SDF-1 released from CD14 or CD11b MNCs play a role during in vitro generation of ECFCs colonies\textsuperscript{77,78}. In vitro conversion of human induced pluripotent stem cells (hiPSCs) and embryonic stem cells into the cells that completely resemble the in vitro and in vivo biological features of ECFCs pointed out the importance of neuropilin-1-mediated activation of VEGFR-2 signaling through VEGF-A in endothelial differentiation\textsuperscript{139}. In agreement with previous reports\textsuperscript{89}, the same study also identified FGF-2 and BMP-4 as important regulators of endothelial differentiation of hiPSCs into ECFCs-like cells\textsuperscript{139}.

### 2.4 Phenotypic plasticity of ECFCs

Exposure of in vitro isolated ECFCs to mesenchymal-stem cell inductive environment composed of TGF-β or PDGF-BB activates a genetic program which triggers these cells to acquire a mural cell phenotype. This shift of endothelial to mesenchymal phenotype of ECFCs and other endothelial cell is known as endothelial-mesenchymal transdifferentiation (EndoMT). Since ECFCs and other ECs belong to mesodermal lineage it might be that the EPCs are also susceptible to EndoMT depending on the demands of microenvironment. ECFCs implanted in fetal model of sheep acquired phenotype
of tissue-resident progenitor cells with mural phenotype. Notwithstanding, this hypothesis implicates that the phenotypic plasticity actually is one of the hallmarks of tissue resident progenitor that give rise to EPCs. It is possible to envisage that phenotypical plasticity of tissue-resident EPCs is crucial for neovascularization and regeneration. The tissue resident EPCs undergoing endothelial differentiation would give rise of ECFCs for building of vascular networks while in same time by EndoMT and differentiating toward mural cells will provide cells that are crucial for stabilization and maturation of newly-formed blood vessels.

2.5 Conclusion
The identification of EPCs contributed to important changes of the scientific landscape in the field of vascular biology. As a result of vast research it became clear that vasculogenesis is not only restricted to fetal but also occurs during post-natal life. Although it has been reported that several types of EPCs exist in the adult organism, the collective evidence suggests that ECFCs most likely are the only cell type of all identified EPCs that is clearly confined to the phenotype of truly endothelial EPCs. Scientific reports suggest that blood vessels as well as vascular beds within different tissues are places where the EPCs resides in adult life. It is also not excluded that these cells reside in bone marrow or interstitial connective tissue of solid organs. ECFCs, which can be isolated from the blood and as well as from tissues, do not belong to hematopoietic cell lineage, possess a phenotype and functional characteristics indistinguishable from other endothelial cells types and most importantly exhibit robust in vitro and in vivo vascularization potential. The potent neovascularization potential of ECFCs makes these cells to be considered as a preferential cell source for treatment of ischemic pathologies by tissue neovascularization and regeneration.

3. PB-ECFCs AS A CELLULAR PLATFORM FOR INVESTIGATION OF THE PATHOPHYSIOLOGY OF DISEASES
Emergence of ECFCs opened new venues for investigating how pathological mechanisms that underlie certain diseases can affect the biological functioning of the endothelium as well as its regenerative potential. As these cells can be isolated with minimal invasive procedure and grown in large numbers in vitro, ECFCs at present represent a cellular platform that is used to investigate endothelial dysfunction in cases of venous thromboembolic disease (VTD), pulmonary arterial hypertension (PAH), gestational and post-natal diabetes, hypertension and preeclampsia, hereditary haemorrhagic telangiectasia, chronic obstructive pulmonary disease (COPD), von Willebrand disease,
end-stage renal failure, cancer or burns. However, as can be expected, at child delivery and during adult life one may expect a different response of ECFCs with respect to their emergence in cell culture, proliferation, migration, adhesion and angiogenic capacity.

### 3.1 Biological features of ECFCs in diseases during fetal life

Accumulating evidence indicates that the disbalance in perinatal environment during fetal life alters the competence of ECFCs for regeneration and maintenance of vascular homeostasis in post-natal life. Quantitative and functional abnormalities of cord blood ECFCs have been reported in preeclampsia\(^91,92\), gestational diabetes\(^93,94\), and in preterm low-birth-weight infants\(^95\) or in premature neonates with normal weight\(^96\).

Prolonged time of emergence and reduction in the number of ECFC colonies from cord blood as well as premature senescence of the cells were reported in preeclampsic pregnancies\(^91,92\) and in low-birth-weight preterm infants\(^95\). Decreased functionality of SIRT1 was the underlying mechanism that increased the senescence rate of ECFCs in premature neonates\(^96\) while hyperactivation of p38MAPK contributed in case of gestational diabetes\(^94\). Further, ECFCs obtained from cord blood of premature low-birth-weight neonates\(^95\), preterm infants with normal birth weight\(^96\) or from gestational diabetes\(^93\) exhibited reduced in vitro and in vivo angiogenic potential suggesting impairment of neovascularization competence of ECFCs. ECFCs from premature low-birth-weight infants displayed a phenotype characterized by upregulation of mRNA levels of antiangiogenic factors such as thrombospondin 1 and 2, collagen type XVIII alpha 1 chain tumstatin, brain angiogenesis inhibitor 1, platelet factor 4, and angiopoietin 1 and reduced levels of proangiogenic genes such as epidermal growth factor, neuropilin 1 and fibroblast growth factor\(^95\). Interestingly, not all pathological conditions during pregnancy have detrimental effect on the biological features of ECFCs. Ingram et al reported very similar biological functions between the ECFCs obtained from preeclampsic and healthy pregnancies, including the ability to form vascular networks in vivo\(^95\).

Therefore, while gestation diabetes reduced, the preeclampsic condition did not alter the angiogenic capacity of ECFCs. This indicates that the biological competence of ECFCs depends on the pathophysiological mechanism that underlies a certain pathological condition during pregnancy.
3.2 Biological features of ECFCs in diseases during adult life

Quantitative and functional abnormalities have also been reported in ECFCs obtained from adult patients (Table 1). Metabolic diseases such as diabetes mellitus reduce the outgrowth of ECFCs from peripheral blood MNCs of diabetic patients in comparison to healthy controls [97]. Jarajapu et al. reported similar findings since they were able to isolate ECFCs only from 15% of the diabetic individuals while PB-MNCs from nondiabetic individuals gave ECFCs colonies nearly in 90% of the cases [98]. ECFCs isolated from diabetic patients showed reduced proliferation, migration and in vitro tube formation [99]. Additional research identified that hyperglycemia was the main cause of reduced in vitro and in vivo biological functions of ECFCs in post-natal diabetes. The high glucose levels decreased eNOS, FoxO1, and Akt phosphorylation as well as bioavailability of nitric oxide (NO) undermining the biological activity of ECFCs [100]. Moreover, ECFCs isolated from diabetic patients exhibited reduced neovascularization capacity in immunodeficient mice compared with healthy donor and age-matched ECFCs [101]. These studies exemplify that exposure to increased glucose levels during post-natal life significantly affects the biocompetence of ECFCs with respect to their regenerative abilities as already observed in prenatal life. On the other hand, pretreatment of ECFCs isolated from diabetic patients or exposed to hyperglycemia with adiponectin [101] or vitamin D [102] or far infrared radiation [99] prior in vivo administration reversed the detrimental effects of increased glucose levels on angiogenic ability of ECFCs. Therefore, the strategies that aim to harness the neovascularization capacity of ECFC for regenerative purposes should include methods that aim to restore the impaired functional competence of the cells obtained from diabetic patients prior in vivo application.

Vascular diseases also affect biological functions of ECFCs. ECFCs obtained from hemorrhagic telangiectasia patients revealed deficiency in endoglin. Abnormalities in TGF-β pathways, mediated by ALK1 and ALK5, underlined the appearance of disorganized and depolymerized actin fibers [103]. ECFCs exhibiting this phenotype have impaired angiogenic capacity which may lead to fragility of small vessels and bleeding characteristic of this type of vascular dysplastic disease. Biological and functional abnormalities of ECFC in patients with recurrent, unprovoked venous thromboembolic disease (VTD) was also reported [104]. Surprisingly, more colonies were enumerated in the cultures established with peripheral blood collected from VDT patients than controls. The cells from patients with VTD showed similar capacity to form tube-like structures but exhibited significant abnormalities in the structure of mitochondrial membrane and increase in ROS and pro-inflammatory cytokine production. Therefore, it is possible that inherited susceptibility for hypercoagulation of ECFCs in patients with VTD might limit the
use of these cells to facilitate the reparative processes such as vessel recanalization and repair after an episode of thromboembolic event. Nevertheless, observed abnormalities in ECFCs from VTD patients offer a possibility to establish an assay to measure the endothelial dysfunction in this patient population or for estimation of susceptibility for thromboembolic event in population prone to this condition. However, the study of Brittan et al.\textsuperscript{105} calls for caution in case if such an assays would be developed. Namely,

**Table 1. Quantitative and functional abnormalities in ECFCs obtained from adult patients.**

Most of the pathological conditions and diseases mainly have detrimental effect on ECFCs. On the other hand, recent studies also indicated that it’s likely that ECFCs play a role in the development or maintenance of vicious pathophysiological cycle, especially in diseases driven by pathological mechanism which involves deregulation of endothelial integrity and function.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes and hyperglycaemia</td>
<td>reduces the outgrowth</td>
<td>97, 98, 99, 100, 101</td>
</tr>
<tr>
<td></td>
<td>reduced migration</td>
<td></td>
</tr>
<tr>
<td></td>
<td>reduced proliferation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>reduced in vitro angiogenicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>reduced nitric oxide (NO)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>reduced in vivo</td>
<td></td>
</tr>
<tr>
<td>Hemorrhagic Telangiectasia</td>
<td>reduced in vitro tube formation</td>
<td>103</td>
</tr>
<tr>
<td>Venous Thromboembolic Disease (VTD)</td>
<td>increased outgrowth</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>increased ROS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pro-inflammatory cytokine profile</td>
<td></td>
</tr>
<tr>
<td>PAH</td>
<td>increased outgrowth</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>reduced in vitro angiogenicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>reduced in vivo angiogenicity</td>
<td></td>
</tr>
<tr>
<td>COPD</td>
<td>increased the DNA damage and premature senescence</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>decreased angiogenic ability</td>
<td></td>
</tr>
<tr>
<td></td>
<td>increased apoptosis</td>
<td></td>
</tr>
<tr>
<td>End-stage Renal Failure</td>
<td>increased outgrowth</td>
<td>112</td>
</tr>
<tr>
<td>Burns</td>
<td>increased outgrowth</td>
<td>113</td>
</tr>
<tr>
<td>von Willebrand Factor Disease</td>
<td>decreased synthesis of vWF</td>
<td>114, 115, 116</td>
</tr>
<tr>
<td></td>
<td>increased migration</td>
<td></td>
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<tr>
<td></td>
<td>increased proliferation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>increased in vitro angiogenicity</td>
<td></td>
</tr>
</tbody>
</table>

PAH: pulmonary arterial hypertension; COPD: chronic obstructive pulmonary disease
in this study it was observed that in patients with premature coronary artery disease endothelial cells derived of the local vessel wall, rather than ECFCs, were impaired with respect to proliferation, adhesion and migration, as well as expression of microRNAs which are known to regulate endothelial function. This indicates that in certain diseases not the inherited but the local environment and the pathogenesis of disease affects the biological response of endothelial cells as well as ECFCs.

In lung diseases such as hereditary and idiopathic pulmonary arterial hypertension (PAH) and chronic obstructive pulmonary disease (COPD), ECFCs display alternations in their biological features. PAH is a progressive vascular disease characterized by dysregulated EC growth and survival and hyperproliferation\(^{106}\), leading to remodeling and obliteration of pulmonary arterioles that finally lead to heart failure\(^{107}\). Genetic studies pinpoint that defects in BMP-2 receptor (BMPR-II) play important role in pathobiology of PAH. Up until now, the pathological mechanisms underlying excessive cell proliferation in PAH were mainly investigated in human pulmonary arterial endothelial cells (hPAEC). Several studies have identified existence of highly proliferative ECFCs in cultures of hPAEC\(^ {59,55} \). In addition, ECFCs have successfully been isolated from PB-MNCs from PAH patients\(^ {59} \). Our data as presented in Chapter 6 revealed that the colonies of ECFCs from PB-MNCs of PAH patients emerged earlier and in greater number compared to healthy controls. These cells either obtained from PAH hPAEC or peripheral blood exhibited higher proliferative potential but reduced in vitro and in vivo angiogenic potential\(^ {108} \) which is in line with our observation. Interestingly, irrespectively whether the cells were obtained from PAH patients bearing mutations of BMPR-II or not, the expression of this receptor was reduced in both groups of PAH patients further strengthening the concept that disbalance of BMP-2 signaling plays important role in pathogenesis of this lethal disease\(^ {109} \). Proteomic analysis of PAH ECFCs identified that translationally controlled tumor protein (TCT1) mediates the hyperproliferative phenotype of PAH ECFCs and acts as a prosurvival factor\(^ {109} \). Identification of this protein that promotes tumor cell growth and survival further pinpoints that the pathophysiology of PAH might share some common aspects which are hallmark of tumor biology.

In addition to genetic dysregulation, the lifestyle habits that have detrimental effect on cardiovascular system also affect ECFCs. Smoking is the major risk factor for the development of COPD which affects 20% of smokers\(^ {110} \). Investigation of whether ECFCs are dysfunctional in smoking individuals and patients with COPD revealed that exposure to cigarette smoke leads to reduction of SIRT-1 activity which increased the DNA damage and triggered premature senescence\(^ {111} \). Further, the same study reported that the senescent ECFCs from COPD patients exhibited impaired angiogenic ability
and increased apoptosis compared to cells from healthy nonsmokers. Therefore, epigenetic changes might play an important role in the dysfunction of ECFCs obtained from smokers and COPD patients as well as in patients with other diseases.

Not all pathological conditions have detrimental effect on biological functions of ECFCs. Patients with end-stage renal failure or burns generated more ECFCs colonies displaying normal endothelial cell phenotype as well as unaltered in vitro and in vivo neovascularization. ECFCs isolated from patients with von Willebrand factor disease although showed decreased synthesis and storage of vWF yet exhibited similar or even higher in vitro proliferation, migration and angiogenic capacity than the healthy controls.

### 3.3 Conclusion

Scientific literature clearly pinpoints that the dysfunction of ECFCs is part of the phenotype or underlies the pathophysiological mechanism of certain diseases. Changes of biological features of ECFCs in pathological conditions offers an opportunity to employ these cells to study human diseases by using in vitro-based models. ECFCs have been either exposed to well defined in vitro conditions that are relevant for the pathogenesis of disease (forward approach) or have been isolated and thoroughly investigated in patients in order to study or identify a mechanism that underlies certain disease (reverse approach).

The forward approach by using healthy ECFCs offers an opportunity to recapitulate the early stages of the pathophysiology of disease. Exposure of ECFCs to hyperglycemia illustrates this research strategy. On the other hand, the identification of TCT1 protein in PAH ECFCs is a typical example of reverse approach.

Comparison between patient-derived and healthy-control ECFCs enables identification of the differences between diseased- and non-diseased cells. Data presented in Chapter 6 is a typical example of reverse-type of study wherein the ECFCs isolated from PAH patients were compared with healthy-control ECFCs with respect to outgrowth, proliferation, reaction to shear stress and tube-forming ability.

Both approaches are complementary and should be employed in case when ECFCs are used as a cellular platform for disease modeling. Biological characteristics of ECFCs warrants that these cells will be often used to study the cellular and molecular mechanisms of cardiovascular disease in future. This will potentially lead to the discovery of novel treatments for this group of detrimental pathological conditions.
4. PB-ECFCs as a Therapeutic Tool for Tissue Regeneration and Repair

4.1 Neovascularization Capacity of ECFCs

In the field of regenerative medicine, the therapeutic neovascularization consists of interventions or procedures including soluble factors, cells and biocompatible materials that create conditions permissive for formation of new blood vessels. In vivo studies identified that the endothelial-colony forming cells (ECFCs) represent a promising cell source for therapeutic neovascularization. ECFCs are able to initiate and facilitate neovascularization in diseased tissue and, by acting in paracrine manner, actively participate in the creation of favorable conditions for efficient and appropriate differentiation of tissue-resident stem or progenitor cells. The intrinsic neovascularization ability of ECFCs facilitated the restoration of blood perfusion in ischemic tissue in the mouse hindlimb model, sustained the survival of tissue-resident somatic cells and improved hemodynamic parameters after myocardial infarction. These cells have been used to create in vitro or in situ pre-formed vascular networks within tissue-engineered scaffolds that efficiently interconnected with host vasculature and facilitated tissue regeneration after in vivo implantation. Their neovascularization ability was more pronounced when combined with stem/progenitor cell types that differentiate toward a mural cell phenotype. Indeed, the neovascularization and tissue regenerative ability of PB-ECFCs have been confirmed in different animal models of ischemia or diseases where the cells have been applied as a cell bolus, embedded alone or in combination with other cell types in tissue-engineered scaffold or as a vehicle for gene therapy.

4.2 PB-ECFCs for Cell-based Therapies

Systemic or tissue targeted application of ECFCs promoted neovascularization-dependent tissue regeneration in animal models of peripheral ischemia or vascular damage, traumatic brain injury or stroke. However, systemic application of ECFCs unraveled that the cells were often accumulated into the tissues that were not primary targets. In addition, intravenous application was accompanied by low engraftment and short survival rate of ECFCs into targeted area. On the other hand, when ECFCs were applied directly as a cell bolus into targeted tissue, the cells improved the organ function and facilitated vascularization in animal models of myocardial infarction, traumatic brain injury or retinal ischemia. Application of cell bolus of ECFCs into the affected tissue circumvents some of the drawback of systemic
With this approach, it is less likely that the cells will be entrapped in other organs and tissues and also it grants presence of a higher number of viable cells into the desired region.

### 4.3 PB-ECFCs for pre-vascularization of tissue-engineered scaffold or tissue regeneration

In vivo studies indicate that the pre-vascularization of tissue-engineered constructs by embedding of ECFCs might represent the most feasible therapeutic approach for harnessing of neovascularization and regeneration ability of ECFCs. The advances of this approach are multifold: 1) it offers greater control of the microenvironmental conditions that regulate blood vessel formation; 2) it provides a possibility to employ other cell types that either facilitate neovascularization or differentiate toward tissue-specific cell lineage; 3) it allows to modulate the regenerative ability of ECFCs by altering the structure or composition of biocompatible scaffold; and 4) it gives opportunity to add different trophic mediators into the scaffold that support neovascularization and regeneration. ECFCs embedded in biocompatible scaffolds formed functionally long-lasting and host-interconnected vascular networks in vivo. Addition of mesenchymal stem cells (MSCs), adipose tissue-derived stem cells (ADSCs), fibroblasts or immune cells further increased the overall neovascularization efficiency of ECFCs. In addition, ECFCs promoted differentiation of stem or progenitor cells toward a desired somatic phenotype. Finely tuned changes of the composition and the structure of biocompatible scaffolds as well as addition of pro-angiogenic growth factors to tissue-engineered constructs also increased the regenerative effect of ECFCs in vivo. Therefore, it can be envisaged that ECFCs combined with other cell types embedded into biocompatible scaffolds which are enriched with pro-regenerative trophic factors can be applied as a tool for tissue regeneration in near future.

### 4.4 PB-ECFCs as vehicle for gene therapy

Long persistence of physically incorporated ECFCs in newly formed vasculature suggests that these cells can be used for sustainable delivery of recombinant proteins into the systemic circulation. The ECFCs, independently of their neovascularization features, can be used as a gene vehicle for treatment of diseases such as diabetes mellitus, cancer, or hemophilia A. Genetically-engineered ECFCs embedded in collagen/fibrin gels together with BM-MSCs were able to deliver erythropoietin (EPO) in murine model of radiation-induced anemia and nephrectomy-induced kidney failure mouse model. The mice that received ECFCs-EPO have shown restoration of hematocrit levels, RBC count, and hemoglobin concentration. ECFCs transfected with a complementary DNA for human coagulation factor VIII efficiently secreted this protein.
Chapter 1

into circulation achieving therapeutic levels relevant for clinic treatment of hemophilia even after 5 months. Genetically manipulated ECFCs able to express cytotoxic or inhibitory gene products have also been used to induce anti-tumor responses in animal models of glioma, lung or pancreatic tumors. ECFCs expressing anti-angiogenic factors such as soluble VEGFR-1 (sVEGFR-1) and angiostatin–endostatin (AE) fusion proteins reduced the tumor size and growth in C3TAG mice with spontaneous mammary tumors and in mice bearing established lung or pancreatic tumors. These studies showed that ECFCs can be attractive cellular vehicles for gene delivery.

4.5 Conclusion

ECFCs exert their regenerative potential by inducing neovascularization, by triggering local tissue response that involves differentiation of precursor cells toward somatic phenotype, and if genetically manipulated, by releasing proteins that can skew the disease progression. In vivo studies outlined the basic premises for use of ECFCs for tissue repair. First, these cells can be used as a cell bolus either into the systemic circulation or directly into target tissue for induction of tissue repair which mainly is neovascularization-driven process. Second, ECFCs will form functional long-lasting vasculature within a biocompatible scaffold if they are combined with cell types that have or can acquire a mural cell phenotype. Third, ECFCs can induce differentiation of tissue-resident precursor cells into mature somatic cells. And finally, these cells also represent a suitable cell source for sustained delivery of gene products.

However, prior to implementation of ECFCs for regenerative purposes several important in vitro aspects of ECFCs biology and function should be also addressed. It is necessary to have a standardized isolation procedure that should increases the yield of ECFCs from tissue material, that does not imposes changes of the phenotype and functionality of isolated ECFCs as well as prevents possible contamination of isolated ECFCs cultures with other cell types. In addition, the generation of sufficient number of ECFCs for in vivo application through in vitro expansion should be performed with methodology that grants intact cell viability, phenotype, functionality, and immunogenicity as well as that avoids changes or alternation of karyotype. An interesting approach that might increase the beneficial outcome of in vivo implantation of ECFCs is the selection of the most angiogenic cells from cultures of ECFCs based on specific cell surfer markers such as the stem/progenitor marker CD34. Notwithstanding, accumulated data strongly indicate that ECFCs should be added as a feasible and promising tool to the current armory of different approaches that intend to regenerate and restore the function of diseased tissue.
5. SCOPE OF THE THESIS

The seminal work of Lin et al.\textsuperscript{53} and the research that followed identified the existence of circulating progenitor cells committed to endothelial lineage that give rise to ECFCs. These cells possess all the characteristics of EPCs and are indistinguishable of mature endothelial cells with respect to phenotype and in vitro and in vivo biological functions. The emergence of ECFCs was a proof that the de-novo blood vessel formation is not only restricted to fetal development but also occurs in adulthood. Therapeutic strategies that harness the regenerative potential of ECFCs were soon outlined and at present these cells are considered as the most suitable cell type for induction of therapeutic neovascularization and tissue repair. Additional research also pinpointed of the importance of ECFCs in vascular homeostasis and disease pathogenesis. Therefore, the prospects of using ECFCs are two-fold: ECFCs can either be used for regenerative neovascularization and tissue regeneration in clinics or as an in vitro model to investigate human disease mechanisms.

6. AIM OF THE STUDY

This thesis sought to examine several crucial aspects regarding in vitro isolation and manipulation of autologous peripheral blood isolated ECFCs prior clinical application. Therefore, the research presented in this study is centered around investigation of:

1) whether the isolation of PB-ECFCs and the consecutive in vitro manipulation in xenogeneic-free conditions affect the angiogenic potential of the cells

2) the effect of hypoxia and mononuclear cells on outgrowth and proliferation of PB-ECFCs

3) the role of stem/progenitor cell surface marker on angiogenic and barrier function of ECFCs

4) the phenotypic and functional differences between ECFCs isolated from healthy and patients with pulmonary arterial hypertension.
7. CHAPTERS OF THIS THESIS

Figure 2. Bench-To-Bedside Cycle of ECFCs as covered in this thesis

Clinical application of ECFCs requires isolation and amplification of ECFCs in xenogeneic-free conditions. Functional and phenotypic characterization, and tumorigenicity testing should guarantee that the cells are viable, safe and with unaltered vasculogenic properties. In this cycle, new concepts for application of ECFCs should be tested and new mechanisms or therapeutic targets must be identified.

CVD: cardiovascular diseases

Chapter 2 provides an overview of the published literature regarding the in vivo regenerative potential of ECFCs. In this chapter we outlined the main areas where and how these cells might be implemented as a routine treatment in clinic in future.

Study of endothelial cell biology using in vitro-based models usually requires large quantities of cells. Chapter 3 presents a reliable and efficient isolation and in vitro expansion procedure that increases the yield of ECFCs by using a method which excludes the use of animal-derived products. This approach grants generation of sufficient number of ECFCs for in vivo application with preserved cell viability, phenotype, and functionality in a relatively short period. Most importantly, this method reduces the risk of contamination of ECFCs with animal-derived noxious material as well as it more closely resembles human physiology than the standardized in vitro methodologies based on use of xenogeneic products.
Hypoxia and cell-cell interaction governs physiological or post-ischemic neovascularization. The effect of hypoxia, trophic factors and PB-MNCs on the outgrowth and pro-angiogenic features of ECFCs were investigated in Chapter 4. In Chapter 5 we took a step further to increase the beneficial effect of eventual therapy based on ECFCs. We selected the most angiogenic cells from cultures of ECFCs based on the surface expression of stem/progenitor marker CD34 and investigated their in vitro phenotypic and functional characteristics relevant to sprouting angiogenesis. In addition, in this chapter the role of stem/progenitor cell surface marker CD34 during sprouting and maintenance of barrier function in PB-ECFCs was elucidated. Being able to isolate ECFCs from PAH patients in our laboratory, in Chapter 6 ECFCs have been used as a cellular platform to investigate the biological features of these cells in comparison with the cells obtained from healthy donors. The future implications of the research presented in this study are summarized and discussed in Chapter 7.
REFERENCES

General introduction


General introduction


Chapter 1


CHAPTER 2

REVIEW: THERAPEUTIC POTENTIAL OF HUMAN- DERIVED ENDOTHELIAL COLONY-FORMING CELLS IN ANIMAL MODELS

Dimitar Tasev
Pieter Koolwijk
Victor W.M. van Hinsbergh

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ABSTRACT

Purpose of review
Tissue regeneration requires proper vascularization. In vivo studies identified that the endothelial-colony forming cells (ECFCs), a subtype of endothelial progenitor cells that can be isolated from umbilical cord or peripheral blood, represent a promising cell source for therapeutical neovascularization. ECFCs not only are able to initiate and facilitate neovascularization in diseased tissue but can, by acting in paracrine manner, contribute to the creation of favorable conditions for efficient and appropriate differentiation of tissue-resident stem or progenitor cells. This review outlines the progress in the field of in vivo regenerative and tissue engineering studies and surveys why, when and how ECFCs can be used for tissue regeneration.

Recent findings
Reviewed literature that regards human derived ECFCs in xenogeneic animal models implicates that ECFCs should be considered as an endothelial cell source of preference for induction of neovascularization. Their neovascularization and regenerative potential is augmented in combination with other types of stem or progenitor cells. Biocompatible scaffolds pre-vascularized with ECFCs interconnect faster and better with the host vasculature. The physical incorporation of ECFCs in newly-formed blood vessels grants prolonged release of trophic factors of interest, which also makes ECFCs an interesting cell source candidate for gene therapy and delivery of bioactive compounds in targeted area.

Summary
ECFCs possess all biological features to be considered as a cell source of preference for tissue engineering and repair of blood supply. Investigation of regenerative potential of ECFCs in autologous settings in large animal models prior to clinical application is the next step to clearly outline the most efficient strategy for using ECFCs as treatment.
1. INTRODUCTION

Cell-based therapies and tissue engineering are therapeutic modalities that can be used to replace or restore the function of damaged or diseased tissues. Accumulated data from in vivo animal studies and clinical trials pointed out that the efficacy of cell-based therapeutic approaches was often undermined either by insignificant accumulation and retention, or due to poor cell survival of the applied cells in the targeted tissue. In addition, an inadequate vascularization of tissue-engineered construct was also reported as a reason for the reduction of therapeutic efficacy of cell-based treatments.

The efficiency of cell-based tissue regeneration and complete restoration of functionality relies on the cell type that either 1) has to possess the ability to induce proper establishment of a new vascular bed; 2) exhibits a behavior that evokes a regeneration-related switch of the phenotype of tissue-resident cells or differentiation of resident stem/progenitor cells; or 3) possesses a feature that can be modulated by gene manipulation in such way that it will further enhance the regenerative potential of the cell or can be used as gene delivery vehicle to modulate the behavior of other cells. Mesenchymal stem cells are fulfilling some of the above mentioned criteria and at present are the most often used cell type for tissue regeneration and its application in disease treatment in pre-clinical animal models and clinical trials. However the lack of an intrinsic propensity for formation of new blood vessels seriously hampers their applicability especially in tissue engineering. Emergence of endothelial-colony forming cells (ECFCs), a subset of circulating endothelial progenitor cells with a high proliferative potential, has opened new modalities for cell-based therapies and tissue engineering.

This survey discusses the reported findings from in vivo studies that aimed to explore the potential use of ECFCs to induce neovascularization, to facilitate new tissue formation during tissue regeneration or to target diseased tissues specifically.

2. WHAT ARE ENDOTHELIAL COLONY FORMING CELLS?

ECFCs originate from progenitor cells present in the peripheral or umbilical cord blood and once isolated in culture they possess all phenotypical and functional characteristics specific for endothelial lineage. These cells also referred as late outgrowth endothelial progenitor cells (late EPCs) or blood-outgrowth endothelial cells (BOECs) differ from the early outgrowthCD14+/CD45+ EPCs (early EPCs) which originate from myeloid-monocytic lineage and participate in the processes of vascular regeneration in a paracrine manner. In contrast to early EPCs, ECFCs possess robust proliferative and
clonal ability granting generation of sufficient number of cells for clinical application in a relatively short period. Most importantly, these cells incorporate into the endothelial lining of newly formed blood vessels and exhibit pronounced vascularization ability in vivo. Especially their neovascularization potency is augmented when ECFCs are used in combination with stem/progenitors cells, such as mesenchymal stem cells or adipose-tissue derived stem cells. In the past decade many in vivo studies outlined ECFCs as promising cell type for new cell-based improvement of disease outcome. They regard different animal models of ischemic diseases as well as animal studies for validation of novel tissue engineered concepts for tissue regeneration and reconstruction. However, little progress was offered with respect to the translation of those proof-of-concept findings into clinical settings.

Notwithstanding, ECFCs have been successfully isolated from healthy donors and patients paving the path for use of these cells as a cellular platform to study the pathophysiology of certain diseases as well as for autologous treatment of various pathologies.

In this review we use the term ECFCs for the entire spectrum of names by which these cells are known. It specifies peripheral or cord blood derived or tissue-derived cells belonging to the endothelial lineage, which possess robust proliferative and clonal ability, that exhibit in vitro and in vivo angiogenic capacity and express endothelial cell markers such as CD31, CD146, CD309, CD144, vWF, eNOS and are negative for hematopoietic (CD45, CD14) or mesenchymal stem cell markers (CD70, SMA). In addition, the clinical applicability of reported findings was a criterion for this survey, thus excluding in vivo studies that were performed with materials that pose safety concerns and have little application perspective for patients.

3. THERAPEUTIC USE OF ECFCS IN NEOVASCULARIZATION AND TISSUE REGENERATION

Accumulated evidence indicates that ECFCs can exert their therapeutical potential 1) by physically incorporation into neo-vessels of the target tissue, thus improving oxygenation and delivery of nutrients into the affected area; 2) in a paracrine manner by creating a niche for differentiation of stem/progenitor cells towards needed somatic cell type; or 3) if genetically manipulated, to skew the disease progression toward a more positive outcome. A large pool of in vivo studies reported that the intrinsic neovascularization ability of ECFCs underlies the restoration of blood perfusion in ischemic tissue in the mouse hindlimb model, sustained the survival of tissue-resident
somatic cells and improved hemodynamic parameters after myocardial infarction\textsuperscript{20,21}, or induced symptom relief in case of stroke\textsuperscript{12}. Embedded into biocompatible materials, ECFCs have been used to create pre-formed vascular networks within tissue-engineered scaffolds that efficiently interconnected with host vasculature and facilitated tissue regeneration after in vivo implantation\textsuperscript{23}.

Recent advances in the field of regenerative medicine introduced the concept to use ECFCs, often independently of their neovascularization features, for treatment of diseases such as diabetes mellitus\textsuperscript{24}, cancer\textsuperscript{25}, or hemophilia A\textsuperscript{26}. These latter aspects of ECFC applications are discussed below (sections 5.2 and 6). The next sections focus on the therapeutic approaches that hold promise to use ECFCs for tissue repair and regeneration. Two major applications forms have been used in vivo, namely as cell bolus into the systemic circulation or targeted tissue, or embedded in biocompatible scaffolds. Since the identification of ECFCs, most research that aimed to improve regeneration using the neovascularization ability of ECFCs was performed by injecting a cell bolus alone or in combination with other cell types either into systemic circulation or directly into targeted tissue affected by ischemia. In vitro or in situ pre-vascularization of engineered-biocompatible scaffold by ECFCs represent an alternative approach using the neovascularization ability of ECFCs for tissue-engineering assisted regeneration of tissues and organs. Both approaches can be used to deliver modified ECFCs as a vehicle for gene therapy (Figure 1).

\textbf{Figure 1.} Application of ECFCs for tissue regeneration and repair
Data obtained from in vivo studies delineated three main approaches of use of ECFCs for regenerative purposes. Tissue regeneration and repair can be accomplished by injecting ECFCs either into systemic circulation (1.a) or directly into the tissue affected by ischemia (1.b). These cells have also shown beneficial therapeutic effect when used for *in vitro* or *in situ* pre-vascularization of tissue-engineered constructs (2.a,b) or for *in vitro* endothelialization of vascular grafts prior implantation (2.c). In vivo studies performed with changing the properties of ECFCs by gene manipulation have outlined the principles of use of these cells for gene therapy as well (3).

### 4. BOLUS APPLICATION OF ECFCS

Systemic application of ECFCs induced neovascularization-dependent tissue regeneration in animal models of peripheral ischemia, vessel damage, traumatic brain injury, stroke or lung-related diseases. However, the encouraging results from those studies were blunted by the observation that intravenous bolus application of ECFCs was accompanied by entrapping the ECFCs in tissues that were not primary targets and low engraftment and short survival rate into targeted area. Furthermore, those studies did not provide further insight in the mechanism of how ECFCs participate in neovascularization and tissue regeneration. Thus, intravenous bolus injection of ECFCs has drawbacks that impair the therapeutic efficacy of these cells suggesting that this application route is not the most efficient approach for administration of ECFCs.

Targeted application of a cell bolus into the affected tissue circumvents the low engraftment of ECFCs and imposes greater control over the systemic distribution of the cells and the cell number needed to establish the desired therapeutic effect. Studies performed with rodent animal models of myocardial infarction, traumatic brain injury or retinal ischemia reported promising improvement of organ function and increased vascularization after application of ECFCs by intramyocardial, intracerebroventricular or intravitreal injection, respectively.

#### 4.1 Supporting the angiogenic efficacy of bolus injected ECFCs

The initial animal studies where ECFCs where applied as bolus cell injection for treatment of pathological conditions pointed to the necessity to develop other strategies that will increase the therapeutic potency of ECFCs by using stem or progenitor cells in addition to ECFCs or to modulate the ECFCs function by pretreatment with soluble bioactive components, environmental clues or gene transfer.
Co-implantation of ECFCs with cell types that can support neovascularization by acquiring mural cell phenotype and stabilize the newly-formed vasculature resulted in even greater regeneration benefits. A combination of ECFCs with adipose tissue-derived stem cells (ADSCs)\textsuperscript{36}, MSCs\textsuperscript{7}, or SMCs\textsuperscript{37,38} significantly improved neovascularization score by forming functionally long-lasting new-blood vessels that are interconnected with host vasculature, alleviated symptoms and improved overall organ or tissue functionality in different models of ischemia. Interestingly, similar results were obtained when ECFCs were co-implanted with host myeloid cells\textsuperscript{39}. While in cell-therapy studies ECFCs were indispensable for neovascularization, the accessory cells are not, as already reported for MSCs\textsuperscript{5} and myeloid cells\textsuperscript{39}.

4.2 Support of ECFC-mediated vascularization by growth factors and hypoxia

Augmentation of the therapeutic ability of ECFCs can also be achieved by exposing the cells to pro-survival or pro-angiogenic trophic factors or exposing the cells to environmental conditions that usually are present in ischemic areas. Pretreatment of ECFCs with the chemokine SDF-1\textsuperscript{40}, fucoidan\textsuperscript{61,19}, the formyl peptide receptor-2 agonist WKYMVm\textsuperscript{42}, and erythropoietin\textsuperscript{43} improved hindlimb blood flow and capillary density, and showed less necrosis and better muscle preservation than the animals treated with control ECFCs in the mouse hindlimb ischemia model. Intramyocardial application of ECFCs pre-treated with a very low concentration of genistein(10\textsuperscript{-10} M) induced cellular proliferation and secretion of angiogenic cytokines at the ischemic sites of an murine acute myocardial infarction model\textsuperscript{20}. It was accompanied by enhanced the capillary formation, decreased myocardial fibrosis and improved overall cardiac function over period of 28 days\textsuperscript{20}. Stimulation of ECFCs with adiponectin significantly improved hindlimb perfusion in streptozotocin-induced hyperglycemic nu/nu mice\textsuperscript{44}. Most importantly, pre-treatment of ECFCs isolated from DM patients with adiponectin completely reversed their reduced neovascularization ability in vivo. Although the duration of this improvement needs further evaluation, this pretreatment offers a novel and promising approach to counteract the functional impairment of ECFCs in diabetic patients.

Hypoxia, acidic environment as well as abundance of fibrinous exudate are usual components of an inflammatory milieu. Preconditioning of ECFCs with these environmental clues prior their use as a therapeutic means to induce regeneration is an interesting approach for increasing of their angiogenic efficacy. Exposure of ECFCs to 1\%\textsuperscript{45} or 2\% O\textsubscript{2}\textsuperscript{46} up to 24h enhanced the proliferation and survival of transplanted ECFCs, and triggered a pro-angiogenic cytokine secretion at ischemic sites through HIF-1\alpha/TWIST\textsuperscript{45} and the JAK2/STAT3 axis\textsuperscript{46}. These cellular effects improved the blood flow
ratio and the capillary density in ischemic mouse hindlimb. Similar results of improved neovascularization into ischemic tissue driven by activation of activation of AKT and ERK1/2 have been reported after pre-exposure of ECFCs to pH6.6 acidic environment or shortly cultured in the presence of human platelet lysate.

Increased survival and pro-angiogenic ability by activation of either AKT or the JAK2/STAT3 axis has also been used as an approach to genetically modulate these pathways in ECFCs in order to increase their therapeutic efficacy. Restoring Akt1 activity in ECFCs from South Asian men rescued their inherited reduced vascular reparative potential in mouse hindlimb ischemia, while ex vivo transduced ECFCs with Aktandhemoxygenase-1 have improved cardiac performance and reduced negative remodeling after myocardial infarction in nude mice. Furthermore, selective silencing of Lnk adaptor protein activated the JAK2/STAT3 pathway in ECFCs which accelerated angiogenesis and promoted the tissue repair in ischemic hindlimb. Therefore, strategies that increase the therapeutic efficacy of ECFCs offer a way to circumvent drawbacks of bolus application of ECFCs in clinical settings and might provide better therapeutic outcome.

Notwithstanding, bolus application of ECFCs remains a clinically relevant application route especially for treatment of pathological conditions when a targeted intra-tissue use is the only way to introduce cell-based therapy, such as in myocardial infarction, or by systemic application in intimal hyperplasia or lung repair.

5. USE OF ECFCS IN PRE-VASCULARIZED TISSUE-ENGINEERED CONSTRUCTS

ECFCs can also be used as a means for generation of pre-vascularized tissue-engineered constructs. This approach offers greater control of the microenvironmental components that govern blood vessel assembly. It also provides the possibility to introduce other cell types that either facilitate angiogenesis or differentiate toward tissue-specific cell lineage. Furthermore, it allows to modulate the biological activity of ECFCs by changes of the structure of biocompatible scaffold or by addition of trophic mediators that support neovascularization and regeneration. Earlier studies with ECFCs embedded in scaffold made of Matrigel showed that the cells formed functionally long-lasting and host-interconnected vascular networks in vivo especially in combination with MSCs or ADSCs. These findings provided a platform for researchers in the tissue-engineering field to start evaluating the potential of ECFCs for tissue regeneration and reconstruction in conditions that can be applied in clinical practice for treatment of different pathological conditions.
5.1 Effect of scaffold materials on neovascularization properties of ECFCs

A tissue-engineered graft essentially consists of tissue cells supported by a (bio)matrix. Both natural biomatrices and biocompatible synthetic matrices can be used, some of which even display self-organizing properties towards the embedded or surrounding tissue cells. Both type of materials are relatively safe for clinical use, mimic structural and functional properties of the natural extracellular matrix and can be modulated by physicochemical processes to improve the biomechanical properties of the scaffold to maintain cell function, the development of the vascular network and tissue regeneration52. The in vivo research studies that evaluated the potential of ECFCs for tissue engineering are discussed in the next sections according to the use of natural or synthetic biocompatible materials.

5.1.1. Natural materials, either chemically modified or combined with peptides

When embedded in hyaluronan acid (HA)53,54,55, gelatin56, alginate57, collagen or fibrin hydrogels58,59,60,61, ECFCs exhibited potent neovascularization effects. Nevertheless, not all natural biopolymers are suitable for tissue engineering. As reported previously, gelatin lacks mechanical stability62, alginate by itself has limited intrinsic propensity to facilitate angiogenesis because of uncontrolled degradation63, while ECFCs embedded in HA hydrogels exhibited similar neovascularization effects as the empty control scaffolds55. However, addition of integrin-binding RGD-peptides and VEGF121 or HGF57,64 to alginate gels created a permissive pro-angiogenic environment which resulted in increased neovascularization driven by ECFCs after intramuscular application of the scaffold in the mouse ischemic hindlimb.

Similarly, addition of RGD and MMP-sensitive peptides to HA scaffolds laden with human ECFCs resulted in vascular networks formation in tumor bearing mice composed of 60% of the blood vessels containing both human ECFCs and host endothelial cells, while the remaining vessels contained only human ECFCs55. The newly-formed vascular bed in ECFC-laden scaffolds anastomosed with the host’s circulation and supported blood flow in the hydrogel after 14 days transplantation, while human microvasculature could not be detected in implants of HA hydrogels without ECFCs.

Chemical modification of biomaterials can also influence the behavior of ECFCs in the matrix. Addition of vinyl methacrylate groups to gelatin gels (GelMA) induced neovascularization in implants laden with ECFCs and MSCs in immunodeficient mice, but also revealed that the extent of vascular network formation was different between the GelMA constructs with final concentrations of 1 and 10% methacrylate65. In the 1%GelMA construct, numerous blood vessels containing erythrocytes were uniformly distributed throughout the implants while there were fewer perfused blood vessels...
in the 10%GelMA constructs with microvessels mainly located at the periphery of the constructs. Gelatin-phenolic hydroxyl gels loaded with ECFCs and ADSCs induced a different extent of in situ vascularization, which was dependent on the concentration of H₂O₂ that was used for enzymatic crosslinking. The number of lumens inside the construct decreased with increasing the H₂O₂ concentration that was in direct correlation with the reduced proteolytic degradability of the scaffold in nude mice. Taken together, by changing the biochemical and physical properties of the local microenvironment within the scaffolds composed of natural polymers, it is possible to influence the vasculogenic ability of ECFC and to guide neovascularization in vivo as was also reported for collagen scaffolds.

5.1.2. Synthetic materials, combination of them or modified by physicochemical procedures

Synthetic polymers such as poly-L-lactide (PLLA), poly(D,L-lactic-co-glycolic acid) (PLGA), biphasic calcium phosphate (BCP), β-tricalciumphosphate (βTCP) or polycaprolactone (PCL) possess several advantages over natural ones, since they are inexpensive, FDA approved, and have mechanical properties that can be modulated to the desired degree of inducing optimal neovascularization in vivo. Addition of natural biopolymers like fibrin, fibronectin or RGD sequences circumvents the lack of cell adhesion peptides in the molecular structure of synthetic materials while crosslinking with growth factors such as VEGF, HGF, Ang-1 or BMP-2 enables them to acquire permissive pro-angiogenic properties.

ECFCs embedded within scaffolds composed of synthetic polymers were used successfully to improve neovascularization and tissue regeneration. ECFCs embedded in heparin-immobilized PCL scaffolds loaded with VEGF induced significantly high density of blood vessel formation after 7 days of subcutaneous implantation in immunodeficient mice compared to empty scaffolds. Subcutaneously implanted polycaprolactone-tricalcium phosphate (PCL-TCP) scaffolds loaded with ECFCs and human fetal MSC induced robust neovascularogenesis and ectopic bone formation in vivo after 12 weeks. Besides neovascularization, ECFCs demonstrated an osteo-inductive effect by increasing the bone-formation ability of human fetal MSC through BMP/TGF-β signaling. Robust neovascularization accompanied by increased bone induction was also reported when ECFCs were embedded with bone marrow-derived MSCs in hydroxyapatite (HA)-polylactide-co-glycolide (PLG) scaffolds and applied for treatment of orthopic calvarian defect in albino nude rats. Subcutaneous application of ECFCs combined with primary osteoblasts into starch polycaprolactone fiber meshes (SPCL) scaffold also induced bone formation. An interdependent relationship between
increased neovascularization and efficient osteogenesis was also reported when ECFCs alone were loaded into β-tricalciumphosphate (βTCP) coated with fibronectin in nude rat calvaria model. Similar result were reported when ECFCs were embedded into biphasic calcium phosphate (BCP) constructs enriched with BMP-2 induced bone formation in immunocompetent mice. These studies indicate that ECFCs are suitable for bone repair.

In mouse model of full-thickness excision wound transplantation of ECFCs-seeded RGD-g-PLLA scaffold resulted in better neovascularization compared to empty control scaffolds. In addition, the ECFCs-seeded RGD-g-PLLA scaffolds promoted superior dermal wound healing when compared with the intradermal ECFCs injection indicating that the scaffolds-application route of ECFCs significantly improved cells’ localization, survival and retention in the injured tissue (Table 1).

Early studies that used ECFCs for regenerative purposes emphasized the need to investigate and adopt different strategies in order to increase the therapeutic potential of ECFCs as well as to expand the list of possible indications for treatment of pathological conditions.

1- Pretreatment of ECFCs with bioactive compounds or factors increased the cell survival, tissue engrafting as well as angiogenic capacity of ECFCs.

2- Gene manipulated ECFCs exhibited better therapeutic effect than source cells.

3- ECFCs combined with other cell types have shown facilitated angiogenesis as well as tissue regeneration and repair.

4- Embedding of ECFCs in natural and synthetic biocompatible scaffolds enables efficient neovascularization and induction of tissue regeneration and repair.

5.2 Other approaches that utilize ECFCs for tissue regeneration

In addition to their vasculogenic potential, ECFCs can also have a direct regenerative effect by secreting soluble regulators that govern tissue repair. ECFCs combined with tissue or organ derivatives such as pancreatic islands, skin sheets or decalcified bone were also used for tissue-engineered regeneration.
Table 1. Strategies that improve therapeutic potential of ECFCs

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<tr>
<th>Strategies that improve therapeutic efficiency of ECFCs</th>
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<tr>
<td><strong>1) Pre-treatment of ECFCs with bioactive compounds or factors</strong></td>
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<tr>
<td>SDF-1</td>
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<td><strong>4) Embedding in biocompatible scaffolds</strong></td>
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<tr>
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<td>poly-D, L-lactide (PLLA)</td>
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<td>biphasic calcium phosphate (BCP)</td>
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<tr>
<td>polycaprolactone (PCL)</td>
<td>16, 72, 76</td>
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Intraportal transplantation of ECFCs-covered porcine pancreatic islets exhibited better graft function and survival, reduced inflammatory response and better regulation of increased glucose levels in diabetic nude mice. Reduced graft loss and dysfunction was reported when ECFCs were co-transplanted with a rat marginal islet graft under the kidney capsule of hyperglycemic NOD/SCID mice. In vivo cell tracing demonstrated that ECFCs remained at the site of transplantation inducing beta cell proliferation, which was accompanied with improvement of glycemia and increased plasma C-peptide compared to control animals. ASCs and ECFCs combined with either freshly isolated mature human adipocytes or pig pancreatic islets in collagen/fibronectin gels established a functional vascular network after 2 weeks of subcutaneously application at the flank region of NOD/SCID mice. The implants loaded with adipocytes exhibited typical morphology of vascularized adipose tissue, while the implants carrying islets contained vascular networks with clusters of cells staining positive for insulin. These studies support the use of autologous ECFCs in translational studies that aim to improve current islet-transplantation protocols for the treatment of type 1 diabetes.

In another study ECFCs were embedded together with MSC-derived smooth muscle cells in collagen/fibronectin spheroids and loaded into porous decalcified bovine cancellous bone (PBCB, Tutobone) for treatment of calvarian critical size defects in SCID mouse model. Spheroids composed of ECFCs alone or in combination with smooth muscle cells were unable to form blood vessels but once loaded into PBCB triggered ingrowth of host blood vessels into the implants which correlated positively with bone formation within the implants. Furthermore, incorporation of ECFC into tissue-engineered human skin substitutes resulted in formation of vascular conduits enabling perfusion and survival of human bioengineered tissue, while combination of dermal fibroblast sheets with ECFCs induced robust vasculogenesis, adequate epithelial coverage and normal matrix organization which prevented excessive wound contraction.

Endothelialization of synthetic small-diameter vascular grafts using ECFCs opens another new venue for application. The pitfalls of previous studies performed with other endothelial cell types such as umbilical vein endothelial cells were mainly related to graft failure due to intimal hyperplasia or thrombus formation followed by vessel occlusion. ECFCs attenuate intimal hyperplasia contributed by mesenchymal stem cells after vascular injury in mouse femoral wire-injury model, but a modulating effect has to still to be evaluated in appropriate vascular grafts. In animal studies this type of application has been combined with gene transfer to improve anticoagulant properties. To that end, control ECFCs or ECFCs transfected with an adenoviral vector containing the gene for human thrombomodulin were sodded into the lumens of small diameter ePTFE vascular grafts which were implanted into femoral arteries of...
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athymic rats. After 28 days the patency rate of control ECFCs and ECFCs+AdTM was 75-88%, while intimal hyperplasia was observed near both the proximal and distal anastomoses. However, these regions of anastomoses were negative for human vWF staining indicating that the host cells but not the ECFCs contribute to this negative side effect of graft implantation.

Published literature indicates that ECFCs affect the differentiation of stem/progenitor cells toward a somatic cell type in a paracrine manner by releasing soluble trophic factors such as BMPs, TGF-β, PDGF-BB, VEGF, or angiopoietins. Recent findings suggest that exosomes represent a paracrine mechanism through which ECFCs also exert a therapeutic effect. These microvesicles with a diameter ranging from 30 up to 100 nm, are involved in cell-cell communication as vehicles that transfer information (mRNA, miRNA, DNA) as well as proteins between the cells. Intravenous application of exosomes derived from ECFCs attenuated renal injury in mouse model of experimental acute kidney injury (AKI) by reducing plasma creatinine, tubular necrosis, macrophage infiltration, oxidative stress and apoptosis. Therefore, the use of exosomes released from ECFCs under stringent in vitro conditions represents a promising therapeutic modality for tissue regeneration that warrants further investigation in different animal models of diseases.

These studies emphasized that ECFCs possess desirable tissue-regenerative attributes that are not strictly dependent on cells' neovascularization ability but also on the trophic capacity to create a microenvironment that induces a pro-regenerative phenotypic switch in tissue-resident somatic cells.

6. GENETICALLY MODIFIED ECFCs AS A VEHICLE FOR TREATMENT

While ECFCs possess appealing therapeutic potential by virtue of their ability to induce neovascularization and release of trophic factors that induce regeneration into affected tissue, these cells also represent an attractive cell source for vehicles for gene therapy.

ECFC-based gene-delivery strategy was used as a feasible technology for administration of recombinant proteins in case of anemia or hemophilia A. It was shown that ECFCs transfected with a nonviral plasmid carrying complementary DNA for modified human coagulation factor VIII pre-dominantly accumulated in bone marrow and spleen of NOD/SCID mice from where efficiently secreted FVIII into circulation achieving therapeutic levels relevant for clinic treatment of hemophilia even after 5 months.
Similarly, genetically-engineered ECFCs able to express controlled delivery of erythropoietin (EPO) were embedded in collagen/fibrin gels together with BM-MSCs and subcutaneously implanted in nephrectomy-induced kidney failure mouse model or in a murine model of radiation-induced anemia. ECFCs-EPO combined with MSCs formed extensive vascular networks inter-connected with the host vasculature and once engrafted, the cells expressed and secreted functional EPO in vivo. After 5 weeks the mice with implanted ECFCs-EPO have shown enhanced hematocrit, RBC count, and hemoglobin concentration, reactive splenomegaly, and downregulation of endogenous EPO expression in the kidneys.

Systemic application of ECFCs encoding a cytotoxic or inhibitory gene products has been used to induce anti-tumor responses in animal models of glioma, lung or pancreatic tumors. ECFCs expressing anti-angiogenic factors such as soluble VEGFR-1 (sVEGFR-1) and angiosstatin–endostatin (AE) fusion proteins induced favorable disease outcome by reducing the tumor size and growth in C3TAG mice with spontaneous mammary tumors and in mice bearing established lung or pancreatic tumors while in an orthopic model of glioma authors reported doubling of the median survival of mice treated with sVEGFR-1. In Lewis lung carcinoma, tumor growth was suppressed only after the application of combination of cells producing sVEGFR-1 or AE proteins. Replicating oncolytic viruses such as measles viruses of the Edmonston B strain (MV-Edm) have shown promise for treatment of cancer. Interestingly, ECFCs express CD46 and can be easily infected by MV-Edm while being resistant to MV-Edm-mediated cell death and protected virus replication from host immune response. Infected ECFCs releasing attenuated measles viruses of the Edmonston B strain (MV-Edm) efficiently contaminated the U87 glioma cells leading to focal infection and cytopathic effects that reduced tumor size and prolonged survival of mice. The above given studies showing that ECFCs can be attractive cellular vehicles for gene delivery.

**7. AUTOLOGOUS IMPLEMENTATION OF ECFCS IN NON-MOUSE MODELS**

Tissue regeneration is a complex yet finely tuned spatio-temporal process of continuous interaction between immune cells, vascular and tissue-resident stem or progenitor cells that actively occurs during lifetime. It is markedly accelerated after tissue injury. Exclusion of one component of this system in experimental animal models could lead to misleading interpretation of collected data compromising the clinical translation of reported proof-of-concept finding. Most of our knowledge about ECFCs is gathered from studies performed in immunocompromized murine animal models. However,
interaction of implanted human ECFCs and immune cells showed that this interaction is crucial for regeneration. Lack of immune response in immunocompromized animals interferes with hampers evaluating the immunogenicity of ECFCs in vivo as well as the interplay between ECFCs and immune cells. Only one study reported low immunogenicity of human PB-derived ECFCs in rat skin transplantation model with immunocompetent WKY rats which warrants further investigation of ECFCs behavior in autologous or other intact animal models.

Other factors also influence the evaluation of therapeutical efficacy of ECFCs in mice models. Concomitant pathologies often present in mice animal models, such as diabetes mellitus in NOD mice, hinders the clear extrapolation of collected knowledge for development of new treatments based on use of ECFCs. Many examples showed that ECFCs can be used to treat peripheral ischemia in a hindlimb ischemic mouse model. However, the lack of an explanation whether the observed beneficial effect of ECFCs is due to direct involvement in angiogenesis or paracrine stimulation of arteriogenesis hampers the objective evaluation whether the obtained data are clinically relevant or not. In addition, despite its large flexibility, the mouse is less suitable for isolation and evaluation of the use of autologous ECFCs for disease treatment as its blood volume is relatively small. Therefore, these simplified murine animal models are unlikely to accurately represent the in vivo performance of ECFCs in humans.

Successful isolation of ECFCs from larger than murine animals offers an opportunity to test ECFCs in the autologous environment of intact organism. This approach offers a more reliable and accurate insight of how ECFCs behave during neovascularization and tissue regeneration. It may be preferable to start with a medium sized animal, such as rat or rabbit, to test the feasibility of the treatment and to work out the eventual technical problems, and then to investigate the same concept into large animal models.

ECFCs have been successfully isolated and characterized from peripheral blood or organs of rats, rabbits, pig, dog, sheep, goat as well as non-human primate opening new ventures to investigate the possibility of using ECFCs for treatment of different pathological conditions in autologous settings. Isolated cells from different animals were compliant with the present criteria for characterization of ECFCs such as belonging to endothelial cell lineage, absence of markers characteristic for HSC or MSC, robust proliferation and clonal ability as well as in vitro and in vivo neovascularization capability. Application of autologous ECFCs promoted bone fracture repair and bone regeneration, attenuated the effect of acute lung injury, and rescued infracted myocardium in different disease models in rat. ECFCs might represent a preferential cell source for endothelialization of vascular grafts and for treatment
of cardiovascular\textsuperscript{90,103,104,102} or lung\textsuperscript{105} related pathologies as reported in various disease models in rabbit. Subsequent in vivo testing in large and relevant animal models offer a better opportunity to evaluate the performance of ECFCs in clinically-relevant conditions, i.e. closely resembling human pathophysiology. Autologous transplantation of ECFCs has shown promising results in treatment of different pathological conditions in porcine\textsuperscript{106,107,91,108,110,111}, canine\textsuperscript{112,92,113,114,115,116}, ovine\textsuperscript{93,117,118}, goat\textsuperscript{94} animal models. Published reports of beneficial role of autologous-implanted ECFCs in large animal models offers also an opportunity for developing in vitro assays and markers that might that have a scientifically sound correlation with clinical relevant parameters while in same time can monitor and predict the performance of ECFCs in vivo.

8. CONCLUSION AND FURTHER PERSPECTIVES

The research performed after the identification of ECFCs\textsuperscript{12} suggests that harnessing the robust angiogenic potential of ECFCs holds promise to use these cells for regenerative purposes. ECFCs are expressing cell surface markers characteristic for endothelial lineage and are devoid of markers such as CD45 or CD14 which suggest that ECFCs are not derived from hematopoietic stem cell pool. At present, little is known about the origin of these cells as well as the pathways that govern endothelial differentiation of stem or progenitor cells that give rise of ECFCs.

These cells can be isolated from cord or peripheral blood as well as adipose tissue using clonal assays. Previous research reported that ECFCs isolated form cord blood possess different angiogenic and endothelial cell characteristics compared to the cells isolated form peripheral blood. CB-ECFCs proliferate more and are able to form new vasculature that last longer in vivo than PB-ECFCs\textsuperscript{119}. Thus, selection of CB-ECFCs seemed to be a more appropriate cell type for regenerative treatments than PB-ECFCs. However, the latter but not the former are more resistant to karyotype abnormalities that can occur during in vitro manipulation. This indicates that ECFCs isolated from peripheral blood are safer and therefore overall a better cell type for regeneration than CB-ECFCs. An alternative was suggested by Prasain et al\textsuperscript{120} who reported successful generation of ECFCs from iPSCs using an induction protocol based on the presence of trophic factors that govern endothelial differentiation. These cells were able to form human vessels in mice and to repair the ischemic mouse retina and limb, and most importantly they lack teratoma formation potential. This approach offers a platform to generate a clinically relevant number of ECFCs in controlled manner as well as cells from patients with pathologies which are reported to impair angiogenic ability of ECFCs\textsuperscript{121,122,9,123} but demands further investigation especially in large animal models.
Animal studies with ECFCs undoubtedly pointed to improved neovascularization and regeneration in different disease models, but little is known about the ability of these cells to home to different organs, how the microenvironment clues affect their phenotype and functionality, how ECFCs contribute to tissue-specific cell populations, and to what extent they contribute to tissue repair. After systemic application of ECFCs in rodent models, it was reported that most of the cells were mainly entrapped in the lungs, while only modest accumulation occurred in the kidneys, liver, and spleen. Intra-peritoneal or intra-hepatic administration of ECFCs in a fetal sheep model showed that the cells were either located in the perivascular regions of the liver still expressing endothelial makers or migrated to the intestinal crypt region contributing there significantly to the myofibroblast population.

Labeling of ECFCs with magnetic particles and guiding the circulating cells with magnetic field into targeted tissue might represent an example of the strategy how ECFCs can better be accumulated and engrafted into the desired region. This will allow to follow up the phenotype and the functionality of implanted cells over a longer period of time which will help to better understand the processes that regulate the fate of ECFCs during regeneration in specific environment and disease.

Conversely, to better neovascularization and regeneration, the experience with application of ECFCs in immunocompromized mice bearing small human lung tumors or fibrosarcoma calls for caution since the implanted cells facilitated the disease progression possibly through a paracrine mechanism.

Extensive research confirmed that biocompatible scaffolds laden with ECFCs contribute to tissue regeneration by neovascularization as well as by inducing differentiation of implanted or tissue-resident stem or progenitor cells toward desired somatic phenotype. Promising results were achieved in treatment of bone-related or skin pathologies which warrants translation of this approach into clinical setting in near future. However, the plethora of biocompatible materials used for TE studies as well as not yet well understood how additional trophic factors regulate the fate of ECFCs in scaffolds blurs the vision how and when we can use ECFCs for regeneration. For example, treatment of skin defects caused by burns by TE skin equivalents that incorporate ECFCs will demand different approach than the treatment of the skin ulcers caused by diabetic vasculopathy. Therefore, before the use of ECFCs for TE purposes it is important to clearly underline the scaffold design parameters (cell retention, control cell fate and differentiation, stimulation of tissue formation and remodeling, biodegradability, hemocompatibility, bioactivity in case of natural biomaterials) which should be tailored by the requirements of specific tissue-regenerative or pathological milieu.
Accumulated evidence strongly suggests that ECFCs represent a promising and suitable source for pre-vascularization of tissue engineered constructs for bone and skin regeneration, endothelialization of tissue-engineered vascular grafts, vascular repair, treatment of myocardial infarction and other ischemic-induced diseases as well as a vehicle for gene therapy. However, irrespectively of the animal model used to investigate the therapeutic efficacy of ECFCs, it is necessary to clearly define experimental outcomes that can significantly contribute to translation of collected data in clinical relevant situations. In addition, by choosing an appropriate application route that easily can be to translated in clinical settings, the regenerative efficacy of ECFCs can be tuned to the demands of local tissue microenvironment.

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REFERENCES


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Review: Therapeutic potential of human-derived endothelial colony-forming cells in animal models


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Chapter 2


LONG-TERM EXPANSION IN PLATELET LYСATE INCREASES GROWTH OF PERIPHERAL BLOOD-DERIVED ENDOTHELIAL COLONY-FORMING CELLS AND THEIR GROWTH FACTOR-INDUCED SPROUTING CAPACITY

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ABSTRACT

Efficient implementation of peripheral blood-derived endothelial-colony cells (PB-ECFCs) as a therapeutic tool requires isolation and generation of a sufficient number of cells in ex vivo conditions devoid of animal-derived products. At present, little is known how the isolation and expansion procedure in xenogeneic-free conditions affects the therapeutic capacity of PB-ECFCs.

The findings presented in this study indicate that human platelet lysate (PL) as a serum substitute yields twice more colonies per mL blood compared to the conventional isolation with fetal bovine serum (FBS). Isolated ECFCs displayed a higher proliferative ability in PL supplemented medium than cells in FBS medium during 30 days expansion. The cells at 18 cumulative population doubling levels (CPDL) retained their proliferative capacity, showed higher sprouting ability in fibrin matrices upon stimulation with FGF-2 and VEGF-A than the cells at 6 CPDL, and displayed low β-galactosidase activity. The increased sprouting of PB-ECFCs at 18 CPDL was accompanied by an intrinsic activation of the uPA/uPAR fibrinolytic system. Induced deficiency of uPA or uPAR by siRNA technology completely abolished the angiogenic ability of PB-ECFCs in fibrin matrices. During the serial expansion, the gene induction of the markers associated with inflammatory activation such as VCAM-1 and ICAM-1 did not occur or only to a limited extent. While further propagation up to 31 CPDL proceeded at a comparable rate, a marked upregulation of inflammatory markers occurred in all donors accompanied by a further increase of uPA/uPAR gene induction. The observed induction of inflammatory genes at later stages of long-term propagation of PB-ECFCs underpins the necessity to determine the right time-point for harvesting of sufficient number of cells with preserved therapeutic potential.

Thus, the presented isolation method and subsequent cell expansion in platelet lysate supplemented culture medium permits suitable large-scale propagation of PB-ECFC. For optimal use of PB-ECFCs in clinical settings, our data suggest that 15-20 CPDL is the most adequate maturation stage.

Keywords: endothelial-colony forming cells, platelet lysate, angiogenesis, fibrin matrices, proteolysis
Long-term expansion in platelet lysate increases growth of peripheral blood-derived endothelial colony-forming cells and their growth factor-induced sprouting capacity

1. INTRODUCTION

Prospective vascular regenerative therapies rely on suitable sources of endothelial cells that can be propagated in ex vivo conditions devoid of the presence of animal-derived products. Isolation and generation of sufficient numbers of endothelial cells required for delivery into patients demands identification of defined cell populations which growth and function can be monitored in xenogeneic-free cell culture conditions. Limited proliferative and vasculogenic potential of mature endothelial cells such as human umbilical vein endothelial cells (HUVEC)[1-2] and human dermal microvascular endothelial cells (HMEC)[3-4] restrain the use of endothelial cells derived from autologous tissue for future tissue engineering and cell-based therapies. A specific subgroup of circulating endothelial progenitor cells (EPCs), present in cord and adult peripheral blood, represent a promising source for in vitro expansion and obtaining sufficient endothelial cells for clinical application.

Recent advances in the field of vascular biology have identified two distinct types of EPCs, depending on the time of appearance during isolation period. Early outgrowth EPCs, which are derived from the myelo-monocytic lineage, appear in culture during the first seven days of isolation and participate in the processes of vascular regeneration in a paracrine fashion[5]. On the contrary, the late outgrowth EPCs also known as endothelial-colony forming cells (ECFCs) appear in the cell culture usually after 10 days of isolation and are not derived from myelo-monocytic lineage. The peripheral and cord blood-derived ECFCs, characterized by robust proliferative capacity[6] and vessel forming ability in vivo[7], are currently considered as a viable cell source for future clinical application, both as a source for tissue repair and to study pathophysiological mechanisms at cellular level in patients. These cells originate from circulating stem/progenitor cells which are easily encountered in cord blood, yet exceedingly rare in peripheral blood[8].

At present, efficient isolation of ECFCs relies on creating an in vitro environment that favors differentiation of the progenitor cell into an endothelial lineage. Current protocols for isolation and expansion of peripheral blood-derived ECFCs (PB-ECFCs) depend mostly on supplementing the cell culture medium with 10-20% (v/v) of FBS. However, the transfer of potentially harmful xenogeneic material[9] and the induction of an immune response[10-11] in human recipients to FBS restricts the use of animal-derived products for isolation and ex vivo expansion of PB-ECFCs. Therefore, alternative xenogeneic-free approaches for isolation and ex vivo manipulation of PB-ECFCs prior to clinical application are greatly needed. Previous experience with ex vivo expansion
of mesenchymal stem cells (MSCs) suggests that human blood-derived products such as platelet lysate (PL)[12], serum[13], and platelet-rich plasma (PRP)[14] are feasible FBS substitutes.

Successful application of ECFCs from adult blood as a therapeutic tool relies on prolonged expansion in culture conditions that might have impact on cell physiology. Serial passaging during expansion affects the response of cells to the environmental factors. Previous studies have identified that long-term expansion reduces the therapeutic efficiency of hematopoietic stem cells (HSC)[15]. ECFCs expanded in medium supplemented with FBS are prone to high incidence of karyotype changes[16] and exhibit a decreased angiogenic ability[7,17]. On the contrary, long-term expansion of ECFCs in medium supplemented with a FBS alternative, in particular platelet lysate, preserved genomic stability of the cells[18]. However, we had to modify this procedure since we and others[19] were not able to isolate ECFCs from peripheral blood directly. Furthermore, little is known whether the expansion procedure in animal-free conditions affects the angiogenic ability of PB-ECFCs.

In this study, we compare the efficiency of initiation of PB-ECFCs cultures in xenogeneic-free settings based on use of platelet lysate as serum supplement with the current widespread used procedure which relies on FBS. In addition, we investigate whether the angiogenic ability of PB-ECFCs is affected by long-term expansion in medium supplemented with platelet lysate. Finally, the underlying mechanism that governs the sprout formation in fibrin matrices was studied. The findings presented in this study indicate that 1) platelet lysate is a better culture medium supplement than FBS for isolation and ex vivo propagation of PB-ECFCs, 2) cells expanded in platelet lysate retain their endothelial phenotype, 3) extensive propagation increases the angiogenic capacity of PB-ECFCs, and 4) sprout formation in fibrin matrices depends on PAI-1-balanced activity of the uPA/uPAR system.

2. MATERIALS AND METHODS

2.1 Preparation of pooled human platelet lysate
Platelet-rich plasma (PRP) for platelet lysate was prepared by the blood transfusion service (Department of Hematology, VU University Medical Center, Amsterdam, The Netherlands) accordingly the protocol of Korte et al[20]. Upon arrival, the bags of 5 whole blood donations, each containing 10^9 platelets per mL was stored at -80°C. A batch of pooled PL consisting whole blood donations obtained from 35 healthy donors were prepared after thawing the bags of PRP followed by centrifugation at 4075x g for
15 minutes to remove the residual platelet fragments. The platelet lysate for cell culture was aliquoted and stored at -20°C. Prior to preparation of cell culture medium, the PL was thawed and centrifugated again at 4075 x g for 15 minutes. Similar results were obtained with a second pooled PL preparation based on blood of 70 healthy donors.

2.2 Isolation and culture of ECFCs from adult peripheral blood
Peripheral blood samples (PB) were collected from 10 healthy donors (5 males and 5 females, age range 21-50 years, average age 30±8SD years) after obtaining written informal consent in accordance with the institutional guidelines. To isolate mononuclear cell (MNCs) fraction, the blood was pre-diluted 1:1 with pre-warmed phosphate buffered saline solution (PBS) and transferred into LeucoSept tubes (Greiner Bio-one, The Netherlands) previously filled with FicolPaque Plus (GE Healthcare Europe GmbH, The Netherlands). After centrifugation at 740 x g for 30 min, the interphase with MNC was collected and washed three times with M199 (Lonza, Verviers, Belgium) supplemented with 0.1% penicillin/streptomycin (Invitrogen, The Netherlands). After final washing, the MNC were re-suspended either in complete EGM-2 (Lonza, Walkersville, MD, USA) supplemented with 10%FBS (FBS-EGM) or with 10% platelet lysate (PL-EGM), 0.1% penicillin-streptomycin, 2 mM L-glutamine and 10 U/mL heparin, and seeded at density of 1.3*10^6 cells/cm² (~1mL blood per one well) into 48 well-plates pre-coated with 3µg/cm² human collagen type I prepared according to manufacturer's instructions (#C7624, Sigma Aldrich). After 24 hours, nonadherent cells were carefully removed and fresh medium was added to each well. Medium was changed daily for 7 days and then every 2 days. After 10-30 days distinct ECFC colonies with regular cobblestone morphology appeared in the cell culture. The outgrowth ECFC colonies were enumerated by daily visual inspection using phase-contrast microscopy. Primary clones of PB-ECFCs were detached using Accutase (#A6964, Sigma Aldrich) and cells re-plated onto one well of a 12 well plate were designated as passage 0. For further expansion and experimental purposes cells were detached with 0.05% trypsin/EDTA and seeded at density of 5000 cells/cm²onto cell-culture plates pre-coated with 5µg/cm² rat-tail collagen type I (BD Biosciences, Erebodegem, Belgium) for each passing step in PL-EGM or FBS-EGM.

2.3 Immunophenotyping of PB-ECFCs and uptake of Dil-Ac-LDL
Isolated cells were characterized for uptake of Ac-Dil-LDL (Biomedical Technologies Inc, USA) according to the manufacturer’s protocol. Expression of endothelial lineage surface markers was evaluated by flow cytometry (FACS Calibur; BD Biosciences) at 18 CPDL using PE mouse anti-human CD34 (IgG1, 555822), PE mouse anti-human CD144 (IgG1, 561714), PE mouse anti-human CD31 (IgG1, 555446), PE mouse anti-human CD309 (IgG1, 560872), all purchased from BD Biosciences, as well as FITC mouse anti-
human CD146 (IgG1, 130-092-851, MiltenyBiotec) and PE mouse anti-human CD105 (IgG1, MHCD10504, Invitrogen). Flow cytometry detection of hematopoietic cells was performed using antibodies against hematopoietic cell–specific surface antigens such as PE mouse anti-human CD133/1 (AC133) (IgG1, 130-080-801, MiltenyBiotec), FITC Mouse Anti-Human CD45 (IgG1, 555748, BD Bioscience), FITC mouse anti-human CD14 (IgG2a, 555397, BD Bioscience). Appropriate isotype controls PE mouse anti-human IgG1 isotype (IgG1, 555749), FITC mouse anti-human IgG1 isotype (IgG1, 555748), and PE mouse anti-human IgG2a (IgG2a, 559319) were purchased from BD Bioscience. Data were analyzed by using the FCS Express 4 software package (De Novo Software, Toronto, Ontario, Canada).

For immunocytochemistry phenotyping, cells were seeded on gelatin coated glass cover slips, fixed with 4% paraformaldehyde/EDTA and permeabilized with 0.5% Triton X-100/PBS. After washing, cells were incubated with unconjugated primary antibodies (goat anti-VE-Cadherin clone C-19, rabbit anti-vWF clone H-300, and goat anti-PECAM-1 clone M-20, all from Santa Cruz Biotechnology, USA) prepared in PBS containing 0.1% human serum albumin (HSA) for 24 h at 4°C. The next day, after two washing steps, cells were incubated with appropriate FITC labeled secondary antibodies (rabbit anti-goat IgG or donkey anti-rabbit IgG Alexa 488, Molecular probes, Invitrogen, USA) prepared in 0.1% HSA for 2 h in dark room. Cell nuclei were visualized with DAPI VectashieldHardSet present in the mounting medium (VectorLabLtd, BrunschwigChemie, The Netherlands) prior to immunofluorescence examination using 4D-digital imaging microscope (DIM).

2.4 Growth kinetics of PB-ECFCs and proliferation assay

To determine the proliferative capacity of PB-ECFCs in PL-EGM or FBS-EGM, viable cells were enumerated at passage 1 using hemocytometer and trypan blue exclusion assay. For each subsequent passage, 5000 cells/cm² were seeded on 6-well plates pre-coated with rat-tail collagen type I. The cell number after each passaging step was used to calculate the total number of yielded cells, the CPDL, and the population doubling times (PDT) as previously reported[6]. CPDL values were calculated during this propagation period (40-42 days) and did not include the cell proliferation during initial clonal outgrowth.

For short-term proliferation assays 500 cells/cm² were seeded onto 12-well plate pre-coated with rat-tail collagen type I in PL-EGM or FBS-EGM. Renewal of culture medium was performed every other day. After 7 days in culture, the cells were washed with PBS and fixed with pre-warmth 2% paraformaldehyde/HBSS. Crystal violet staining was used to visualize the cell nuclei and 5 pictures from each well were taken using phase contrast microscopy. The number of cells was determined using ImageJ software.
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2.5 Tube-formation fibrin assay

Assessment of sprouting ability of PB-ECFCs expanded in PL-EGM was performed at 6, 18, and 31 CPDL by seeding 20,000 cells on 3D human fibrin matrices prepared as previously described[21]. Following overnight incubation in M199 supplemented with 10% inactivated human serum and 10% new-born calf serum, tube formation was induced by stimulating the cells with either 10ng/ml TNF-α (T), 10ng/ml FGF-2 (F) or 25ng/ml VEGF₁₆₅(V) alone, or the combinations of them (TF: TNF-α+FGF-2, TV: TNF-α+VEGF₁₆₅, FV: FGF-2+VEGF₁₆₅). All growth factors were purchased from ReliaTech GmbH, Wolfenbuttel, Germany. To investigate the effect of FBS and PL on tube-formation of PB-ECFCs in fibrin matrices, the cells were stimulated twice with 25ng/mL VEGF-A prepared in M199+10%FBS+10U/mL heparin or M199 +5%PL+10U/mL heparin. After 48h stimulation, the cells were fixed with 2% paraformaldehyde/HBSS and quantification of the length of formed tube-like structures was performed using Optimas image analysis software as previously described[21]. The tube formation ability of PB-ECFCs of 3 donors was determined in triplicate wells.

2.6 siRNA transfection tube-formation assay

To investigate the involvement of uPA, uPAR and PAI-1 in sprout formation in fibrin matrices by PB-ECFCs expanded in PL, siRNAs against uPA (Hs_PLAU_6 FlexiTube siRNA, cat.no. SI02662135) or uPAR (Hs_PLAUR_6 FlexiTube siRNA, cat.no. SI03048458) were purchased from QiagenBenelux B.V., the Netherlands and prepared according to manufacturer’s instructions. Pool of target-specific siRNAs against PAI-1 (sc-36179) was purchased from Santa Cruz Biotechnologies, USA. ON-TARGETplus Non-targeting Pool siRNA (cat.no. D-001810-10-05) was purchased from GE Dharmacon, Lafayette, CO. Prior transfection experiments cells were starved for 4h in M199 and were transfected using DharmaFECT4 reagent (Dharmacon). All siRNA and DharmaFECT4 were prepared in M199 + 10% inactivated human serum supplemented with 10ng/mL FGF-2 at final concentration of 20nM. The transfection medium was replaced by fresh standard PL-EGM medium, 24h post-transfection. Transfection efficiency was evaluated by qRT-PCR after additional 24h period of recovery. At the same time point, the cells were seeded onto fibrin matrices and sprout formation was initiated by stimulating the cells with combination of 10ng/mL TNF-a and 10ng/mL FGF-2 every day during 3-days period in medium as already described in the previous section. Inhibition of tube formation was accomplished by preventing the conversion of plasminogen to plasmin by adding 100U/mL aprotinin (100U/mL) to the stimulation medium consisting TNF-a and FGF-2. The tube formation ability of PB-ECFCs of 4 donors was determined in triplicate wells. Quantification of the length of formed tube-like structures was performed as already described in the previous section.
2.7 ELISA of urokinase-type plasminogen activator (uPA) and Serpin E-1 (PAI-1)

For ELISA determination of soluble uPA antigen in conditioned medium, the PB-ECFCs of three donors at 6, 18, and 31 CPDL were previously starved in EBM-2 + 5%PL for 24h. After starvation period the conditioned medium was collected and centrifuged to remove the cell debris. Collected supernatant was used to determine concentration of soluble uPA as previously described[22]. The concentration of soluble human Serpin E-1 (PAI-1, cat.num.DY1786, R&D systems, Minneapolis, MN) in conditioned medium was determined by ELISA following manufacturer’s instructions.

2.8 Real-Time Polymerase Chain Reaction

For RNA isolation the PB-ECFCs of three donors at 6, 18, and 31 CPDL prior to collection of cell lysates were previously starved in EBM-2 + 5%PL for 24h. The total RNA was isolated using RNeasyMinElute Cleanup Kit (Qiagen, The Netherlands) and the RNA quality was tested with a Nanodrop 1000 spectrophotometer. Copy DNA (cDNA) was synthesized using the Cloned AMV First Strand cDNA Synthesis Kit from Invitrogen. The sequences of primers used for determination of genes of interest are given in S1 Table. Quantitative RT-PCR was performed using SYBR Green in an ABI 7500 sequence detection system (Applied Biosystems, Foster City, USA) and the following protocol: 2 min 50°C, 10 min 95°C and 40 cycles (15 sec 95°C, 1 min 60°C) and dissociation curve. The relative expression levels of target genes were calculated with the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by following equation as previous described[23]: Δ Ct sample = (Ct sample GENE) – (Ct sample HKG). The relative gene expression = \(2^{(\Delta \text{Ct sample} - \Delta \text{Ct Sample})}\).

2.9 Determination of telomere length

The telomere length of PB-ECFCs from three different donors at 6, 18, and 31 CPDL was determined by quantitative real-time polymerase chain reaction (QPCR) as previously described[24]. DNA was isolated using DNeasy kit (Qiagen) following the manufacturer’s protocol. The sequences of the primers for single-copy gene (36B4) and telomeres are specified in literature[24]. QPCR was performed in duplicate wells using SYBR Green in an ABI 7500 sequence detection system (Applied Biosystems, Foster City, USA). The relative telomere length was determined using the telomere/single gene (T/S) ratio with the calculation \(2^{(\text{Ct (telomeres)} - \text{Ct (single-copy-gene)})} = 2^{-\Delta \text{Ct}}\).
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### 2.10 Senescence-Associated β-Galactosidase Staining

Senescence-associated β-galactosidase (SA-β-Gal) activity in PB-ECFCs was evaluated with a Cellular senescence assay (#KAA002, Merck Milipore) following the manufacturer’s protocol.

### 2.11 Statistical analysis

Data are expressed as means ± SEM. At least three independent experiments were performed for all analyses. Single comparisons were made with Student’s t tests for normally distributed data or the Wilcoxon matched-pairs signed rank test for data not normally distributed. Significance was defined as a p value of <0.05. Comparisons between multiple groups were performed using one- or two-way ANOVA with Bonferroni post hoc test.

### 3. RESULTS

#### 3.1 Isolation of PB-ECFCs in medium supplemented with platelet lysate

Mononuclear cells collected from adult peripheral blood of 10 individual donors (5 males, 5 females) were divided and inoculated on human collagen type-I pre-coated plates in EGM-2 medium supplemented either with 10% FBS or 10%PL. After 10 days, both culture conditions gave rise to the appearance of colonies with a cobblestone morphology characteristic for ECFCs (Fig. 1A, FBS and Fig. 1B, PL). During the first 10 days of initial plating, the MNCs in EGM-2 medium supplemented with 10%PL generated more colonies per total volume of collected blood than MNC in EGM-2 with 10%FBS (2.3 vs. 1.2 colonies in PL-EGM and FBS-EGM, respectively; p=0.053; Fig. 1D). At the end of the isolation period of 30 days, two times more colonies per mL of PB were counted in the cultures initiated with PL-EGM than the cultures plated in FBS-EGM (0.16 vs. 0.08 colonies/mL PB, p=0.04; Fig. 1E; or 0.11 colonies/10⁶MNC vs. 0.06 colonies/10⁶MNC). This indicates that PL is a more efficient serum supplement for isolation of PB-ECFCs than the current standard protocol based on the use of FBS.
3.2 Phenotypic characterization of PB-ECFCs isolated in platelet lysate-containing medium

To determine the endothelial phenotype of PB-ECFCs isolated in PL-EGM, the primary colonies from the donors were serially expanded by seeding 5000 cells/cm² in PL-EGM. Immunofluorescence cytochemistry confirmed the expression of endothelial marker CD31, CD144, vWF in the PB-ECFCs as well as the uptake of Ac-Dil-LDL (Fig.2A-D). The flow cytometric immunophenotyping with antibodies specific for endothelial cell lineage markers confirmed the endothelial nature of PB-ECFCs (Fig.2E). The cells were positive for CD31, CD34, CD144, CD146, CD309, CD105 and negative for hematopoietic cell surface antigens CD14, CD45, and CD133 which is in accordance with previously published data[6]. Altogether, these data show that PB-ECFCs isolated and expanded in platelet lysate resembles the immunophenotype of the ECFCs isolated and expanded in FBS[6].

Figure 1. Comparison of ECFCs colony outgrowth in fetal bovine serum and platelet lysate. Representative images of ECFC colonies that appeared in FBS-EGM (A) and PL-EGM (B,C) between 10-30 days. (D): The average number of colonies that grew in FBS (open triangle) and PL (black triangle) from 10 isolations at day 10. (E): The number of colonies yields in FBS-EGM-2 (open triangle) or PL-EGM (black triangle) during the isolation period in all donors relative to the number of mL of peripheral blood. Data are expressed as mean±SEM. Statistical analysis was performed with non-parametric Wilcoxon matched pairs test (* p<0.05).
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Figure 2. Phenotypic characterization of PB-ECFCs
PB-ECFCs monolayers expanded in EGM-2 medium supplemented with 10% PL were assessed for the presence of endothelial cell markers by immunofluorescence cytochemistry (A-C) and flow cytometry as well as for uptake of Dil-Ac-LDL (D). For immunofluorescence cytochemistry staining, cells were seeded on glass cover slips, fixed and stained with antibody against CD31, VE-cadherin or vWF. Cell nuclei were visualized with DAPI staining. Cells were positive for CD31 (A, green), VE-cadherin (B, green), and vWF (C, green). Cell nuclei appear blue. (D): Incorporation of Dil-Ac-LDL by PB-ECFCs (red spots, cell nuclei stained blue with DAPI). Panel E: Flow cytometry characterization of PB-ECFCs for CD31, CD34, CD309, CD144, CD146, CD105, CD14, CD45, and CD133. Plots depict control isotype IgG staining (black histograms) versus specific antibody staining (empty histograms).

3.3 Increased proliferation of PB-ECFCs by platelet lysate
The proliferative ability of PB-ECFCs during the short-term (7 days) growth was significantly faster rate in PL-EGM than in FBS-EGM (Fig. 3A). Subsequently, the growth kinetics of PB-ECFCs from three donors were evaluated simultaneously in FBS or PL during the same period of 40 days. PB-ECFCs serially expanded in FBS generated less cells (4.11*10^11 cells vs. PL: 2.9*10^15 cells, Fig. 3B) and showed lower CPDL (Day 40: CPDL_{FBS} 20.7 vs. CPDL_{PL} 33.6, Fig. 3C) than their counterparts expanded in PL. These results show that PL is more suitable as a serum supplement than FBS, not only for isolation, but also for long-term expansion of PB-ECFCs.

3.4 Loss of progenitor status reduces proliferation of PB-ECFCs during ex vivo expansion
The ability to maintain telomere length during consecutive cell divisions as well as the high expression of the progenitor-related CD34 antigen on the cell surface are two hallmarks of progenitor cells. We subsequently evaluated whether these parameters become altered during serial propagation of PB-ECFCs at 6CPDL (7 days of culture),
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18CPDL (19 days), and at 31CPDL (38 days) in PL-EGM containing medium. PB-ECFCs can serially be expanded in PL-EGM for more than 30 CPDL within 40 days with an average population doubling time (PDT) of 23.8±1.0 h per cell cycle. Comparison of the PDT at different time intervals during the culture period showed that the cells at 31 CPDL exhibit significant longer PDT (28.6±1.5 h) than the cells at 6 CPDL (19.8±1.1 h) or 18 CPDL (22.8±1.7 h) respectively, indicating a decline in proliferation rate (Fig.4A).

![Figure 3. Proliferative potential of PB-ECFCs in FBS and PL medium](image)

For growth kinetics as well as cumulative population doubling determination experiments, cells were serially expanded by seeding 5000 cells/cm² in EGM-2 supplemented with either 10%FBS or 10%PL during period of 40 days. (A): Comparison of proliferative potential of PB-ECFCs maintained in FBS-EGM (open bar) or PL-EGM (closed bar) during 7 days. Results represent the mean ± SEM of counted number of cells relative to cm² of plating surface of 3 independent experiments of three different donors. *p < 0.05 by Student paired t test. (B): Total number of cells yielded during long-term expansion of PB-ECFCs in FBS-EGM (open symbol, n:3) or PL-EGM (closed symbols, n:3). Each symbol indicates total number of cells at each passaging step. (C): Cumulative population doubling levels (CPDL) of PB-ECFCs in PL-EGM (closed bars) or FBS-EGM (open bars) after 10, 20, and 40 days of expansion. Results represent the mean ± SEM of CPDL at three different time points of 3 independent experiments of 3 different donors. Two-way ANOVA with Bonferroni post hoc test.

The reduction in proliferation was reflected in the dynamics of telomere shortening. A significant telomere shortening was detectable only in the cells at 31 CPDL (Fig.4B). To investigate if this decrease was caused by an accumulation of senescent cells, β-galactosidase staining was performed. Although at 6, 18 and 31 CPDL a few β-galactosidase positive cells were detectable, there was no difference in their numbers between these conditions (data not shown).
Long-term expansion in platelet lysate increases growth of peripheral blood-derived endothelial colony-forming cells and their growth factor-induced sprouting capacity.

In addition, we investigated if the observed reduction in the rate of cell division at 31 CPDL might be reflected in a decline of CD34+ cells in the culture. Flow cytometric analysis revealed a gradual decrease in the percentage of CD34+ ECFCs during the 40 days of culture; 31.0%, 32.3% and 15.2% CD34+ cells at 6, 18 and 31 CPDL, respectively (Fig.4C). Additional analysis of mean fluorescence intensity by flow cytometry and gene expression of CD34 by QPCR confirmed the decline of expression of this antigen at protein and gene level in time (Supplemental Fig.2). These data indicate that the moderate attenuation of proliferation of PB-ECFCs at 31 CPDL is associated with detectable telomere shortening and reduced numbers of CD34+ cells during in vitro expansion, which reflects a gradual loss of progenitor hallmarks during serial propagation.

**Figure 4. Comparison of proliferation-related features in PB-ECFCs during long-term cell expansion.**

During long-term expansion of PB-ECFCs in PL-EGM, a set of experiments at different CPDLs (white bar 6CPDL, grey bar 18CPDL, black bar 31CPDL) were performed to investigate proliferation rate, telomere length and expression of the progenitor-cell marker CD34. (A) Comparison of population doubling time (PDT). (B) Telomere (T) and single copy gene (S) were amplified by quantitative real-time PCR, with the T/S ratio proportional to telomere length at different stages of cells’ age. (C): Flow cytometry data of percentage of cells positive for CD34. Results represent the mean ± SEM of 3 independent experiments each performed with different donor at indicated CPDL. Comparison between each CPDL was performed by one-way ANOVA with Bonferroni post hoc test (*p<0.05).

**3.5 Effect of long-term expansion on the sprouting response of PB-ECFCs**

Fibrin is the most abundant protein present in the provisional extracellular matrix during angiogenesis. Therefore, we evaluated whether long-term expansion affects the sprouting capacity of PB-ECFCs in 3D fibrin matrices at different CPDL (6, 18, and 31 in PL-EGM). Quantification of the sprouting capacity of PB-ECFCs at 6, 18, and 31 CPDL (Fig.5B-C) showed that cells at 18 CPDL responded much stronger to the sole addition
of FGF-2 or VEGF-A than their counterparts at 6 CPDL. Combined addition of FGF-2 and VEGF-A induced more sprout formation in cells at 18 and 31 CPDL than in 6 CPDL cells (Fig 5D). TNF-α also stimulated sprouting (Supplemental Fig. 3A). Interestingly, addition of TNF-α to FGF-2 or VEGF-A or FGF-2+VEGF-A completely abolished the difference in angiogenic sprouting between cells at 6 CPDL and 18 or 31 CPDL that was observed when sole growth factors were used (S3 Fig.B-D). In aggregate, the data indicate that PB-ECFCs give a direct sprouting response upon exposure to TNF-α, and that 18 and 31 CPDL PB-ECFCs respond more avidly to single angiogenic growth factors than 6 CPDL cells.

Figure 5. Comparison of tube-forming capacity of PB-ECFCs at different time-points of ex vivo expansion upon stimulation with VEGF-A and FGF-2.

PB-ECFCs obtained from 3 different donors were serially expanded in medium supplemented with PL and the sprouting ability of cells in fibrin matrices was assessed at 6, 18, and 31 CPDL. Cells at indicated CPDL (white bar 6 CPDL, grey bar 18 CPDL, black bar 31 CPDL) were either unstimulated (A) or stimulated with FGF-2(B), and VEGF-A(C), FGF-2+VEGF-A (D), TNF-α. Results represent the mean ± SEM of mean tube length of tube-like structures of the 3 donors each performed at indicated CPDL. Comparison between each CPDLs was performed using one-way ANOVA with Bonferroni post hoc test. (*p < 0.05).
3.6 Increased angiogenic response of PB-ECFCs in fibrin matrices is accompanied by intrinsic upregulation of the fibrinolytic system

A finely tuned degradation of the fibrin matrix via the PAI-1-balanced activity of uPA/uPAR/plasmin system at the surface of endothelial cells ensures proportional fibrinolysis and adequate angiogenesis. We therefore evaluated the mRNA expression of uPA, uPAR, tPA and PAI-1 in ECFCs at 6, 18, and 31 CPDL. The basal expression of uPA, uPAR and PAI-1 genes was considerably upregulated in PB-ECFCs at 18 and 31 CPDL compared to the cells at 6 CPDL (Fig.6A, 6B, and 6D).

To evaluate whether the increased mRNA levels of uPA and PAI-1 induce proportional protein synthesis we assessed the amount of these two soluble antigens in conditioned medium (CM) collected at various CPDL. As illustrated in Fig.6E, soluble uPA antigen was prominently present in CM of PB-ECFCs at 18 CPDL and 31 CPDL. With respect to the amount of released soluble PAI-1 in CM, cells at 31 CPLD produced significantly more PAI-1 only in comparison to 6 CPDL cells but not to the cells at 18 CPDL (Fig.6F). In addition, cells at 6 and 18 CPLD produced comparable amount of soluble PAI-1 in CM under basal, unstimulated conditions. MMP-1, MMP-2 and MMP-14 were also clearly expressed in PB-ECFCs (Ct values at 6 CPDL 16.2±0.4, 20.0±0.1 and 22.1±1.3, mean±SEM, 3 donors), while MMP-9 expression was very low (Ct values at 6 CPDL 30.8±0.4, mean ± SEM, 3 donors). There was no statistical difference or general trend of the changes of these MMPs during serial propagation (data not shown).

3.7 Activation of uPA/uPAR/PAI-1 system directs sprout formation in fibrin matrices

Given the increased expression of the uPA, uPAR and PAI-1 genes during serial propagation of PB-ECFCs in PL, we next assessed their impact on sprout formation in fibrin matrices. The siRNA technology was used to knockdown uPA, uPAR and PAI-1 expression in PB-ECFCs. qRT-PCR confirmed a significant decrease of ~98% in the mRNA levels of uPA, uPAR and PAI-1 when siRNA-uPA, siRNA-uPAR or siRNA-PAI-1 transfected cells were compared with the cells transfected with non-targeting siRNA pool (Supplemental Fig.4A). The transfection procedure didn’t alter significantly the mRNA levels of uPAR and MMP-14 in siRNA-uPA cells, the uPA and MMP-14 gene expression in siRNA-uPAR cells or uPA, uPAR and MMP-14 in siRNA-PAI-1 cells (Supplemental Fig.4B,C,D). During the course of a 3-day stimulation period, the siRNA-NT cells exhibited similar sprouting response as the control, non-transfected cells (Supplemental Fig. 4E). On the other hand, siRNA-uPA or siRNA-uPAR transfected cells completely failed to form sprouting structures in fibrin matrices (Fig.7A,B). In contrast, silencing of PAI-1 mRNA induced an increase of sprouting in TNF-α+FGF-2 stimulated ECFCs, while non-targeting siRNA had
Figure 6. Expression of genes and soluble antigens involved during sprouting in fibrin matrix in PB-ECFCs at different maturation stages

Quantitative RT-PCR analysis was performed on total cellular mRNA isolated from PB-ECFCs at different CPDL (open bar 6 CPDL, grey bar 18 CPDL, black bar 31 CPDL). Gene expression levels of uPA (A), uPAR (B), tPA (C), and PAI-1 (D) in PB-ECFCs. Data are expressed as n-fold difference of expression of genes in cells at 6 CPDL. One-way ANOVA with Bonferroni post hoc test (p<0.05).

Evaluation of the production of uPA (E) and PAI-1 (F) antigens in CM by PB-ECFCs was performed at 6 CPDL (open bar), 18 CPDL (grey bar), and 31 CPDL (black bar) using ELISA. Results represent the mean ± SEM of uPA or PAI-1 concentration in ng relative to mL of conditioned medium of 3 independent experiments each performed at indicated CPDL. Comparison between different groups was performed one-way ANOVA with Bonferroni post hoc test (p<0.05).
Long-term expansion in platelet lysate increases growth of peripheral blood-derived endothelial colony-forming cells and their growth factor-induced sprouting capacity.

**Figure 7. Effect of uPA, uPAR and PAI-1 knockdown on angiogenic response of PB-ECFCs in fibrin matrices**

The involvement of uPA/uPAR/PAI-1 system during sprouting in fibrin matrices was assessed in PB-ECFCs obtained from 3 different donors at 18 CPDL. A and B: diminished angiogenic ability of PB-ECFCs transfected with siRNA-uPA or siRNA-uPAR in fibrin matrices. C: sprout formation by cells transfected with non-targeting, control siRNA (si-RNA-NT). D: sprout formation by cells transfected with siRNA-PAI-1 (si-RNA-PAi-1). E: Comparison of angiogenic response of PB-ECFCs transfected with non-targeting siRNA (siRNA-NT) and siRNA targeting uPA (siRNA-uPA), uPAR (siRNA-uPAR) or siRNA-PAI-1 (si-RNA-PAi-1) expressed as mean ± SEM of mean tube length of tube-like structures of 3 independent experiments of 3 different donors (open bars: unstimulated cells, black bars: cells stimulated with 10ng/mL TNF-α + 10ng/mL FGF-2). Comparison between different groups was performed using two-way ANOVA with Bonferroni post hoc test (p<0.05).
no effect (Fig.7C,D). In line with an involvement of the fibrinolytic system, the plasmin inhibitor aprotinin also fully prevented sprout formation (data not shown). These data indicate that sprout formation in fibrin matrices by PB-ECFCs expanded in PL requires pericellular proteolysis involving uPA/uPAR/plasmin activity, which is balanced by PAI-1.

3.8 Induction of activation markers during serial propagation of PB-ECFCs

Progressive upregulation of VEGFR2 in cord blood-derived ECFCs during the expansion process might underlie the increased activation of uPA/uPAR[25]. However, we could not detect a significant upregulation of VEGFR2 mRNA level during long-term expansion of PB-ECFCs in PL-EGM (Supplemental Fig.5). As uPA can be induced by several factors including the inflammatory mediator TNF-α, we subsequently evaluated whether a general inflammatory activation accompanied serial propagation of PB-ECFCs. To that end, we compared the induction of uPA in PB-ECFCs cultures of three different donors with that of VCAM-1, ICAM-1, E-selectin, IL-8, and MCP-1, five factors that can also be induced by TNF-α in endothelial cells. We indeed observed activation of these genes after extensive serial propagation, but the three donors tested differed in the onset of activation as reflected by the five markers, of which VCAM and ICAM-1 are shown in Fig.8. Cells at 18 CPDL displayed limited induction of inflammatory markers, but in two donors marked activation was observed at CPDL31. Comparison of the patterns of these five markers with that of the induction of uPA shows a similar pattern for uPA at several time points indicating that the increase of mRNA levels of uPA runs parallel with the other proteins (Fig.8).

Figure 8. Expression of inflammatory activation-related markers

Quantitative RT-PCR analysis was performed on total cellular mRNA isolated from 3 donors at 6, 18, and 31 CPDL. Gene expression levels of ICAM-1 (A), VCAM-1 (B), and uPA (C) in individual donors PB15 (◆), PB84 (●), and PB224(▲), during long-term expansion. Analysis of uPA expression was performed with the same data set as depicted in Figure 7A. Data are expressed as n-fold difference of expression of same genes in cells at 6 CPDL.
4. DISCUSSION

In the present study we demonstrate an improved method for the isolation of ECFCs from adult peripheral blood. The presented procedure is based on use of human platelet lysate as a serum substitute and yields more colonies per mL collected blood compared to the conventional isolation with FBS. Isolated PB-ECFCs displayed a higher proliferative ability in medium supplemented with platelet lysate than cells expanded in medium with FBS allowing a faster generation of sufficient number of cells for clinical applications. Furthermore, our data showed that an increase of sprouting capacity of PB-ECFCs in fibrin matrices during long-term expansion in platelet lysate is accompanied by an increase at protein and mRNA level of the fibrinolytic system. Thus, presented isolation method and further cell expansion in platelet lysate permits optimal large-scale propagation of angiogenic potent PB-ECFCs.

In the present manuscript we demonstrated that the initial yield and expansion of ECFCs from adult peripheral blood by a renewed method using platelet lysate (PL) is stimulated to a larger extent by PL- than by FBS-containing medium. The obtained endothelial cells showed limited telomerase shortening, continued CD34+ expression, and maintenance or increase of sprouting activity in fibrin matrices, which was inhibited by blocking expression of uPA and uPAR. Furthermore, they displayed a limited but gradual increase in expression of inflammation-associated genes in particular after 18 CPDL. Thus the use of platelet lysate provides a humanized condition that enables isolation and large scale propagation of angiogenic potent PB-ECFCs.

4.1 Isolation and characterization of PB-ECFCs in platelet lysate-supplemented medium

The conventional method for the isolation and expansion of peripheral blood-derived ECFCs include supplementation of cell culture medium with 10-20% (v/v) of FBS. However, such procedures yield cells possibly inapt for future clinical application due to the safety concerns related to the use of FBS in preparation of adult stem cells[9]. Isolation of ECFCs in animal serum-free conditions according to the requirements of good manufacture practice has been previously reported in human serum[26-27] and platelet lysate[18]. Data from our lab suggest that isolation of PB-ECFCs in 5% autologous plasma is feasible, yet the limited amounts of donor-derived plasma hampers long-term expansion of ECFCs to numbers sufficient for clinical treatment. It should be noted that - in our attempts to replicate the method with PL proposed by Reinisch et al.[18] - we and others[19] did not succeed in isolating ECFCs from peripheral blood obtained from various individual donors. However, by employing density gradient separation of MNCs from peripheral blood as well as human collagen as coating substrate we were able to
isolate ECFCs from adult blood in in vitro settings based on the use of platelet lysate. Immunofluorescence phenotyping and functional assays confirmed that PB-ECFCs isolated and cultured in platelet lysate exhibit endothelial lineage markers, and are able to form tube-like structures which is in accordance with previous reports[6]. In addition, these cells are devoid of CD45 and CD14 antigen indicating that PB-ECFCs isolated by the presented procedure do not represent cells from a hematopoietic/monocytic lineage.

4.2 Clonal outgrowth and proliferative capacity of PB-ECFCs in platelet lysate
The number of colonies generated in FBS-EGM and PL-EGM is higher compared to the current widespread procedure for isolation of ECFCs in FBS. In FBS-EGM we obtained two-fold more colonies compared to previous reports[8-28]. Coating with human collagen type I instead rat-tail collagen type I and use of 48-well instead standard 6-well plates may contribute to the difference in number of counted colonies. The presence of heparin in FBS-EGM might also contribute to better clonal outgrowth observed in our study via modulating the activity of heparin-binding sites on VEGF-A[29] and FGF-2[30] which are also present as a supplement in isolation medium. Nevertheless, the abundance of the many growth factors that are released from the platelets, such as VEGF, EGF, HGF, PDGF, and IGF[31] might contribute to enhanced clonal outgrowth of ECFCs in PL compared to FBS and increased proliferation rates. This grants harvest of sufficient number of 10^9-10^11 cells for clinical use after 15-20 CPDL (for comparison see reference [32]). The cultures of PB-ECFCs at indicated CPDL exhibit a rather stable telomere length and are devoid of significant presence of senescent cells, in accordance with previously reports on a low rate of intrinsic senescence in ECFCs[6,30]. Moreover, the expression of the tip-cell related surface protein CD34 by a significant portion of PB-ECFCs suggests the presence of cells with robust sprouting capacity. Therefore, with respect to the isolation and further expansion of PB-ECFCs in platelet lysate, our improved method offers yield of enough cells with robust angiogenic potency for clinical application within 30 days or less after the blood collection.

4.3 Effect of serial passage on in vitro sprouting ability of PB-ECFCs
Serial passage during expansion may affect the response of cells to the environmental stimuli (e.g. growth factors, hypoxia) that are commonly used to study the physiological process such as angiogenic sprouting[34]. Previous comparison of angiogenic ability of ECFCs expanded in medium containing FBS or PL has unraveled that the serum supplement modulates sprouting ability of these cells[35]. Data from our lab is also in line with that observation since ECFCs formed more sprouts when 5%PL was introduced
Long-term expansion in platelet lysate increases growth of peripheral blood-derived endothelial colony-forming cells and their growth factor-induced sprouting capacity in our fibrin-based tube-formation assay compared to 10% FBS (S6 Fig). However, it should be noted that Hofbauer et al.[35] used a Matrigel assay, which reflects the rapid reorganization of endothelial cells into a network, while we have used a human fibrin matrix, which reflects the body’s own temporary repair matrix, provides true capillary-like endothelial tubules and is suitable for tissue engineering purposes.

In contrast to our expectation, the data from the sprouting assay suggest that extended cell expansion at 18 and 31 CPDL increases tube formation by FGF-2- and VEGF-A stimulated PB-ECFCs in fibrin matrices as compared to their counterparts at 6 CPDL. Similar findings using the Matrigel angiogenesis assay have also been reported by other investigators[25] who suggested a role for VEGFR2 in VEGF-enhanced sprouting. We found no effect of serial passaging on VEGFR2 mRNA, but we cannot excluded an effect on surface density or turnover of VEGFR2. On the other hand, Basire et al.[36] first reported that the robust angiogenic capacity of ECFCs might be contributed to high intrinsic uPA/uPAR proteolytic ability of these cells. The siRNA data in our study further point to the important role of receptor-bound uPA activity in the formation of tubular structures by PB-ECFCs expanded in PL. This finding is also in line with the previous studies from our group that showed that tube formation by cord blood ECFCs expanded in FBS conditions was inhibited by anti-uPA as well as anti-uPAR IgG antibodies[37]. Furthermore, preceding the enlargement of endothelial cell diameter during long-term propagation an increased expression of uPA has been previously observed[38]. Our data is in the line with this observation since PB-ECFCs at 18 and 31 CPDL displayed an increase of fibrinolytic system at gene and protein level and much higher sprouting ability compared to the cells at 6 CPDL. Interestingly, the amount of PAI-1 antigen markedly exceeds the amount of u-PA antigen produced, as can be observed in the Figure 7. This is comparable with earlier findings in endothelial cells[39]. However, although the amount of PAI-1 does modulate u-PA activity and u-PA-dependent tube formation by ECFC, its effect is minor as compared to the contributions of u-PA and UPAR, as these molecules are rate limiting. In contrast, PAI-1 is produced in excess but only part of it is encountered in an active form[40]. The trend of progressive upregulation of uPA, uPAR and PAI-1 genes runs parallel with a general inflammatory activation of ECFCs since the inflammatory markers such as VCAM, and ICAM-1 become upregulated at mRNA level at a later time point during the serial propagation. From these data we conclude that use of expanded ECFCs at CPDL 15-20 will provide cells with no or limited inflammatory activation.
4.4 Sprouting response of PB-ECFCs under inflammatory condition

Our data revealed that addition of TNF-α augments the basal sprouting induced by FGF-2 or VEGF in PB-ECFCs. Foreskin-derived microvascular endothelial cells (hMVEC) also require the simultaneous presence of TNF-α and FGF-2 or VEGF-A to form tube-like structures in fibrin matrices [21] but application of solely TNF-α to the cells inhibits cell growth and is not sufficient to induce sprouting. However, the PB-ECFCs expanded in platelet lysate were able to form tube-like structures upon stimulation with solely TNF-α at any stage of cellular age when sprouting was assayed. In vivo TNF-α can induce sprouting angiogenesis [41], but mutual interaction between different cell types cannot be excluded. Why TNF-α displays this ability in PB-ECFCs and not in hMVEC is unclear, but one may hypothesize that PB-ECFCs produce an additional growth factor either constitutively or after induction by TNF-α, which is able to induce sprouting together with TNF-α.

In conclusion, the cell expansion of PB-ECFCs in medium supplemented with platelet lysate permits generation of sufficient number of cells in less than 30 days after the initial blood collection. For optimal use of PB-ECFCs in clinical settings, our data suggest that 15-20 CPDL is the most adequate maturation stage. The cells at this time point retained their proliferative capacity and showed increased sprouting ability in fibrin matrices, were devoid of significant presence of senescence as well as exhibited unaltered expression of the markers associated with inflammatory activation. Thus, the presented isolation method and subsequent cell expansion in platelet lysate supplemented culture medium permits suitable large-scale propagation of angiogenic potent PB-ECFCs.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Dr. Angelika M. Dräger from Department of Hematology, VUmc for providing platelet-rich plasma, and Nicky Boontje for technical assistance during blood collection. The project was financially supported by a grant of the Netherlands Initiative for Regenerative Medicine (NIRM). The authors can exclude any conflict of interest and financial relationship related to the content of the presented project.
Long-term expansion in platelet lysate increases growth of peripheral blood-derived endothelial colony-forming cells and their growth factor-induced sprouting capacity

REFERENCES


Long-term expansion in platelet lysate increases growth of peripheral blood-derived endothelial colony-forming cells and their growth factor-induced sprouting capacity


Chapter 3

Long-term expansion in platelet lysate increases growth of peripheral blood-derived endothelial colony-forming cells and their growth factor-induced sprouting capacity

SUPPLEMENTARY MATERIAL

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Supplemental Figure 1. Outgrowth of PB-ECFCs at the end of isolation procedure

(A): The average number of ECFCs colonies that appeared in culture at the end of isolation. (B): Distribution of colony outgrowth in FBS (open bars) or PL (black bars) after 10 days, between 10-20 days and at the end of isolation period.

Supplemental Figure 2. Comparison of expression of progenitor-cell marker CD34 on PB-ECFCs at different maturation stages.

A): Flow cytometry data represents mean fluorescence intensity calculated as CD34 antibody fluorescence intensity minus autofluorescence of matched isotype antibody. (B):qRT-PCR validation of mRNA levels of CD34 gene in PB-ECFCs at 18 and 31 CPDL are expressed as a n-fold difference of expression of the same genes in the cells at 6 CPDL.
Long-term expansion in platelet lysate increases growth of peripheral blood-derived endothelial colony-forming cells and their growth factor-induced sprouting capacity.

Supplemental Figure 3. Tube-forming capacity of PB-ECFCs at different time-points of ex vivo expansion upon stimulation with TNF-α.
PB-ECFCs obtained from 3 different donors were serially expanded in medium supplemented with PL and the sprouting ability of cells in fibrin matrices was assessed at 6, 18, and 31 CPDL. Cells at indicated CPDL (white bar 6 CPDL, grey bar 18 CPDL, black bar 31 CPDL) were stimulated with TNF-α(A), TNF-α+FGF-2 (TF, B), TNF-α+VEGF-A (TV, C), and TNF-α+FGF-2+VEGF-A (TFV, D). Results represent the mean ± SEM of mean tube length of tube-like structures of 3 independent experiments each performed at indicated CPDL. Comparison between each CPDLs was performed using one-way ANOVA with Bonferroni post hoc test.(*p < 0.05).
Supplemental Figure 4. qRT-PCR validation of transfection efficiency.

A: Quantitative RT-PCR analysis was performed on total cellular mRNA isolated from PB-ECFCs of three donors transfected with non-targeting siRNA (siRNA-NT) and siRNA targeting uPA (siRNA-uPA), uPAR (siRNA-uPAR) or PAI-1 (siRNA-PAI-1). Gene expression levels of uPA in siRNA-uPA cells (white bar), uPAR (grey bar) in siRNA-uPAR and PAI-1 (dark grey bar) in siRNA-PAI-1 cells expressed as n-fold difference of expression of the same genes in cells transfected with non-targeting siRNA (black bar).

B: Gene expression levels of uPAR and MMP-14 (white bars) in siRNA-uPA cells expressed as n-fold difference of expression of the same genes in cells transfected with non-targeting siRNA (black bar).

C: Gene expression levels of uPA and MMP-14 (white bars) in siRNA-uPAR cells expressed as n-fold difference of expression of the same genes in cells transfected with non-targeting siRNA (black bar).

D: Gene expression levels of uPA, uPAR and MMP-14 (white bars) in siRNA-PAI-1 cells expressed as n-fold difference of expression of the same genes in cells transfected with non-targeting siRNA (black bar).

E: Comparison of angiogenic response of PB-ECFCs transfected with non-targeting siRNA (siRNA-NT) to control, untransfected cells expressed as mean ± SEM of mean tube length of tube-like structures of 3 independent experiments of 3 different donors (open bars: unstimulated cells, black bars: cells stimulated with 10ng/mL TNF-α + 10ng/mL FGF-2, gray bars: cells stimulated with 10ng/mL TNF-α + 10ng/mL FGF-2 + 100U/mL aprotinin).
Long-term expansion in platelet lysate increases growth of peripheral blood-derived endothelial colony-forming cells and their growth factor-induced sprouting capacity.

**Supplemental Figure 5.** Gene expression levels of VEGFR2 in PB-ECFCs at different maturation stages.
Quantitative RT-PCR analysis was performed on total cellular mRNA isolated from PB-ECFCs of three donors at different CPDL (open bar 6 CPDL, grey bar 18 CPDL, black bar 31 CPDL). Data are expressed as n-fold difference of expression of the same genes in cells at 6CPDL. One-way ANOVA with Bonferroni post hoc test (p<0.05).

**Supplemental Figure 6.** Tube-forming capacity of PB-ECFCs in presence of FBS or PL
The tube-forming effect of FBS or PL on PB-ECFC isolated and expanded in PL was investigated after stimulation with 10ng/mL VEGF-A prepared in M199+10%FBS+10U/mL heparin or M199+5%PL+10U/mL heparin. Results represent the mean ± SEM of mean tube length of tube-like structures of 3 independent experiments. Comparison between each CPDLs was performed using one-way ANOVA with Bonferroni post hoc test (*p < 0.05).
CHAPTER 4

HYPOXIA DECREASES OUTGROWTH OF ENDOTHELIAL COLONY-FORMING CELLS FROM CORD AND PERIPHERAL BLOOD

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Henk Broxterman
Pieter Koolwijk
Victor W.M. van Hinsbergh
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ABSTRACT

Vascular homeostasis and regeneration in ischemic tissue relies on intrinsic competence of the organism to rapidly replenish endothelial cells for new vasculature. It has been shown that the mononuclear cell (MNC) fraction expressing CD34 contains circulating progenitors committed to endothelial lineage. These progenitors give rise to endothelial colony-forming cells (ECFCs) that actively participate in neovascularization of ischemic tissue. At present, the effects of low oxygen tension on the outgrowth and proliferation of ECFCs from MNC precursors is poorly understood.

In this study we investigated the clonal outgrowth of ECFCs from cord blood (CB) and peripheral blood (PB) under hypoxic conditions. MNCs obtained from CB and PB were subjected to 20% and 1% O₂ cell culture conditions and the clonal outgrowth was enumerated during a 30 days incubation period.

Data presented in this study indicate that hypoxia impairs the initial outgrowth of ECFCs from CB and PB MNCs. Re-oxygenation experiments confirmed the dependency of initial ECFCs outgrowth on higher oxygen tension. A low oxygen environment in vitro also reduced the clonal ability of subcultured PB-ECFCs and the proliferation rate of CB-ECFCs. Investigating whether MNCs modulate the clonogenicity of ECFCs in a paracrine or in a direct, cell-cell contact fashion revealed that soluble factors present in conditioned medium collected from MNCs altered the clonogenicity of CB-ECFCs but not that of PB-ECFCs. On the other hand, PB-MNCs placed in close vicinity of PB-ECFCs rescued the hypoxia-induced reduction of clonal capacity of PB-ECFCs.

Findings in this study revealed that hypoxia impairs the initial outgrowth of ECFCs, which can be overcome by specific factors or pre-exposure to oxygen. Further elucidation of the events that regulate the EC differentiation of progenitors within the CD34⁺ MNCs to ECFCs, the cellular response to hypoxia as well as the subtle cell-cell communication in ischemic tissue will contribute to better understand the true nature of these cells and objectively assess their therapeutic potential.
1. INTRODUCTION

Angiogenesis, arteriogenesis and de-novo vessel formation are mechanisms that maintain vascular homeostasis and drive tissue regeneration after an ischemic event in post-natal life. Low oxygen tension and sterile inflammation are the major environmental factors that initiate and further regulate neovascularization and subsequent repair of ischemic tissue. Rapid establishment of a functional and robust vasculature that ensures adequate supply of oxygen and nutrients in a hypoxic area is of utmost importance for tissue repair. Recent advances in the field of vascular biology have identified that the mononuclear cell (MNC) fraction expressing CD34 contains a subset of circulating progenitors committed to the endothelial lineage which are indispensable during the assembly of new vascular networks. These endothelial progenitors give rise to endothelial colony-forming cells (ECFCs) or blood originated endothelial cells (BOECs) that actively participate in neovascularization in particular in ischemic tissue. However, the effects of hypoxia on the outgrowth and proliferation of ECFCs and their precursors is poorly understood.

ECFCs can be isolated from the MNC fraction of both cord (CB) and peripheral blood (PB) as well as from tissue material. ECFCs exhibit high proliferative and colony-forming ability, are phenotypically indistinguishable from other endothelial cells, do not belong to hematopoietic cell lineage, and possess robust in vitro and in vivo neovascularization ability. The formation of primitive vascular networks in ischemic tissue largely depends on the differentiation ability of circulating progenitors to generate ECFCs. The proliferative ability of these ECFCs also plays an important role during vascular formation under hypoxic conditions. Exposure to 1.5% O$_2$ (hypoxia) for 14 days reduces proliferation and angiogenic capacity of ECFCs. Furthermore, the same study also revealed reduced ECFCs colony formation from single-cell plated human umbilical vein endothelial cells (HUVEC) during chronic hypoxia.

Here we investigated the clonal outgrowth of ECFCs from CB and PB under hypoxic conditions. Furthermore, we assessed the effect of low oxygen tension on the proliferative and clonogenic ability of subcultured ECFCs. Co-culture experiments were performed to investigate the manner of how PB-MNCs affect the clonal ability of subcultured PB-ECFCs under hypoxic and normoxic conditions. Findings in this study demonstrated that hypoxia impairs the outgrowth of ECFCs from MNC fraction of cord and peripheral blood.
Chapter 4

2. MATERIALS AND METHODS

2.1 Isolation of CB and PB-ECFCs under hypoxia and normoxia

CB-ECFCs and PB-ECFCs were isolated as previously described with minor modifications\(^\text{11}\). Namely, after isolation of MNCs by Ficoll-Paque density gradient centrifugation, the CB- and PB-derived MNCs were re-suspended in complete EBM-2 (Lonza, Walkersville, MD, USA) supplemented with 10%FBS, 0.1% penicillin-streptomycin, 2 mM L-glutamine, and EGM-2 SingleQuotes (without hydrocortisone and gentamycin/ amphotericin-B). MNCs were divided on two equal inoculation parts and seeded at a density of at least 2.5 x 10^6 cells per cm^2 onto 0.1% gelatin factor V (Sigma) pre-coated 6- or 48-well plates. One culture was placed at 20% O_2/5% CO_2 and the second inoculum at 1% O_2/5% CO_2 atmosphere.

For optimal evaluation of hypoxic conditions cells were cultured inside a custom-designed hypoxia workstation (T.C.P.S., Rotselaar, Belgium) as previously described\(^\text{12}\).

After 3 days, the medium was renewed for the first time, followed by daily renewals during the first week; from day 7 until the end of primary culture the medium was changed every other day.

The renewal medium that was used for the CB- and PB-MNCs cell cultures at 1% O_2 was pre-incubated as a thin layer (2ml/10cm^2) in empty culture dishes in the hypoxia-chamber for at least two hours in order to allow the medium to become fully hypoxic. Renewal of cell culture medium was performed within the hypoxia-chamber preventing the cells being exposed to a normoxic environment. The outgrowth of primary ECFC colonies was daily monitored and counted in the appropriate oxygen environment by phase contrast microscopy on the basis of their characteristic endothelial cobblestone morphology (Supplemental figure 1). Endothelial phenotype of the ECFCs obtained from primary cultures at 20% and 1% O_2 was confirmed using immunofluorescence and flow cytometry as previously described\(^\text{13}\).

To determine the minimally exposure time to 20% O_2 needed for overcoming the lack of colony outgrowth in 1% O_2, three different CB-MNCs were isolated and plated in individual 6-well plates which were transferred after 24, 48, 72 or 96 hours (T1, T2, T3 and T4) from 20% O_2 to 1% O_2. Cells cultured only in 20% O_2 or 1% O_2 served as controls (T0). ECFC colonies were quantified when the colonies had become visible in the culture in 20% O_2 (maximal evaluation 4 weeks).
2.2 Assessment of clonogenic and proliferative capacity of CB- and PB-ECFCs at 20% and 1% $O_2$ conditions

The effect of oxygen tension on clonogenic and proliferative capacity of sub-cultured CB- and PB-ECFCs was assessed at 20% and 1% $O_2$ cell culture conditions. Early passages (2-3) of CB- and PB-ECFCs obtained from the primary colonies at 20% $O_2$ cultures ($n=4$) were seeded at density of 10 cells/cm$^2$ on 0.1% gelatin coated cell culture vehicles in complete EGM-2 and placed at 20% and 1% $O_2$ incubators. Medium change of cultures placed at 1% $O_2$ was performed in the 1% $O_2$ hypoxic chamber as described in the previous section of material and methods.

To investigate whether the soluble factors released by MNCs influence clonal formation ability of sub-cultured CB- and PB-ECFCs, the above described assay was performed under the same conditions except that the cell culture media was composed of 50% complete EGM-2 and 50% conditioned medium collected from CB- and PB-MNCs cultures that were used for investigation of the effect of oxygen tension on outgrowth of ECFCs under 20% and 1% $O_2$. Namely, at every point of medium change, the conditioned medium (CM) from cell cultures was collected, centrifuged at 2000 x g for 5 minutes to remove the cell debris and stored at -20ºC for experimental purposes. Prior experimental procedures, the CM collected after first 72h incubation from 20% $O_2$ and 1% $O_2$ cultures were separately thawed in a water bath and designated as D[0-3] (day0-3). Next, the CM of all 20% $O_2$ cultures [D0-3] was pooled in one batch, aliquoted and used for experiments. The same procedure was applied for 1% $O_2$ CM [D0-3]. The CM obtained from medium change of the cultures at 20% $O_2$ after 72h period (day 5-7) was also pooled after thawing and was labeled as D[5-7]. The same procedure was applied for 1% $O_2$ CM D[5-7].

MNCs, besides being producers of bioactive factors in CM, can also influence the formation of colonies by directly interacting with sub-cultured ECFCs$^{29}$. The clonal co-culture assay was performed by seeding 10 cells/cm$^2$ PB-ECFCs and at least 2 x 10$^6$ MNCs/cm$^2$ PB-MNCs together. The cell culture vehicles were placed at 20% and 1% $O_2$ environment. Medium change of cultures placed at 1% $O_2$ hypoxic chamber was performed as described in the previous section of materials and methods.

After 10 days, the cells from all clonal assays ($O_2$ tension, CM, and co-culture) were washed with PBS and fixed with pre-warmth 2% paraformaldehyde/HBSS. Crystal violet staining was used to visualize the cell nuclei and images from each well were taken using phase contrast microscopy. Enumeration and classification of the colonies as low-proliferative potential (LPP) and high-proliferative potential (HPP) colonies was performed accordingly to previous studies$^7$. 
2.3 Statistical analysis

Data are expressed as means ± SEM. At least four independent experiments were performed for all analyses. Single comparisons were made with Student’s t tests for normally distributed data or the Wilcoxon matched-pairs signed rank test for data not normally distributed. Comparisons between multiple groups were performed using one- or two-way ANOVA with Bonferroni post hoc test. Significance was defined as a p value of <0.05.

3. RESULTS

3.1 Hypoxia impairs the outgrowth of ECFCs from CB- and –PB- derived mononuclear cell fraction

Low oxygen tension in ischemic tissues determines cell fate and proliferation of progenitor or stem cells. To investigate the effect of hypoxia on outgrowth of ECFCs from EPCs that reside within the MNCs fraction, the cell cultures plated with cord- or peripheral blood-derived MNCs were subjected to 1% or 20% O₂ for long-term incubation (up to 30 days) during which the cultures were daily monitored for presence of colonies. Reduction in the number of counted ECFCs colonies compared to the cultures exposed to ambient oxygen levels was detected in hypoxic PB-MNCs cultures. At the end of incubation/isolation period twice more colonies were counted in normoxic than in hypoxic cultures (Fig. 1A, Supp.table 1). This difference was even more pronounced when CB-MNCs were used for ECFCs isolation under hypoxic and normoxic conditions (Fig. 1B, Supp.table 2). Under 1% O₂ 80% of the CB-MNCs cultures failed to initiate ECFCs outgrowth while the number of colonies obtained from normoxic cultures of CB-MNCs was comparable to the data already reported by other investigators.

3.2 Reduced clonal ability of ECFCs under hypoxic conditions

Preserved ability to form secondary and tertiary colonies after replating is one of the hallmarks of ECFCs. The proliferation aspect of clonal formation represents the ability of ECFCs to form clones composed of high proliferative cells (HPP-ECFCs) which are able to give rise to secondary and tertiary colonies, and clones with cells characterized with low proliferative potential (LPP-ECFCs). Investigation of whether hypoxia affects the clonal ability of subcultured PB-ECFCs revealed a reduction in the number of clones composed of HPP-ECFCs in cell cultures exposed to low oxygen tension (1% O₂) (Fig. 2A). The number of counted colonies composed of LPP-ECFCs was not altered after 10 days exposure to 1% O₂ (Fig. 2A). Hypoxia also reduced the proliferation rate of CB-derived ECFCs, but less extensively than HPP-ECFCs (Fig. 2B).
Hypoxia decreases outgrowth of endothelial colony-forming cells from cord and peripheral blood

**Figure 1. Clonal outgrowth of ECFCs from MNCs**
Enumeration of outgrowth colonies from peripheral blood (A) or umbilical cord blood (B)-derived MNCs at 20% (open bars) and 1% O₂ (closed bars) expressed as average number of counted colonies per donor. Statistical significance was determined by paired t-test if p<0.05

**Figure 2. Effect of O₂ concentration on clonal and proliferation capacity of subcultured ECFCs**
A: Clonal formation ability of subcultured PB-ECFCs under 20% and 1% O₂ conditions. HPP indicates colonies composed of cells with high proliferative potential whilst LPP stands for colonies consisting of cells with low proliferative potential. Statistical significance between two oxygen concentration conditions was determined by unpaired t-test if p<0.05.
B: Proliferation of subcultured CB-ECFCs under 20% and 1% O₂.

**3.3 Prior exposure to oxygen overcomes hypoxia-induced impairment of CB-ECFC proliferation**
Hypoxia fully impaired initial colony formation by CB-ECFCs, while it had only a limited effect on the proliferation of subcultured CB-ECFCs that were initially cloned in ambient oxygen atmosphere. Therefore, we evaluated whether exposure to oxygen contributed
to initiating the process of ECFC colony formation by CB cells. To that end, freshly isolated CB-MNC fractions of three donors were seeded and exposed for varying days (0-96h) to ambient oxygen (20% O₂) and subsequently transferred to 1% O₂ atmosphere for additional culture. Colony formation was monitored for several weeks. Figure 3 shows that 2-3 days of 20% O₂ exposure, but not less, was sufficient to initialize outgrowth of ECFC colonies, an ability that was maintained at 1% O₂ at a nearly similar success rate as in 20% O₂ (mean of 18 colonies in normoxia, and mean of 17 colonies after 4 days of priming at 20% O₂).

Figure 3. Effect of prior exposure to 20% O₂ on the induction of CB-ECFC colony formation in 1% O₂

The graph depicts enumeration of ECFC colony outgrowth from umbilical cord blood-derived MNCs obtained from three donors after 24, 48, 72 or 96 hours (T1, T2, T3 and T4) exposure to 20% O₂. Cells cultured only in 20% O₂ or 1% O₂ served as controls (T0). ECFC colonies were quantified when the colonies had become visible in the culture in 20% O₂. Symbols ▲, □, ● positioned at the left end of x-axis represent the number of colonies in MNC cultures that were continuously cultivated at 20% O₂.

3.4 Different response of subcultured CB- and PB-ECFCs in presence of soluble factors derived from MNCs

The MNC fraction from umbilical cord and adult peripheral blood is composed mainly of cells belonging to hematopoietic cell lineage. MNCs actively participate in modulation of angiogenic response of ECFCs. However, little is known in which manner the MNCs affect clonal and proliferative ability of ECFCs under hypoxic conditions. Therefore, we investigate whether the MNCs modulate the clonogenicity of ECFCs in paracrine or in direct, cell-cell contact fashion.
Hypoxia decreases outgrowth of endothelial colony-forming cells from cord and peripheral blood

The paracrine aspect of communication between MNCs and PB-ECFCs was investigated by exposing early passages of PB-ECFCs which were cultured at 20% O₂ to conditioned medium (CM) collected from PB-MNCs cultures that were previously subjected to 1% O₂ and 20% O₂ for 3 (day 0-3) and 7 days (day 5-7 CM). Subsequently, the number of colonies was evaluated as a readout of the clonal ability of ECFCs in presence of conditioned medium. Conditioned medium collected from PB-MNCs exposed to hypoxia, both after 3 and 7 days did not alter the total number of counted ECFC colonies compared to their normoxic counterparts (Fig. 4A). Colony formation was reduced only in the cell cultures incubated with CM20%D[5-7] in comparison to control, non-treated cells or to the cells exposed to CM20%D[0-3], but not in comparison with its hypoxic counterpart with CM1%D5-7).

In contrast to PB-ECFCs, subcultured CB-ECFCs treated with conditioned medium collected from CB-MNCs after 3 days exposure to hypoxia (CM1%D[0-3]) and normoxia (CM20%D[0-3]) exhibited reduced clonal ability (Fig. 4B). Incubation of CB-ECFCs with CM collected from CB-MNCs after 7 days exposure to hypoxia (CM1%D[5-7]) and normoxia (CM20%D[5-7]) did not affect the clonal capacity of the cells. These data implicate that it is unlikely that PB-MNCs produce growth inhibitory factors that might be accountable for the overall hypoxia-induced inhibition of ECFC colony formation. On the other hand, the results suggest that the CB-MNCs initially secrete trophic factors that transiently induce clonal impairment of CB-ECFCs irrespectively of oxygen concentration.

To investigate whether cell-cell interaction between MNCs and PB-ECFCs affects the clonal ability of the former, early passages of PB-ECFCs were co-cultured with freshly obtained MNCs at 20% and 1% O₂ for 10 days. At the end of the incubation period the number of counted colonies revealed that the addition of MNCs to the cell cultures of PB-ECFCs significantly increased the clonal formation irrespectively of oxygen concentration (Fig. 4C). Interestingly, PB-ECFCs co-cultured with PB-MNCs under hypoxic condition responded in similar manner as the control cultures at 20% O₂ which were devoid of MNCs. These data indicate that MNCs placed in close vicinity of PB-ECFCs rescues hypoxia-induced reduction of clonal ability of ECFCs obtained from peripheral blood.
Figure 4. Effect of paracrine and cell-cell regulation of clonogenicity of ECFCs
Clonal formation ability of sub-cultured PB-ECFCs (A) or CB-ECFCs (B) in presence of conditioned medium (CM) collected from PB-MNC cultures after 3 and 7 days inoculation with endothelial differentiation medium. CM[D0-3] stands for conditioned medium collected after 3 days whilst CM[D5-7] is conditioned medium collected after 7 days. Multiple comparison between experimental groups was performed by one-way ANOVA with Bonferroni’s test (p<0.05). *: significance to 20% O2 CM[D0-3]; #: significance to 1% O2 CM[D0-3]

C: Evaluation of clonal formation ability of early passages of PB-ECFCs in presence of MNCs under 20% and 1% O2 (C). Open bars represent PB-ECFCs cultures without PB-MNCs whilst closed bars depict cell cultures with PB-MNCs. Multiple comparison between experimental groups was performed by one-way ANOVA with Bonferroni’s test (p<0.05). #: significance to 20% O2; *: significance to 20% O2 MNC; +: significance to 1% O2 MNC.
4. DISCUSSION

Data presented in this study indicate that hypoxia impairs the initial outgrowth of ECFCs from cord and peripheral blood. Low oxygen tension also reduces clonal ability of subcultured PB- but not of CB-derived ECFCs. Soluble factors present in conditioned medium collected from MNCs altered the clonogenicity of CB-ECFCs but not in case of PB-ECFCs. On the other hand, PB-MNCs placed in close vicinity of ECFCs rescued the hypoxia-induced reduction of clonal capacity of PB-ECFCs.

The MNC fraction of cord or peripheral blood contains circulating progenitor cells with ability to differentiate toward EC lineage. Cultivation of freshly isolated CB- or PB-derived MNCs in endothelial differentiation condition under standard cell culture oxygen tension of 20% O2 yields ECFCs. Our findings suggest that upon exposure to low oxygen tension this MNC fraction exhibited reduced clonal outgrowth of ECFCs irrespectively of blood source indicating less extensive EC differentiation from circulating precursors. However, the difference in the ability of CB-MNCs and PB-MNCs to generate primary clones under hypoxia suggests that beside oxygen tension other factors play an important role in outgrowth of ECFCs from MNCs. The microenvironment which imprint stem and progenitor cells during the fetal and post-natal life can be accountable for observed difference between CB-MNCs and PB-MNCs in their ability to generate ECFCs colonies. Once isolated, ECFCs obtained from umbilical cord blood differ with respect to proliferation, in vivo vessel formation, telomere length, sensitivity to ROS, and response to hypoxia from PB-ECFCs.

Notwithstanding, the crucial question raised by our findings is ‘Why is there poor outgrowth of circulating ECFCs in hypoxia, a property that would be essential for vessel repair or new vessel forming abilities in hypoxic conditions?’ Three possibilities might explain the outgrowth arrest of ECFC colonies in hypoxia: (1) differentiation arrest of EPCs toward mature endothelial phenotype; (2) direct retardation of ECFC proliferation by oxygen deprivation; and (3) suppressive effect of cell-cell interaction between EPCs and accessory MNCs.

Observed inhibition of ECFC outgrowth might be a hypoxia-induced differentiation arrest. Exposure of CB-MNC to 20% O2 for at least 48h support this hypothesis. Several lines of research pointed to an important effect of oxygen tension on cell differentiation. Hypoxia reversibly arrested stem cells in an undifferentiated state while low oxygen tensions have also been used to maintain the pluripotency of different progenitors. Therefore, it is not excluded that hypoxia by maintaining a quiescence phenotype of putative EPCs suppresses their endothelial differentiation toward ECFCs especially...
if the microenvironment is devoid of pro-neovascularization clues. It is plausible that 
in ischemic tissues - once the pro-angiogenic environment is established - growth 
factors that are essential for EC differentiation such as VEGF, FGF or HGF in conjunction 
with pro-angiogenic myeloid cells such as circulating angiogenic cells (CACS) would 
eventually overcome the hypoxia-induced inhibition of differentiation of EPCs toward 
EC phenotype.

Hypoxia not only inhibited the initial outgrowth of ECFCs from CB- and PB-MNCs 
but also has an impact to the clonal and proliferative ability of subcultured cells. Interestingly, the CB-ECFCs displayed reduced proliferation at 1% O₂ which is in 
agreement with previously reports whilst the clonal assay performed with PB-ECFCs 
revealed that oxygen tension modulates the proliferation and growth of these cells. HIF- 
1α activation under hypoxic conditions with subsequent cell cycle arrest in G1/S phase 
and inducement of apoptosis has been reported as a mechanism that restricts the growth of EC obtained from post-natal tissues. Whatever the transcriptomic background is of 
observed hypoxia-induced cell proliferation in CB- and PB-ECFCs, future investigation is 
warranted.

During neovascularization, ECFC proliferation matches the growth of the neovascular 
tree. Our finding that hypoxia reduces ECFC proliferation is contra-intuitive suggesting 
that other factors that play a crucial role during in vivo neovascularization and are 
accountable for proper in vivo multiplication of EC are absent in our in vitro hypoxic 
assay. Indeed, rapid neovascularization reported in animal studies of addition of VEGF to 
ECFCs as well as co-implantation of ECFCs with myeloid cells or MSCs pinpoint to 
the necessity to include these clues during in vitro assaying in order to unravel the true 
behavior of ECFCs under hypoxia.

When we tested whether direct contact with MNCs under hypoxia influences clonal 
formation and cell growth of ECFCs we observed that the CM didn't induce proliferation 
of ECFCs under hypoxic conditions that matches the cell division rate at 20% O₂. 
Interestingly, when cultured in presence of PB-MNCs, the ECFCs exhibited a similar 
increase in growth rate irrespectively of oxygen concentration. This finding suggests 
that cell-cell interaction with MNCs plays an important role in ECFCs proliferation 
and that this effect is not oxygen dependent. The MNCs fraction of blood contains 
cell populations whose role during in vivo and in vitro models of neovascularization 
is well documented. CD34+ MNCs improved in vitro tube-formation of hMVEC while 
CD11b+ myeloid cells play active role during steps that precede formation of functional 
anastomoses and perfused vessels. Importantly, depletion of CD11b+ myeloid cells 
significant reduced neovascularization of the implants indicating that these cells are
Hypoxia decreases outgrowth of endothelial colony-forming cells from cord and peripheral blood

important for ECFCs proliferation. Which cell fraction of MNCs is mainly responsible for observed increase of clonal and proliferative ability of ECFCs under our experimental conditions remains unknow.

Cord-blood or peripheral-derived ECFCs are considered to be a main endothelial cell type for cell-based or tissue engineering therapies. These therapeutically approaches aim to induce neovascularization and tissue regeneration in conditions where ischemia is one of the major pathological events. A delicate network of signaling events induced by lack of oxygen regulates the process of neovascularization. Outlining of the mechanisms that govern EC differentiation of CD34+ MNCs, cellular response to hypoxia as well as intricate cell-cell communication in ischemic tissue will facilitate the implementation of ECFCs-based therapeutically strategies in clinical practice.
REFERENCES


Supplemental Figure 1. Clonal outgrowth of ECFCs from peripheral blood-derived MNCs at 20% and 1%O₂
Phase-contrast images of ECFC colony outgrowth under 20%O₂ (A,B) and 1%O₂ (C,D).
Hypoxia decreases outgrowth of endothelial colony-forming cells from cord and peripheral blood.

**Supplemental Table 1**

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**Supplemental Table 2**

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Supplemental Tables 1 and 2.

Clonal outgrowth of ECFCs from cord- and peripheral blood-derived MNCs at 20% and 1%O₂.
CD34 EXPRESSION MODULATES TUBE FORMING CAPACITY AND BARRIER PROPERTIES OF PERIPHERAL BLOOD-DERIVED ENDOTHELIAL COLONY FORMING CELLS (ECFCS)

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Victor W.M. van Hinsbergh

Endothelial colony forming cells (ECFC) are grown from circulating CD34+ progenitors present in adult peripheral blood, but during in vitro expansion part of the cells lose CD34. To evaluate whether the regulation of CD34 characterizes the angiogenic phenotypical features of PB-ECFCs we investigated the properties of CD34+ and CD34- ECFCs with respect to their ability to form capillary-like tubes in 3D fibrin matrices, tip cell genes expression and barrier integrity.

Selection of CD34+ and CD34- ECFCs from subcultured ECFCs was accomplished by magnetic sorting (FACS:CD34+:95% pos; CD34-:99% neg). Both fractions proliferated at same rate while CD34+ ECFCs exhibited higher tube-forming capacity and tip cell gene expression than CD34- cells. However, during cell culture CD34- cells re-expressed CD34.

Cell seeding density, cell-cell contact formation and serum supplements modulated CD34 expression. CD34 expression in ECFCs was strongly suppressed by newborn calf serum. Stimulation with FGF-2, VEGF, or HGF prepared in medium supplemented with 3% albumin did not change CD34 mRNA or surface expression. Silencing of CD34 with siRNA resulted in strengthening of cell-cell contacts and increased barrier function of ECFC monolayers as measured by ECIS. Furthermore, CD34 siRNA reduced tube formation by ECFC, but did not affect tip cell gene expression.

These findings demonstrate that CD34+ and CD34- cells are different phenotypes of similar cells and that CD34 (1) can be regulated in ECFC; (2) is positively involved in capillary-like sprout formation; (3) is associated but not causally related to tip cell gene expression; and (4) can affect endothelial barrier function.
1. INTRODUCTION

Adequate regeneration of the tissue affected by ischemic insult relies on efficient angiogenesis that can be facilitated by tissue engineering or cell-based therapeutics involving endothelial cells (ECs). Endothelial cells from different vascular beds differ in their angiogenic ability to induce neovascularization in vivo[1]. Therefore, selection of an adequate EC type is a pre-requisite step for successful construction of tissue-engineered vascular constructs.

Endothelial-colony forming cells (ECFCs) are considered as the most suitable ECs type for regenerative angiogenesis[2]. These cells, also named as late outgrowth endothelial progenitor cells (EPCs) or blood-outgrowth endothelial cells (BOECs) differ from the early outgrowth CD14+ /CD45+ EPC which originate from the myeloid-monocytic lineage and participate in vascular regeneration in a paracrine fashion[3]. The ECFCs, which can be obtained from cord or peripheral blood, exhibit pronounced vascularization ability in vivo by physically incorporating into newly formed blood vessels[4]. Especially their neovascularization potency is accentuated in combination with stem/progenitors cells like mesenchymal stem cells or adipose-derived stem cells[5]. Previous studies revealed that ECFCs originate from the blood-derived mononuclear progenitor cell fraction expressing CD34 among other stem/progenitor cell surface markers such as CD31 or CD146[6-7] and are negative for CD14 or CD45.

CD34 is a widely used cell surface marker for detection and isolation of stem and progenitor cells with robust regenerative potential for therapeutical purposes[8]. CD34 is a 115-kDa transmembrane glycoprotein rich with O- and N-glycans. Depending on the cell type, specific modifications by sulfation or sialylation[9-10] modulate the function of the CD34 structure in such manner that different ligands in different environments can interact with this surface protein[11]. Although the CD34 function remains elusive, its expression is modulated by growth factors TGF-β1 and TNF-α [12,13], by oxygen concentration[14], as well as by several transcription factors [15,16]. Beside on HSCs[17], it is expressed on small vessel endothelial cells[18], mast cells[19], epidermal stem cells[20] as well as tumor cells[21]. In endothelial cells CD34 has been used to identify sprouting endothelial tip-cells during neovascularization in vitro[22] and in vivo[21-23]. Previous publications on other cells reported that CD34 modulates cyto-adhesion[24,25,26], cell shape[27] or is critically involved in L-selectin-mediated transendothelial migration of T-lymphocytes in high venular endothelia [28,29]. However, the function of CD34 remains still obscure.
When ECFCs are propagated in culture, they retain stable expression of endothelial specific markers on their surface, but CD34 declines during serial propagation[30]. Monolayers of peripheral blood derived ECFCs (PB-ECFCs) comprise mixture of cells that do or don’t express CD34 on cell surface implying that phenotypical differences might exist between CD34+ and CD34- cells. Previous reports that ECFCs which originate from CD34+ colonies exhibit reduced vascularization properties than the cells isolated from CD34- ECFCs colonies[31] further underpins the necessity to investigate the angiogenic features of CD34+ and CD34- ECFC. It might be that the CD34 expression in ECFCs marks a specific state of an endothelial phenotype that might has different angiogenic properties.

Therefore, we investigated whether the regulation of CD34 characterizes the angiogenic phenotypical features of PB-ECFCs. Findings presented in this study revealed that the CD34 expression is inducible and regulated in PB-ECFCs by the state of confluency and serum factors in the growth medium. By separation of CD34+ and CD34- cells and silencing of CD34 with siRNA we subsequently investigated the contribution of CD34 during the formation of endothelial tubules in a 3D fibrin matrix, the expression of tip cell associated genes, and the endothelial barrier function of ECFCs’ monolayers.

2. MATERIALS AND METHODS

2.1 Cell culture
Peripheral blood-derived ECFCs were isolated, expanded and the endothelial phenotype confirmed as previously described[30]. Briefly, the mononuclear cells (MNC) obtained from adult peripheral blood were plated in EGM-2 medium (Lonza, Walkersville, MD) supplemented with 10% platelet lysate (PL-EGM) and after 10 days the first ECFC colonies emerged. The isolated cells were immunophenotypically characterized by flow cytometry (FC) and were positive for CD31, vWF, CD34, CD144, CD309, and negative for CD14, CD45, and CD133[30]. For experimental purposes the cells were also expanded for 1-2 passages in complete medium (CMi) composed of M199 (Lonza, Verviers, Belgium) supplemented, 10% new-born calf serum (NBCS), 10% human serum (HS), 20µg/mL endothelial cell-growth factor, 2mM L-glutamine, penicillin/streptomycin (100U/ml/100µg/ml), and 5U/mL heparin.

2.2 Magnetic separation and tube-formation assay
Separation of PB-ECFCs on CD34+ and CD34- cells was performed using CD34 MicroBead Kit (clone QBEND/10, #130-046-703, Miltenyi Biotech BV, the Netherlands) according manufacturers’ protocol.
CD34 expression modulates tube forming capacity and barrier properties of peripheral blood-derived ECFCs

To increase the purity of yielded CD34 positive and negative fractions, the cells were twice passed through LS columns and the efficiency of separation was evaluated by flow cytometry (FC) using PE or FITC labeled mouse anti-human CD34 antibody which recognizes different epitope than QBend-10 (clone 581, #555822, BD Biosciences, the Netherlands). Data were analyzed using the FCS Express 4 software package (DeNovo Software, Toronto, Ontario, Canada). The efficiency of separation was also evaluated by determination of the mRNA levels of CD34 in positive and negative fractions by qRT-PCR. After separation the CD34+ and CD34- cells were used for experimental purposes. For assessment of sprouting ability of CD34+ and CD34- PB-ECFCs, 20,000 cells positive or negative for CD34 antigen were seeded on 3D human fibrin matrices prepared as previously described[32]. Following overnight incubation in M199 (Lonza, Verviers, Belgium) supplemented with 10% inactivated human serum and 10% newborn calf serum, tube formation was induced by stimulating the cells with combination of 25ng/mL vascular endothelial factor-A (VEGF-A) + 10ng/mL fibroblast growth factor (FGF-2). All growth factors were purchased from ReliaTech GmbH, Wolfenbuttel, Germany. After 48h stimulation, the cells were fixed with 2% paraformaldehyde/HBSS and quantification of the length of formed tube-like structures was performed using Optimas image analysis software as previously described[32]. The tube formation ability of PB-ECFCs of 3 different donors was each determined in triplicate wells.

2.3 Proliferation ability of CD34+ and CD34- cells

For short-term proliferation assays 500 CD34+ or CD34- cells/cm² were seeded onto 12-well plate precoated with rat-tail collagen type I (BD Biosciences, Erebodegem, Belgium) in PL-EGM. Renewal of culture medium was performed every other day. After 7 days in culture, the cells were washed with PBS and fixed with pre-warmed 2% paraformaldehyde/HBSS. DAPI Vectashield HardSet (VectorLabLtd, BrunschwigChemie, The Netherlands) was used to visualize the cell nuclei and 5 pictures from each well were taken using phase contrast microscopy. The number of cells was determined using ImageJ software and the calculations for population doubling was performed as previously described[33].

2.4 Re-expression of CD34 during cell culture

To investigate if CD34 is re-expressed in CD34- PB-ECFCs that were obtained after the magnetic separation from the cell cultures of 4 different donors, the CD34- cells were seeded in CMi medium (CD34-CMi cells) or in PL-EGM medium (CD34-PL cells) and left to reach confluence (Supplemental Figure 1).
Part of CD34- PB-ECFCs were also incubated in M199 supplemented with 3% pyrogen-free human serum albumin (3% HSA, Sanquin, Amsterdam, The Netherlands) for 24h. The CD34 expression was evaluated by FC. After 3-4 days in cell culture, CD34 CMi cells and CD34 PL cells were harvested for experimental purposes. The CD34 expression in CD34 CMi cells and CD34 PL cells was evaluated by qRT-PCR and FC. To investigate the expression of tip-cell associated genes in CD34 PL cells that re-express CD34, the CD34-PL ECFCs were separated on CD34+ and CD34- cells by magnetic separation as previously described and qRT-PCR was performed.

2.5 Effect of serum supplements and growth factors on CD34 expression in PB-ECFCs.

For assessment of the effect of NBCS on CD34 expression in PB-ECFCs over period of 24h, the cells were incubated with 10%NBCS, 10%PL, 10%NBCS+10%PL, and 3%HSA all prepared in M199. The cells were harvested after 9h, 12, and 24h incubation period and the CD34 mRNA levels were evaluated by qRT-PCR.

To investigate the expression of CD34 in cell cultures of PB-ECFCs in CMi and PL-EGM, the ECFCs obtained from 4 different donors were expanded for two passages in CMi or PL-EGM medium and the CD34 surface expression was evaluated by FC.

The effect of pro-angiogenic growth factors and 10%PL on CD34 expression in PB-ECFCs was investigated after 24h incubation of the cells with 10ng/mL fibroblast growth factor-2 (FGF-2, 25ng/mL), vascular endothelial factor-A (VEGF-A, 10ng/mL), hepatocyte growth factor (HGF,10ng/mL) and 10%PL all prepared in M199 supplemented with 3%HSA. In parallel the cells were also incubated with 10ng/mL FGF-2, 25ng/mL VEGF-A, 10ng/mL HGF, and 10ng/mL TNF-α all prepared in EBM-2 medium supplemented with 5%PL for 24h. Cells incubated only in M199+3%HSA or EBM-2+5%PL served as control. The mRNA and surface expression of CD34 was measured by qRT-PCR and FC.

2.6 Effect of cell seeding density and proliferation on CD34 expression in PB-ECFCs.

To investigate whether cell density regulates the CD34 expression during passage of ECFCs, the cells from 4 individual donors were seeded on rat-tail collagen type I pre-coated 6-well plates in PL-EGM at densities of 100, 500, 1000, and 2000 cells/cm². The gene and surface expression of CD34 was evaluated by qRT-PCR and FC after 5 and 6 days proliferation.
Proliferation assay was performed by seeding 100, 500, 1000, and 2000 cells/cm$^2$ in 8 wells per seeding condition using 24-well plate. To enumerate the number of cells in cell cultures after 5 and 6 days proliferation, the cells were fixed and stained with DAPI Vectashield HardSet (VectorLab Ltd, Brunschwig Chemie, The Netherlands). Image acquisition was performed using 4D-digital imaging microscope (DIM) by taking 5 images per well and the cell number was calculated using ImageJ software.

**2.7 siRNA transfection and tube-formation assay**

To investigate the involvement of CD34 during sprout formation in fibrin matrices by PB-ECFCs, the cells were expanded for one passage in CMi to reduce the expression of CD34. After reaching confluence, the cells were expanded at 1:2 ratio and were incubated with ON-TARGET plus human CD34(947) siRNA-SMART pool (#L-019503-00-0005, Thermoscientific, the Netherlands) for silencing CD34 (siRNA CD34 cells) or were incubated with ON-TARGETplus Non-targeting Pool siRNA (cat.no. D-001810-10-05, GE Dharmacon, Lafayette, CO) (siRNA NT cells). Non-transfected cells were used as control. Prior transfection experiments cells were starved for 4h in M199 and were transfected using DharmaFECT4 reagent (Dharmacon). All siRNA and DharmaFECT4 were prepared in M199 + 10% inactivated human serum supplemented with 10ng/mL FGF-2 at final concentration of 20nM. After 24h of the transfection period the transfection medium was replaced by fresh PL-EGM medium. After additional 24h the cells were detached and seeded on fibrin matrices as previously described. The transfection efficiency was evaluated by qRT-PCR and FC prior to seeding the cells on fibrin matrices. Sprout formation was initiated by stimulating the cells with combination of 10ng/mL TNF-α and 10ng/mL FGF-2 every day during 2-days period. Inhibition of tube formation was accomplished by transfecting the PB-ECFCs with siRNAs against uPA (Hs_PLAU_6 FlexiTube siRNA, cat.no. SI02662135, Qiagen Benelux B.V., the Netherlands) that was prepared using the above described procedure. The tube formation ability of PB-ECFCs of 4 donors was determined in quadruplicate wells. Quantification of the length of formed tube-like structures was performed as previously described.

**2.8 siRNA transfection and Endothelial Barrier Function Assays**

To investigate the involvement of CD34 in the maintenance of barrier function the PB-ECFCs from 3 different donors were transfected with siRNA against CD34 or non-targeting siRNA as described in the previous section. Endothelial barrier function was evaluated with electric cell-substrate impedance sensing (ECIS) as previously reported[34]. Briefly, the siRNA CD34 and siRNA NT cells were seeded in 1:1 density on gelatin-coated ECIS arrays, each containing 96 wells with 10 gold electrodes per well (96W10idf plates, Applied Biophysics, Troy, NY) in M199+10%HSi+10ng/mL FGF-2.
Resistance was measured at multiple frequencies to allow for calculation of resistance attributable to cell–cell adhesion (Rb) and to cell–matrix interaction (Alpha) over period of 24h[34].

2.9 Real-Time Polymerase Chain Reaction

The total RNA from PB-ECFCs was isolated using RNeasyMinElute Cleanup Kit (Qiagen, The Netherlands) and the RNA quality was tested with a Nanodrop 1000 spectrophotometer. Copy DNA (cDNA) was synthesized using the Cloned AMV First Strand cDNA Synthesis Kit from Invitrogen. The sequences of primers used for determination of genes of interest are given in Supplemental Table1.

Quantitative RT-PCR was performed using SYBR Green in an ABI 7500 sequence detection system (Applied Biosystems, Foster City, USA) and the following protocol: 2 min 50°C, 10 min 95°C and 40 cycles (15 sec 95°C, 1 min 60°C) and dissociation curve. The relative expression levels of target genes were calculated with the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by following equation as previous described[35]: Δ Ct sample = (Ct sample GENE) – (Ct sample HKG). The relative gene expression = 2 (Δ Ct sample1 –Δ Ct Sample).

3. RESULTS

3.1 Selection of CD34+ and CD34- cells from PB-ECFCs.

While PB-ECFC colonies arise from CD34+ cells[6], during subculturing both CD34+ and CD34- ECFCs are encountered. To investigate whether CD34+ subcultured ECFCs are a better cell source for tissue engineering than their CD34- counterparts, we compared the proliferation capacity of CD34+ with that of CD34- cells as well as their sprouting ability in a 3D-fibrin matrix. Confluent cultures of PB-ECFCs from four donors were separated into CD34+ and CD34- cells using CD34 magnetic beads. After each separation, the purity of yielded cell fraction was evaluated by flow cytometry (FC). Two sequential separations steps yielded highly purified CD34+ (95%±0.8) and CD34- (99%±0.8) cell fractions (Supplemental Fig.2).

During the course of seven days both cell fractions proliferated at same rate indicating that CD34+ ECFCs did not differ to CD34- ECFCs with respect to the proliferation ability (Fig.1A).
CD34 expression modulates tube forming capacity and barrier properties of peripheral blood-derived ECFCs

Figure 1. Angiogenic capacity of CD34+ PB-ECFCs
A: Proliferation capacity of CD34- (open bar) and CD34+ (closed bar) PB-ECFCs expressed as a mean ± SEM of population doubling.
B-C: Representative phase contrast images of sprout formation in fibrin matrices by CD34- (B) and CD34+ (C) ECFCs stimulated with the combination of VEGF-A and FGF-2.
D: Quantification of sprouting ability of CD34- (open bar) and CD34+ (closed bar) PB-ECFCs in fibrin matrices. Data are expressed as a mean ± SEM of mean length of formed sprouts of 4 independent experiments each performed with different donor.
E: mRNA levels of genes associated with tip-cell phenotype in CD34+ PB-ECFC compared to CD34- cells. Open bar depict mRNA levels of tip-cell associated genes in CD34- cells. Closed bars represent mRNA levels of tip-cell associated genes in CD34+ cells.

Stimulation with a combination of VEGF-A and FGF-2 induced sprout formation in fibrin matrices by both cell fractions (Figure 1B,C). Comparison of sprouting ability of CD34+ and CD34- cells revealed CD34+ ECFCs formed more sprouting structures than CD34- ones (Fig.1D). Previously we demonstrated that sprouting by ECFCs required u-PA and uPAR and is modulated by PAI-1 [30]. There was no significant differences observed between CD34+ and CD34- ECFCs in the expressions of u-PA (1.1 ± 0.7), uPAR (1.1 ± 0.3) and PAI-1 (1.5 ± 0.7) (mean±SEM, 4 different donors). Further phenotypical characterization of CD34+ ECFCs unveiled that CD34+ cells displayed an enrichment of
genes usually accepted as markers of tip-cell phenotype (Fig.1E). These data suggest that selection of CD34+ ECFCs might be feasible approach to facilitate the initiation of regenerative angiogenesis in ischemic tissues.

3.2 CD34+ and CD34- ECFCs are interchangeable phenotypes.

As both CD34+ and CD34- ECFCs originate from mononuclear cell fraction positive for CD34[6], we wondered whether CD34- ECFCs represent a separate lineage of cells or merely reflect a phenotype, which can re-acquire CD34 antigen. Therefore we subsequently investigated if the cells in culture that are negative for CD34 can re-express this protein on mRNA and protein level. Using magnetic beads the PB-ECFCs from four donors were separated on CD34+ and CD34- ECFCs and the efficiency of separation was evaluated by flow cytometry (Supplemental Fig.2).

Both CD34+ and CD34- fractions had comparable expressions of VE-cadherin, CD31 and VEGFR2 measured by FC, which were the same as in ECFCs before separation, which confirms the endothelial nature of both fractions (not shown). After separation, the CD34- ECFCs were seeded and cultured in PL-EGM (CD34 PL) or CMi (CD34 CMi) and once the cells reached confluence (3-4 days), the number of CD34+ cells was quantified by FC analysis, while in parallel the mRNA levels of CD34 were evaluated by qRT-PCR. The CD34 PL ECFCs re-expressed significantly more CD34 than the control cells both at gene and protein level (Figure 2A,B).

Incubation of CD34- cells in M199 medium supplemented with 3% human serum albumin (HSA) for 24h also induced upregulation of CD34 on the cell surface (Supplemental Fig.3). This indicates that the expression of CD34 was reversible. To investigate if CD34- ECFCs in the CD34- fraction that re-expressed CD34 on cell surface are also characterized by the enrichment of tip-cell-associated genes (VEGFR2, DLL4, CXCR4, EFNB2, IGFBP3), we subsequently separated CD34 PL that re-express CD34 into positive and negative for CD34 ECFCs and performed qRT-PCR. Indeed, CD34+ ECFCs obtained from CD34 PL cells that re-expressed CD34 on cell surface also expressed significantly more mRNA transcripts of the genes related to tip-cell phenotype (Fig.2C). This finding is in line with the data obtained from freshly separated cells (Figure 1F).

Unexpectedly, the cells cultured in CMi exhibited only slight increases of CD34 mRNA and surface antigen levels compared to the initially seeded CD34- cells (Fig.2A,B). This suggests that serum components of the CMi do not favor the re-expression of CD34 on CD34- ECFCs, indicating that the soluble factors present in serum play a role in regulation of CD34 in PB-ECFCs.
CD34 expression modulates tube forming capacity and barrier properties of peripheral blood-derived ECFCs

Figure 2. Expression of CD34 in CD34+ and CD34- PB-ECFCs fractions after magnetic separation.

A: CD34 mRNA levels in CD34- ECFCs after cell culture in PL-EGM or CMi. Open bar depicts the CD34 mRNA levels in CD34- ECFCs obtained after separation and prior the seeding in PL-EGM or CMi. Closed bars represent CD34 mRNA levels in CD34-CMi and CD34-PL cells after 6 days in cell culture.

B: Flow cytometry evaluation of the number of CD34+ cells in CD34- ECFCs cultures expanded in CMi or PL-EGM. Data are expressed as mean ± SEM percentage of cells positive for CD34 in the cell cultures expanded either in CMi or PL-EGM (closed bars). The control CD34- cells are depicted with open bar.

C: mRNA levels of genes associated with tip-cell phenotype in CD34+ and CD34- PB-ECFCs obtained after magnetic separation of CD34-PL. Open bar depict mRNA levels of tip-cell associated genes in CD34- cells while closed bars represent mRNA levels of same genes in CD34+ cells.

3.3 Serum supplements affect CD34 expression in confluent ECFCs.

We subsequently evaluated which serum supplement(s) might modulate CD34 expression in PB-ECFCs. To that end we compared platelet lysate and serum used in CMi, in particular 10% NBCS and 10% HS. Confluent monolayers of PB-ECFCs grown in CMi were transferred into M199 medium supplemented with 10% NBCS, 10% PL,
10% NBCS/10%PL or 3% HSA and incubated for 9, 12 and 24h. In the presence of only 3% HSA, CD34 mRNA expression increased 5-fold within 9 h and remained constant thereafter (Fig. 3A).

Figure 3. Effect of serum supplements on CD34 expression in PB-ECFCs
A: Time-course of CD34 induction in PB-ECFCs upon incubation with media supplemented with different serum supplements during 24h period.
○ depicts 10% NBCS, □ depicts 10%NBCS+10%PL, Δ depicts 10%PL, ◊ depicts 3%HSA. Data are expressed as mean ± SEM of n-fold difference of CD34 mRNA levels compared to the mRNA levels in the cells incubated with M199+3%HSA at each time point (HSA, p<0.05: # to NBCS, * to NBCS+PL, & to PL; PL, p<0.05: $ to NBCS, ∞ to NBCS+PL).
B: Flow cytometry assessment of CD34 expression in PB-ECFCs cultured in the presence of NBCS or PL.
Data are expressed as a mean ± SEM percentage of cells positive for CD34 in the cell cultures incubated with CMi medium which contains 10%NBCS (open bar) or M199+10%PL (closed bar).
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C: qRT-PCR evaluation of CD34 expression in cells incubated with VEGF-A (25ng/mL), FGF-2 (10ng/mL), FV (10ng/mL, FGF-2+25ng/mL VEGF-A), HGF (10ng/mL), and 10%PL for 24 hours prepared in M199+3%HSA. Data are expressed as mean ± SEM of n-fold difference of CD34 gene levels in stimulated cells (closed bars) normalized to control cells incubated only in M199+3%HSA (open bar).

D: qRT-PCR evaluation of CD34 expression in cells incubated with VEGF-A (25ng/mL), FGF-2 (10ng/mL), HGF (10ng/mL), and TNF-α (10ng/mL) for 24 hours prepared in EBM-2+5%PL. Data are expressed as mean ± SEM of n-fold difference of CD34 gene levels in stimulated cells (closed bars) normalized to control cells incubated only in EBM-2+5%PL (open bar).

E: Flow cytometry evaluation of the effect of VEGF-A (25ng/mL), FGF-2 (10ng/mL), HGF (10ng/mL), and TNF-α (10ng/mL) for 24 hours prepared in EBM-2+5%PL on CD34 expression in PB-ECFCs. Closed bars represent the effect of each of soluble factors prepared in control medium (open bar) on CD34 expression. Data are expressed as mean ± SEM of mean fluorescence intensity (MFI) of CD34 antibody fluorescence intensity minus autofluorescence of matched isotype antibody.

In the presence of PL a slow increase was observed reaching 2- to 6-fold after 9 and 24 h, respectively. However, in the presence of NBCS – a serum component of CMi -, the CD34 mRNA expression remained at the original low level. Also when NBCS and PL were combined the CD34 mRNA expression remained low (Fig.3A). Apparently NBCS did not stimulate CD34 expression, and had a dominant suppressive effect over PL as well. The difference between exposure to medium containing NBCS such as CMi and that to PL was also reflected in the cell surface expression of CD34 antigen (Fig.3B).

To evaluate whether pro-angiogenic growth factors and cytokines might affect the CD34 expression in confluent PB-ECFCs, we compared their effects both in PB-ECFCs that were incubated in M-199 supplemented with 3% HSA as well as EBM-2+5% PL. The growth factors FGF-2, VEGF-A and HGF did not change CD34 mRNA or surface antigen expression significantly in both conditions (Fig.2C-E). Addition of 5-10% PL to HSA-containing medium also did not alter CD34 mRNA expression. The inflammatory cytokine TNF-α reduced the mRNA and antigen expression significantly (Fig.2D,E), in agreement with previous finding on endothelial cells [13].

3.4 Proliferation and seeding density alter CD34 expression in ECFCs.

To investigate whether cell density regulates CD34 expression during passage of ECFCs, the cells from four individual donors were seeded on rat-tail collagen type I in PL-EGM at densities of 100, 500, 1000, and 2000 cells/cm². The CD34 expression was evaluated after five (D5) and six days (D6) of culturing using PCR and FC. During the course of proliferation assay, the conditions with 100, 500, and 1000 cells/cm² contained...
significantly more cells at D6 compared to D5 indicating that the cells continued to proliferate in 24h period between the two time points reaching different levels of confluence (Fig.4A, Supplemental Fig.4A-F).

Figure 4. Effect of cell density and proliferation on CD34 expression in PB-ECFCs
A: Proliferation ability of PB-ECFCs seeded at different cell densities after five (D5, open bars) and six (D6, closed bars) days. Results represent mean ± SEM of counted cells/cm² from 4 independent experiments each performed with different donor.
B: Relation of CD34 surface expression and proliferation in PB-ECFCs seeded at different cells densities. Flow cytometry evaluation of the number of CD34+ cells in cell cultures established with seeding PB-ECFCs at different densities after five (D5, open symbols) and six (D6, closed symbols) days of proliferation. Data represents the percentage of cells positive for CD34 in the cell cultures established by seeding 100 (o), 500 (□), 1000 (Δ), and 2000 (▽) cells/cm² after 5 (open symbols) and 6 (closed symbols) days proliferation plotted against the number of cells counted at the end of proliferation. (*p<0.05). $ indicates p<0.05 to 100 cells/cm² at D5, + indicates p<0.05 to 500 cells/cm² at D5; #, *, & indicate p<0.05 to 100, 500, and 1000 cells/cm² at D6; Φ indicates p<0.05 to 2000 cells/cm² at D5.
C: Relation of CD34 gene expression and proliferation in PB-ECFCs seeded at different cells densities. Comparison of mRNA levels of CD34 at day 5 (D5, open symbols) and day 6 (D6, closed symbols) in cell cultures established by seeding 100 (o), 500 (□), 1000 (Δ), and 2000 (▽) cells/cm² of PB-ECFCs plotted to the number of cells counted at the end of proliferation. Data represent the mean ± SEM of delta Ct values of CD34 mRNA levels at day 6 (D6, closed symbols) compared to delta Ct of CD34 mRNA levels at day 5 (D5, open symbols) which served as control.
CD34 expression modulates tube forming capacity and barrier properties of peripheral blood-derived ECFCs

On the contrary, at 2000 cells/cm² at D5 and D6 similar numbers of cells were counted suggesting that the cell cultures were confluent and the cells had stopped dividing (Fig.4A, Supplemental Fig.4G-H). Before the start of experiment the percentage of CD34⁺ cells in the cultures obtained from different donors was 83.3±11.5% as measured by FC. Strong reduction in the number of CD34⁺ cells was observed in the cultures established with 100, 500, and 1000 cells/cm² at D5 and D6 while a cell density dependent restoration of CD34 content occurred as most clear in 2000 cells/cm² cultures (Fig.4B). These data indicate that proliferation as well as cell density affects the regulation of CD34 in ECFCs probably through establishment of cell-cell contacts. Progressive increase of CD34 mRNA levels between D5 and D6 was also observed in all seeding conditions as measured by qRT-PCR (Fig.4C) suggesting that establishment of cell-cell contact also promotes CD34 transcription irrespectively of proliferation status of cell cultures.

3.5 CD34 alters barrier function of PB-ECFCs.

Previous studies reported that CD34 molecules on two adjacent EC cells play an important role in initiation of lumen formation in mouse embryo by generation of repulsive forces that destabilized cell-cell contacts[36]. Considering that disassembling of the continuity of EC monolayer is one of the first steps in angiogenic sprouting we investigate whether silencing of CD34 changes the barrier properties of ECFC. PB-ECFCs from three donors were transfected with siRNA targeting CD34. Efficient silencing of CD34 at the cell surface and gene level was confirmed by FC and qRT-PCR (Supplemental Fig.5).

The transfection procedure did not alter the gene expression levels of CD31 and VE-cadherin, which play an important role in cell-cell interaction (Fig.5D).

ECIS measurement over period of 24h revealed that silencing of CD34 caused an increase of total resistance of the ECFC monolayer (Fig.5A). Quantification at the end of 24h period confirmed that a significantly higher resistance was observed in the cell monolayers with silenced CD34 than in the controls, non-transfected cells or the cells transfected with non-targeting siRNA (Fig.5B). After computational modeling of the endothelial electrical resistance which enables discrimination between cell-cell and cell-matrix interactions, we detected that the increase of total endothelial resistance upon silencing of CD34 was attributable to increased cell-cell interactions (Fig.5C). The reduction of the strength of cell-cell interaction between PB-ECFCs may be in favor of the suggestion that CD34 might play a role during the initial spouting phase of angiogenesis, which is mainly driven by CD34⁺ endothelial cells.
Figure 5. The role of CD34 during maintenance of barrier function in PB-ECFCs

A: Barrier function of control PB-ECFCs (O), cells transfected with non-targeting siRNA (△), and cells with CD34 silenced by siRNA (□) over period of 24h. Data are expressed as absolute endothelial resistance (Ohm).

B: Absolute endothelial resistance of confluent monolayers of PB-ECFCs of three independent experiments each performed with different donor. Data of control and siRNA CD34 cells (closed bars) are normalized to the values of cells transfected with non-targeting siRNA (open bars) which served as control.

C: Strength of cell-cell interaction in confluent monolayers of control cells, cells transfected with non-targeting siRNA and cells transfected with siRNA CD34. Data of control and siRNA CD34 cells (closed bars) are normalized to the values of cells transfected with non-targeting siRNA (open bars) which served as control.

D: CD31 and VE-cadherin mRNA levels in PB-ECFCs transfected with siRNA CD34. Results represent the mean ± SEM of 4 independent experiments each performed with different donor. Open bar depicts the CD34 mRNA levels in cells transfected with non-targeting siRNA. Closed bars represent the mRNA levels of CD31 and VE-cadherin in the cells transfected with siRNA CD34.
3.6 Silencing of CD34 reduces sprout formation ability of PB-ECFCs.

To further evaluate the contribution of CD34 to sprouting, we assessed the impact of silencing of CD34 expression by siRNA on sprout formation in fibrin matrices. PB-ECFCs transfected with siRNA CD34 (siRNA CD34 cells), or non-targeting siRNA pool (siRNA NT cells) as well as control non-transfected ECFCs were seeded on fibrin matrices and simultaneously stimulated with TNF-α and FGF-2 (TF). siRNA CD34 significantly reduced the mRNA and surface expression of CD34 as validated by qRT-PCR and FC (Supplemental Fig.5).

During the course of 2-days stimulation period, the siRNA-NT cells exhibited similar sprouting response as the control, non-transfected cells indicating that the transfection procedure did not alter the sprouting response of PB-ECFCs (Fig.6A-C) while the siRNA CD34 transfected cells exhibited significantly reduced sprout formation (Fig.6D,E).

Data are expressed as mean ± SEM of n-fold difference of mRNA levels of depicted genes in the siRNA CD34 transfected cells (closed bars) compared to the cells transfected with non-targeting siRNA (open bars) which served as control.

In line with an involvement of the fibrinolytic system in sprouting-associated pericellular proteolysis, the siRNA u-PA-treated cells completely failed to form sprouting structures in fibrin matrices sprout formation (Supplemental Fig.6).

To investigate whether the observed results are related to the difference in expression of genes associated with tip-cell phenotype or involved in pericellular proteolysis accompanying tube formation in fibrin matrices, qRT-PCR was performed to compare the mRNA levels of set of genes in siRNA CD34, siRNA NT and control cells. Silencing of CD34 in ECFCs did not alter the mRNA levels of tip-cell associated genes while uPA and PAI-1 were moderately increased in siRNA CD34 cells compared to siRNA NT cells (Fig.6F). Therefore, the sprouting response of siRNA CD34 cells showed that the CD34 protein might indeed play a role during invasion of fibrin matrices, but its engagement during the sprouting was independent of the induction of tip cell-associated genes in ECFCs.
4. DISCUSSION

Findings in this study demonstrated that compact colonies of ECFCs derived from the mononuclear cell fraction of peripheral blood develop hybrid cellular expression of CD34, i.e. contain CD34+ and CD34- cells, which both express endothelial cell-specific markers. These CD34+ and CD34- ECFCs are not separate cell lineages but merely reflect different but interchangeable phenotypes of endothelial cells. Our data revealed that CD34 is inducible in the CD34- ECFCs and its regulation depends on cell density, and serum supplements. Functional assays and phenotypical characterization of CD34+ and CD34- ECFCs demonstrated that CD34+ cells are characterized by higher sprouting capacity in fibrin matrices and enrichment of tip-cell related genes compared to their CD34- counterparts. Silencing of CD34 by siRNA caused improvement of the barrier
CD34 expression modulates tube forming capacity and barrier properties of peripheral blood-derived ECFCs

function of ECFCs' monolayers and reduced the extent of the tubular structures formation by ECFCs, suggesting a causal role for CD34, but did not affect the expression of tip-cell-associated genes.

4.1 CD34 (re)expression during culture of ECFCs

The re-expression pattern of CD34 in CD34- ECFCs was in agreement with previous reports on the induction of CD34 in human umbilical vein endothelial cells (HUVEC) [22] and hematopoietic stem cells [37]. We observed an increase in CD34 expression in confluent cells, similar as but at a much higher level than previously observed in HUVEC. Furthermore, the present study shows that CD34 was repressed in medium supplemented with NBCS and human serum, and became re-expressed after exposure of ECFCs to medium supplemented with human serum albumin or platelet lysate. Beside by serum, the CD34 expression in ECs might be regulated by pro-angiogenic factors or inflammatory cytokines [34,13]. Although the previous reports suggested that FGF-2 and VEGF-A reduced or increased the CD34 expression in dermal microvascular ECs (DMVEC) or HUVEC, respectively [34,22], both angiogenic factors as well as HGF did not alter the CD34 expression in PB-ECFCs cultured in the presence of either 3%HSA, 5%PL or NBCS. This may be due to the strong suppressive action of NBCS and the much higher CD34 levels that are reached in platelet lysate-exposed ECFCs than in primary cultures of HUVEC and DMVEC. Indeed, according to Barclay et al. [39] ECFCs belong to the endothelial lineage, yet they differ in their response to environmental conditions. Alternatively, the effect of growth factors may influence growth rate in endothelial cells, which it is already very high in ECFCs. However, Hellwig et al. [38] reported that CD34 regulation is independent from the induction of proliferation. Furthermore, the state of confluency affects CD34 expression in ECFCs (this study) as well as in other primary endothelial cells [34,13].

4.2 Increased sprouting of CD34+ ECFCs in fibrin matrices

Functional assessment of sprouting of CD34+ and CD34- separated cells showed that combined use of FGF-2 and VEGF induced more spout formation in the fibrin matrices seeded with CD34+ ECFCs compared to cells that lack this molecule while both cell fractions proliferated at the same rate. Silencing of CD34 in CD34+ ECFCs also reduced formation of endothelial sprouts, suggesting a causal role of CD34 in enhanced tube formation. Increased sprouting was not related to differences in fibrinolytic gene profile of CD34+ and CD34- fractions since both cell subsets have comparable basal mRNA levels of uPA, uPAR, PAI-1, t-PA, and MMP-14. Enrichment of tip-cell related genes in CD34+ ECFCs might underlie increased sprout formation in fibrin matrices due to the increased mRNA levels of VEGFR2 or CXCR4, which are important regulators of the sprouting
phase of angiogenesis[36,37]. In addition, the CD34 re-expression in separated CD34−
cells was accompanied with increased levels of tip-cell related genes suggesting that
CD34 can be used as a marker for selection of ECFCs with distinct phenotype compared
to CD34+ selected cells.

Recently Ferreras et al.[31] reported on two separate types of ECFC colonies, one CD34+
with densely growing cells and a second CD34− type that grows in a more dispersed
way. These authors concluded that only the CD34+ cells were capable of sprouting
when embedded in fibrin or seeded onto Matrigel, while CD34+ were not. In contrast
to Ferreras’s report [31], here presented data show that CD34+ ECFCs are able to form
tubular endothelial structures in a fibrin matrix and to a larger extent than CD34−cells.
Furthermore, our data show that CD34+ and CD34− represent both distinct endothelial but
interchangeable phenotypes rather than separate cell lineages in a mixed population of
ECFCs. Our studies do not exclude the existence of another CD34− population of sparsely
growing late outgrowth cells. We did observe such colonies in our initial cultures, but
these colonies had a poor propagation and could not be cultured further than passage
4 in our experimental conditions.

It is not excluded that in vitro expansion in platelet lysate might have accentuated
the angiogenic phenotype of CD34+ ECFCs. This cell fraction was not only able to
promote sprout formation more than CD34− selected ECFCs, but also displayed a higher
expression of tip-cell-associated genes. However, as silencing of CD34 did not affect the
expression of tip cell-associated genes, another mediator probably controls both CD34
and the other genes. Our observations on tip cell gene expression by CD34+ ECFCs are
in line with observations of Siemerink et al.[22] on CD34+ HUVEC. However, opposite to
the observations of these latter authors both CD34+ and CD34− ECFCs proliferated in our
conditions at similar rates.

4.3 CD34 is involved in regulation of barrier function in PB-ECFCs.

Our observation that CD34 is increased in fully confluent monolayers of PB-ECFCs
and the accumulation of CD34 antigen staining at the cell margins suggests the
involvement of this molecule in modulating cell-cell contacts. Preservation of intact
cell-cell contact is an indispensable component of the barrier function of endothelial
monolayer. However, silencing of CD34 induced increase of the strength of cell-cell
interaction leading to augmentation of the barrier function of monolayers of PB-ECFCs.
The reduction of endothelial barrier function by CD34 may seem counterintuitive as
CD34 is increased in confluent endothelial cells. However, a similar effect was observed
during mouse embryogenesis where the negatively charged CD34 molecules induced
local repulsion of cell contacts between sprouting ECs thus enabling the formation of
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the lumen of future blood vessels[36]. Furthermore, in post-natal life, CD34 is expressed by EC lining the microvasculature in vivo[18]. This antigen, beside on the cell surface, is also distributed within the contact area between adjacent endothelial cells especially on the interdigitating invaginations/processes located close to the luminal side, while the tight junctions appear devoid of CD34[18]. Residing at the entry of tight junctions the CD34 might play a role in the first steps of opening the cell-cell contact area or in preventing closure of junctions once they open. During angiogenesis, it might be helpful for the EC to highly express CD34 on cell surface in order to reduce the stability of the endothelial monolayer thus ensuring favorable conditions for the initiation of sprouting. Data obtained from the sprouting assay with silencing of CD34 by siRNA support this hypothesis.

4.4 CD34+ vs CD34- ECFCs

An interesting observation on CD34 hybrid ECFCs was made by Lee et al.[42], who suggested that the CD34 hybrid colonies contained both CD34+ ECFCs and CD34- ECFCs, the latter acting as a niche supporting cells. The existence of niche supporting cells fits well within current ideas of maintenance of stemness [43]. As we obtained 95% CD34+ cells by cell separation, it is difficult to rule out fully the possibility that potential niche supporting cells escaped the separation. However, we did not observe a difference in the proliferative capacity of CD34+ and CD34- cells, and after a while both populations finally regressed to the same ratio CD34+/CD34- cells, a ratio that was mainly influenced by cell density and culture conditions. The possibility that CD34+ ECFCs can acquire niche supporting properties themselves, or that niche-supporting cells may express temporarily CD34 needs further attention.

5. CONCLUSION

This manuscript demonstrates the plasticity and regulation of CD34 expression in ECFCs cultured from adult human peripheral blood, as well as the stimulatory role of this molecule on endothelial tube formation in a 3D-fibrin matrix. It also pointed to a contribution of CD34 to local barrier destabilization that may accompany sprouting. While deletion of CD34 expression by siRNA demonstrates a causal role of CD34 in improved tube formation – independent of the regulation of several important tip cell genes -, selection of CD34+ cells from CD34+/CD34- hybrid ECFC cultures will only have effect when separation occurs immediately before application of the CD34+ cells. After several days the original hybrid expression pattern of CD34 will be regained by the subcultured PB-ECFCs. Notwithstanding, this mixed phenotype still displays the same sprouting ability and proliferation rate as the purified CD34+ ECFCs.
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REFERENCES


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Supplemental Figure 1. Schematic overview of experimental layout of CD34 separation and re-expression

1) PB-ECFCs cell cultures were separated on CD34⁺ and CD34⁻ cells by MACS.
2) Next, the CD34⁺ cells were further cultured in CMI and PL-EGM until confluence. CD34⁻ cells were also incubated in M199 supplemented with 3%HSA for 24h.
3) Second MACS step was used to obtain CD34⁺ and CD34⁻ cells from CD34⁻ cells that were cultured in PL-EGM. Subsequently, the obtained CD34⁺ and CD34⁻ ECFCs were used for experimental purposes.
Supplemental Figure 2. Expression of CD34 in CD34+ and CD34- PB-ECFCs fractions after magnetic separation.
A-C: Representative flow cytometry histograms of the obtained cell population after CD34 labeled magnetic bead separation. Plots depict control isotype IgG staining (grey histograms) versus CD34 antibody staining (empty histograms). A depicts a representative histogram of CD34+ cells before magnetic separation, B represents the number of cells positive for CD34 in the CD34+ yielded fraction, C depicts the number of cells positive for CD34 in CD34- cells.

Supplemental Figure 3. Flow cytometry histograms of CD34 re-expression in CD34- ECFCs
A-D: Representative histograms of percentage of cells positive for CD34 when CD34- cells (A) were cultured in CMi (B) or PL-EGM (C) medium or incubated in medium with 3%HSA for 24h (D).
Supplemental Figure 4. State of confluence of ECFCs cultures after five and six days proliferation
A-H: Representative phase contrast images of cultures of PB-ECFCs initiated with 100 (A,B), 500 (C,D), 1000 (E,F), and 2000 (G,H) cells/cm² after five (D5) and six (D6) days proliferation.
Supplemental Figure 5. Evaluation of efficiency of silencing CD34 by siRNA
Representative flow cytometry histograms of CD34-FITC positive PB-ECFCs in (A) control, non-transfected cells, (B) in the cells transfected with non-targeting siRNA, and (C) in the cells transfected with siRNA against CD34. Plots depict control isotype IgG staining (grey histograms) versus CD34 antibody staining (empty histograms).

D: qRT-PCR data of efficiency of silencing CD34 with siRNA technology at mRNA level. Data are expressed as a mean ± SEM of n-fold difference of mRNA levels in control and siRNA CD34 transfected cells (closed bars) compared to cell transfected with non-targeting siRNA (open bar).
Supplemental Figure 6. siRNA uPA abolishes sprouting response of PB-ECFCs in fibrin matrices

A: Representative phase contrast images of sprout formation in fibrin matrices by control, non-transfected cells upon stimulation with combination of TNF-α and FGF-2.

B: Representative phase contrast images of absence of sprout formation in fibrin matrices by ECFCs transfected with siRNA u-PA upon stimulation with combination of TNF-α and FGF-2.
CHAPTER 6

PROLIFERATION, SHEAR RESPONSE AND ANGIOGENIC PROPERTIES OF ENDOTHELIAL-COLONY FORMING CELLS OBTAINED FROM PERIPHERAL BLOOD OF PULMONARY ARTERIAL HYPERTENSION PATIENTS

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ABSTRACT

Pulmonary arterial hypertension (PAH) is a devastating lung disease characterized by structural changes of pulmonary arterioles due to disproportionate proliferation and functional incompetence of the endothelial cells (EC). Elevated levels of circulating ECs and CD34+ endothelial precursors detected in the peripheral blood of PAH patients suggest that endothelial progenitor cells (EPCs) are involved in pulmonary vascular remodeling. Highly proliferative endothelial colony-forming cells (ECFC), as putative EPCs, participate in vascular homeostasis in health and disease. However, the role of ECFCs in the pathogenesis, maintenance and development of PAH is largely unknown. In this study we investigate whether the PAH-obtained ECFCs differ from the ECFCs derived from healthy subjects with respect to initial colony outgrowth, proliferation, angiogenesis and reaction to high shear stress (HSS).

Our findings showed an increased number of ECFCs colonies obtained from peripheral blood of a heterogenous group of patients with PAH in comparison to healthy subjects.

A subset of patients (n=7) yielded PAH ECFCs which displayed higher proliferation than the cells obtained from healthy donors and the other PAH donors (n=7). Analysis of the growth pattern of PAH ECFCs revealed that the proliferation status of the cells is significantly correlated with right ventricular end-diastolic volume (RVEDV) and right ventricular end-systolic volume (RV ESV). Functional experiments revealed that PAH ECFCs exhibit altered response to shear flow stress and form less tube-like structures in 3D fibrin matrices in comparison to healthy ECFCs.

Data presented in this study support the hypothesis that excessive proliferation and functional incompetence of PAH ECFCs might be involved in pulmonary vascular remodeling during PAH.
1. INTRODUCTION

Pulmonary arterial hypertension (PAH) is a chronic, irreversible lung disease that leads to right ventricular (RV) hypertrophy, remodeling, and failure of the heart. Severe PAH, both idiopathic and secondary are characterized by structural alterations in the arterioles of the pulmonary tree. Pathologic sections taken from the lungs of patients with PAH show a decreased lumen-size due to aberrant proliferation of endothelial cells (ECs) and smooth muscle cells. These pathoanatomical features lead to excessive expansion of intimal endothelial and smooth-muscle cells compartment followed by subsequent obliteration of the blood vessels and (possibly) vascular rarefaction. Lumen-obliterating, complex plexiform lesions located at the branch points of arteriolar tree are pathognomonic for PAH. Previous research identified a monoclonal origin of proliferating ECs in plexiform lesions of primary PAH but not in secondary disease entity. Interesting, the smooth muscle cell hyperplasia in pulmonary vessels in primary and secondary PAH was polyclonal indicating that genetic or epigenetic alteration in pulmonary ECs similar to that present in neoplastic processes may be responsible for the pathogenesis of PAH.

The structural alterations in the vessel wall coupled with EC-dysfunction, i.e. maladaptive eNOS-mediated vasoconstriction, initiate a vicious cycle of perpetual increase of the blood pressure in the pulmonary vascular tree. Consequently, these events lead to overload of the right ventricle and progressive right heart dysfunction.

PAH is diagnosed according to ESC guidelines when the mean pulmonary artery pressure (mPAP) exceeds above 25mm Hg in absence of left heart disease. Based on similarities regarding the pathological features and hemodynamic findings there are two main groups of pulmonary hypertension (PH): primary PAH which can be manifested as idiopathic and hereditary, and secondary which encompasses pathological conditions (chronic obstructive pulmonary disease, interstitial lung disease, and others) accompanied by elevation of blood pressure in the pulmonary vascular tree. At present, the treatment is symptomatic and based on administration of vasodilatation agents or lung transplantation in end-stage diseased patients.

Structural alterations of pulmonary arterioles caused by excessive proliferation of endothelial cells and functional incompetence of ECs with respect to the regulation of vascular tone underlie the pulmonary hypertension irrespectably of the form how this pathological condition manifests in clinical settings. Selection of survivin-positive, apoptosis-resistant, highly proliferative ECs after the initial apoptotic hit probably drives the initiation of events that finally lead to PAH structural and functional features.
Elevated levels of circulating ECs detected in PAH patients in contrast to chronic thromboembolic pulmonary hypertension patients further underline the hypothesis that the pathogenesis of PAH is rather an event associated with EC deregulation. Furthermore, circulating CD34+CD133+ endothelial precursors were higher in peripheral blood from idiopathic PAH patients than in healthy controls and correlated with pulmonary artery pressure indicating that circulating ECs and EPCs may play a critical role in initiation and development of PAH.

Up until now, the pathological mechanisms underlying excessive cell proliferation in PAH were mainly investigated in human pulmonary arterial endothelial cells (hPAEC). Several studies have identified existence of highly proliferative ECFCs in cultures of hPAEC.

Endothelial colony forming cells (ECFCs) or late outgrowth ECs also known as blood outgrowth ECs represent a type of circulating endothelial progenitor cells present in adult peripheral as well as umbilical cord blood. ECFCs were first identified as outgrowth ECs of the CD34+ MNCs fraction of peripheral blood. These EPCs are characterized by high proliferative ability, cobblestone appearance of cell monolayer, expression of EC-related phenotypic markers (CD31, VEGFR-2, CD144, vWF), robust in vivo and in vitro neovascularization potential and are devoid of markers commonly associated with myeloid cell lineage (CD45, CD14). ECFCs are actively recruited and participate in vascular homeostasis and repair as well as in neovascularization.

Despite the paucity of circulating EPCs in peripheral blood, improved isolation techniques and high proliferation ability make ECFCs a suitable cellular platform for investigation of their involvement in PAH. Several reports investigated the correlation between different phenotypic and functional aspects of circulating EPCs or PAH ECFCs with the clinical hemodynamic read-outs such as mean pulmonary artery pressure (mPAP) or pulmonary vascular resistance (PVR). However, the role of these cells in the pathogenesis, maintenance and development of PAH remains elusive.

In this study we investigate whether the PAH-obtained ECFCs differ from the ECFCs derived from healthy subjects with respect to initial colony outgrowth, proliferation, angiogenesis and reaction to high shear stress (HSS). Data presented in this study showed higher colony outgrowth of ECFCs from blood collected from PAH patients. A subset of patients yielded PAH ECFCs which displayed higher proliferation than the cells obtained from healthy donors. Analysis of the proliferation rate of PAH ECFCs revealed that the proliferation status of the cells is significantly correlated with right ventricular
end-diastolic volume (RVEDV) and right ventricular end-systolic volume (RVESV). Functional comparison revealed that PAH ECFCs exhibit altered response to shear flow stress and form less tube-like structures in comparison to healthy ECFCs.

2. MATERIALS AND METHODS

2.1 Pulmonary arterial hypertension (PAH) patients

PAH patients were recruited from the pulmonary hypertension database of the VU University Medical Center (VUMc, Amsterdam, The Netherlands). In total 43 WHO group 1 PAH patients including different etiologies and genetic backgrounds were included in this study. Control subjects were volunteers, colleagues as well as partners and friends accompanying the patient to the clinic. Patients without PH at diagnostic right heart catheterization were included. Venous blood was drawn either by eligible venous punction or during scheduled right heart catheterization. The IRB of the VU University Medical Center reviewed and approved the study and all subjects gave informed consent.

2.2 ECFC isolation, culture and immunophenotypic characterization

ECFC isolation and culture as previously described23. In brief, a density gradient centrifugation step was used to obtain mononuclear cell (MNC) fraction. Yielded MNC fraction was re-suspended in complete culture medium (EBM-2) plus Single Quots kit (EBM-2, Lonza, Basel, Switzerland) supplemented with 10% human platelet lysate (HPL). Subsequently MNCs were seeded on a rat-tail collagen type I coated 48-well culture plate. Cells were kept at 37°C, 20% O₂, 5% CO₂. After 3 days, the culture plate was washed with PBS to remove non-adherent cells. Medium was refreshed every other day. Colonies appeared after 7 days and were enumerated until the end of the outgrowth period (28 days). When a colony covered 50% of the culture surface, cells were detached and re-plated on 5 cm² (passage 1). Subsequently, when cells reach confluence cells were expanded with a ratio of 1:4. All experiments were performed with cells between passage 4-6. Isolated ECFCs were characterized by the presence of endothelial cell markers (CD31, CD34, CD146, VEGFR2, VE-Cadherin) and lack of CD45, CD14 by flow cytometry as previously described23.

2.3 Proliferation assay

Proliferation ability of ECFCs from healthy subjects and PAH patients was assessed by seeding 1000 cells/cm² in EGM-2 + 10%HPL. The medium was refreshed every other day. Bright field-microscopy images were taken every day. Cell number was determined.
with Image J software, by analysis of 8 randomly taken pictures from each 1 donor/ 24h 
time point, with t=1 being 4 hrs after seeding. Proliferation was followed over a period 
of 9 days. The steepness of individual proliferation curves was analyzed from the linear 
portion of individual proliferation curves (see addendum). Hyper-proliferative subset of 
PAH ECFC was identified by exhibiting more than 3SD from the steepness of the growth 
curve.

2.4 Fluid shear reduced tube-formation ability of PAH ECFC stress 
response of PAH-derived ECFCs

Response of ECFCs obtained from PAH patients and healthy subjects to laminar shear 
flow was evaluated on ibidi 0.6 µ-slides (Integrated BioDiagnostics, Munich, Germany). 
Cells were seeded at a density of 40,000 cells/cm² on a fibronectin coating and allowed 
to attach overnight. Thereafter, unidirectional, pulsatile shear stress was gradually 
increased from 2.5 dyn/cm² (24h), to 21.5-25 dyn/cm² (120h). Shear-adaptation, based 
on cell morphology and orientation, was quantified from phase-contrast images using 
Photoshop CS6 (Adobe, San Jose, CA, USA) and Matlab software (MathWorks, Natick, 
MA, USA). Cells were defined elongated, when twice as long as wide. A subdivision 
was made between not aligned cells and aligned cells orientated within <30° to the 
direction of flow.

2.5 Tube formation assay

Assessment of sprouting ability of ECFCs was done by seeding 20,000 cells on 3D human 
fibrin matrices prepared as previously described. Following overnight incubation in 
M199 supplemented with 10% inactivated human serum (HS) and 10% new-born calf 
serum (NBCS), tube formation was induced by stimulating the cells with a combination 
of 10ng/ml TNF-α (T) and 10ng/ml FGF-2 (F) prepared in M199/HS/NBCS medium every 
day over a 3-days stimulation period. All growth factors were purchased from ReliaTech 
GmbH, Wolfenbuttel, Germany. After stimulation period, the cells were fixed with 2% 
paraformaldehyde/HBSS and quantification of the length of formed tube-like structures 
was performed using Optimas image analysis software as previously described. The 
tube formation ability of PB-ECFCs of all donors was determined in triplicate wells.

2.6 Acquisition of hemodynamic patient measures

Cardiac MRI was performed on a Siemens 1.5-Tesla Avanto or 1.5-Tesla Sonato scanner 
(Siemens Medical Solutions, Erlangen, Germany), equipped with a 6-element phased-
array coil. A stack of short-axis images were obtained at breath-hold per slice, with a 
slice thickness and interslice gap of 5 mm, fully covering both ventricles from base to 
apex. On end-diastolic images (first cine after R-wave trigger) and end-systolic images
Proliferation, shear stress response and angiogenic properties of ECFCs obtained from peripheral blood of pulmonary arterial hypertension patients

(cine with visually the smallest cavity area), endocardial and epicardial contours were manually drawn by using MASS software (Department of Radiology, Leiden University Medical Center, Leiden, The Netherlands) to obtain right ventricular end-diastolic volume (RVEDV), right ventricular end-systolic volume (RVESV), and right ventricular mass. Volume measurements and RV mass were indexed for body surface area. Papillary muscles and trabeculae were included in RV mass. RV ejection fraction is calculated as (RVEDV – RVESV)/RVEDV×100%.

Right heart catheterization was performed using a 7F balloon-tipped Swan Ganz catheter (131HF7, Baxter Healthcare Corp, Irvine, CA). The catheter was inserted via the jugular or femoral vein and brought into position under local anesthesia during continuous electrocardiographic monitoring. The following variables were recorded: mean pulmonary artery pressure (mPAP), right atrial pressure (RAP), mixed venous oxygen saturation (SvO₂), and heart rate. Pulmonary vascular resistance (PVR) was calculated via the following formula: 80×(mean pulmonary artery pressure – pulmonary arterial wedge pressure)/cardiac output. Cardiac output (CO) was determined by either the Fick method or thermodilution, and stroke volume was calculated as cardiac output divided by heart rate. Both cardiac output and stroke volume were indexed for body surface area, shown as cardiac index and stroke volume index, respectively.

2.7 Statistics
All data were tested to have a normal Gaussian distribution. Two tailed student t-test (with Welch’s correction) or One-way ANOVA (with Bonferroni’s or Turkey multiple comparisons test) was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) and p-values ≤ 0.05 were considered significant. Data are presented as mean ± SEM, unless noted differently.

Specific analyses individual growth curves
Slope of the individual growth curve was extracted by performing linear regression on the linear part of the (sigmoidal) growth curve. The best fits (R² >0.97) were selected on an interval between t=2-9 days, with a minimum of 3 data points, and used for further analysis. Slope is termed cell growth/day.

Selection of hyper proliferative and normal proliferative PAH ECFC was based on comparison of PAH ECFC to control ECFC growth slopes. PAH ECFC exceeding the slope of the average control ECFC >3SD were named hyperproliferative. The ECFCs derived from cohort of PAH patients which displayed same proliferation as the cells obtained from healthy subjects were designated as normal proliferative PAH ECFC.
Correlations
Pearson correlations were tested from x, y dotplots. The predicted line, and the 95% confidence interval are showed. Tests were performed for RVEDV, RVESV mPAP and PVR.

3. RESULTS

3.1 Increased outgrowth of ECFCs from peripheral blood obtained from PAH patients
Previous studies have reported increased numbers of endothelial precursors in peripheral blood of PAH patients\textsuperscript{11,12}. Accordingly to these findings, PAH patients should be able to generate more ECFC colonies in comparison with healthy subjects. Comparison of the total number of colonies generated per 100mL blood, confirmed this hypothesis. Namely, we measured an increased outgrowth of ECFCs from MNCs fraction of PAH in comparison to healthy donors (ECFC control (n=10) mean=12.2±4.0 and ECFC PAH (n=15) mean= 28.9±6.6, p=0.029, \textbf{Fig.1}). These colonies were composed of ECFCs growing in cobblestone manner and expressing typical EC-markers, such as CD31, VE-cadherin, VEGFR-2 as evaluated by flow cytometry (data not shown).

\textbf{Figure 1. Endothelial cell colony outgrowth from peripheral blood of healthy and PAH subjects}
Graph represents the number of ECFCs colonies obtained from 100mL whole blood at the end of the isolation and outgrowth procedure. Data are expressed as mean±SEM.
Control (●): n=10; PAH (■): n=15; unpaired t test with Welch’s correction: control vs PAH p=0.029.3.2 PAH ECFCs show donor-dependent alterations in proliferation rates
The hyperproliferative phenotype of ECs is one of the pathophysiological cellular hallmarks of PAH\(^{25,26}\). In order to investigate the proliferation ability of PAH ECFCs, 1000 cells/cm\(^2\) from PAH and healthy subjects were plated on rat-tail collagen I pre-coated dishes and their growth was monitored over a 9 days period. Assessment of proliferative ability of ECFCs from PAH and healthy donors revealed existence of two distinct patterns of proliferation among the PAH group. We observed PAH ECFCs with a hyperproliferative phenotype (hyper-PAH ECFCs) and PAH ECFCs that proliferated at the same rate (normal-PAH ECFCs) as the cells obtained from control, healthy donors (control ECFCs) (Fig.2A). A donor was categorized as hyperproliferative when the steepness of the growth curve of the PAH ECFC exceeds the average steepness of the growth curve of the control ECFC by >3 SD as described in material and methods (Fig.2B). Interestingly, comparison of the clonal outgrowth between hyper-PAH, normal-PAH and control ECFCs unraveled that the proliferation rate is not in direct relationship with the ability to generate ECFCs colonies from peripheral blood. Normal PAH ECFCs exhibited a similar proliferation rate as control ECFCs, but generated more ECFCs colonies per 100mL whole blood than the control, healthy subjects (Fig.2C). A similar trend was observed between normal- and hyper-PAH ECFCs albeit it didn’t reach statistical significance.

The different patterns of proliferation in PAH ECFCs implicate that this biological feature might be used as a biomarker for patient stratification especially when it is correlated with clinical hemodynamic read-outs. It has been shown that the RV function is linked to survival and disease status\(^{27}\). For that reason, we investigated the relation between the proliferation patterns of PAH ECFCs and hemodynamic read-outs RVEDV, RVESV, PVR, and mPAP. Analysis of proliferation curves of PAH ECFCs revealed that the proliferation status of the cells is significantly correlated with RVEDV and RVESV (Fig.2D,E). Correlations of the proliferation slope for PAH ECFCs were tested and found non-significant for PVR and mPAP (data not shown).
Figure 2. Proliferative capacity of PAH ECFCs and its relation to hemodynamic parameters

A: Increased proliferation of ECFCs in a subset of PAH patients
Proliferation rates of control ECFCs (●) and hyper- (■) and normal- (▲) proliferative PAH ECFCs are expressed as mean±SD cell number/cm².
Statistical significance (control: n=4; hyper-PAH: n=7; normal-PAH: n=8) was determined using 2 sided, unpaired t-test (At day 9, p=0.0041; control vs. hyper-PAH ECFC).

B: Quantification of the slope steepness of proliferation curve
The generation of cells per day is given for three groups of ECFCs (● control: n=4; ■ hyper-PAH: n=7; ▲ normal-PAH: n=8) and expressed as mean±SD cells/day. Statistical significance was determined by one-way ANOVA with Bonferroni’s multiple comparisons test (p<0.05).

C: ECFCs outgrowth in PAH subjects
Graph depicts the enumeration of ECFC colony outgrowth from 100mL whole blood expressed as mean±SEM. (control: n=10; hyper-PAH ECFCs: n=8; normal-PAH ECFCs: n=8). Statistical significance was determined by one-way ANOVA with Bonferroni’s multiple comparisons test (p<0.05).

D: Correlation between RVEDV and cell growth. The proliferation rate was estimated from the steepness of the proliferation curve. The steepness of proliferation curve reflects the proliferation rate.
Solid black line indicates correlation prediction; dashed lines represent 95% confidence intervals; ● depicts individual data points. RVEDV: R² 0.5592, p=0.0081, 95% CI -0.9303 to -0.2683

E: Correlation between RVESV and cell growth.
Solid black line indicates correlation prediction, dashed lines represent 95% confidence intervals, ● depicts individual data points. RVESV: R² 0.523, p=0.0119, 95% CI -0.9227 to -0.22177.
3.3 PAH ECFC show donor dependent variation in alignment response to high shear stress (HSS)

PAH is characterized by increased RV and increased pulmonary blood pressure leading to continuous exposure of endothelium to elevated shear stress. Exposure of pulmonary microvascular endothelial cells obtained from PAH patients (PAH MVEC) results in delayed shear adaptation. Since PAH MVEC originate from the pulmonary vasculature we investigated whether ECFCs which are obtained from the systemic circulation react to HSS in same manner as PAH MVEC. Cells were seeded on fibronectin matrix and remained adherent when exposed to 25dyn/cm² HSS (Fig.3). Exposure of control ECFC to HSS for 24h resulted in alignment of the cells in the direction of flow (Fig.3A,4A) which also persisted after 72 and 120h (Fig.3B,C, Fig.4B,C). In PAH ECFC (n=14) we identified two sets of donors with different response to HSS. A subset of donors (n=6-7) exhibited a significantly impaired alignment after 24 h exposure to high shear stress in comparison to control ECFCs (Fig.4A; not aligning PAH ECFC) and this pattern was maintained during the period of 72 and 120 h (Fig.4B,C). On the other hand, among the rest of PAH donors (n=7) we observed that these cells react to HSS in a similar fashion as control ECFCs (Fig.4A,B,C; aligning PAH ECFC). As expected, the PAH MVEC aligned in direction of flow under HSS after 120h in a manner as previously reported. Assessment of proliferation under high shear stress revealed that non-aligning PAH ECFCs exhibited less proliferation than control and aligning PAH ECFCs albeit not statistically significant (Fig.4D). No difference in proliferation was observed between control and aligning PAH ECFCs.
Figure 3. Shear stress response of PB-ECFCs and PAH MVEC.
Top 3 rows: Representative images of PB-ECFC alignment in flow direction after 24, 72, and 120h.
Bottom row: Representative images of alignment response of PAH MVEC after 24, 72, and 120h.
Proliferation, shear stress response and angiogenic properties of ECFCs obtained from peripheral blood of pulmonary arterial hypertension patients

Figure 4. Response of PB-ECFCs to high shear stress
Alignment of PB-ECFCs to direction of flow after 24h (A), 72h (B), and 120h (C). Data is expressed as % of aligned cells. Statistical significance was determined by one-way ANOVA with Tukey’s multiple comparisons test (p<0.05; A, B, C: # vs. ▲ and ○; & vs. ▲ and ○; * vs. ▲ (n=6).

D: Proliferation of PB-ECFCs under prolonged exposure to shear stress. Statistical significance was determined by one-way ANOVA with Tukey’s multiple comparisons test (control n=3, aligning PAH ECFCs n=4, not-aligning PAH-ECFC n=6).

3.4 Reduced tube-formation ability of PAH ECFC
High proliferation of ECFCs is coupled with robust angiogenic response to pro-neovascularization factors in healthy subjects. Whether this observation holds true for PAH-derived ECFCs is not known. In order to investigate the angiogenic ability of PAH ECFCs, healthy and PAH-derived ECFCs (hyper- and normal proliferative) were seeded onto fibrin matrices and their sprouting response to simultaneous exposure to TNF-α and FGF-2 was assessed after 3 days. In general, all three groups of ECFCs formed tube-like structures (Fig. 5). Assessment of the length of formed tube-like structures revealed a trend of reduced sprouting capacity in PAH-derived ECFCs in comparison to the cells obtained from healthy donors. Nonetheless, this trend was not statistically significant.
Figure 5. Reduced tube-formation ability of PAH-ECFCs compared to control ECFCs
Graph represents the tube-formation ability of PB-ECFCs upon stimulation with TNF-α + FGF-2. Data expressed as mean tube length/cm\(^2\) ± SEM (n=4). Statistical significance was determined by one-way ANOVA with Tukey’s multiple comparisons test.

4. DISCUSSION

Data presented in this study showed an increased number of ECFCs colonies obtained from peripheral blood of a heterogeneous population of patients with PAH. Analysis of the proliferation patterns of PAH-derived ECFCs suggests that ECFCs obtained from a group of PAH patients exhibit a hyper-proliferative ability in comparison to the PAH-derived cells, which displayed a similar proliferation rate as ECFCs from healthy subjects. Functional studies showed that PAH derived ECFCs display reduced tube-formation ability in fibrin matrices. Application of high shear stress unravelled the existence of PAH subjects, whose cells aligned in the direction of flow, and a group of patients whose cells did not.

The cellular dysregulation in the pathogenesis of PAH is still largely unknown. Vascular homeostasis and repair relies on rapid differentiation of vessel-wall and circulating progenitor cells into mature ECs. Uncontrolled differentiation of precursor cells and local hyper-proliferation of ECs disturb blood flow and might contribute to obliteration of small blood vessels in the lung microcirculation of PAH patients\(^7,26\). Increased numbers of circulating precursors that exhibit EC-surface markers and may differentiate toward
mature EC phenotype or circulating ECs have been reported in PAH patients\textsuperscript{11,12}. Our finding of increased outgrowth of ECFCs colonies from PAH patients and the presence of a hyper-proliferative profile of PAH-ECFCs of a subset of the patient indicate that indeed PAH cellular pathophysiology might be centered around a change in EC phenotype characterized by a dysregulated pattern of EC differentiation, proliferation and function. PAH pulmonary endothelium is enriched with high proliferative ECFCs\textsuperscript{13}. Toshner et al. reported an increased outgrowth of colonies from patients with pulmonary arterial hypertension (PAH) associated with bone morphogenetic protein receptor type II (BMPRII) mutations displaying a hyper-proliferative phenotype\textsuperscript{20}. Identification of hyper- and normo-proliferative PAH ECFCs points to the presence of diverse levels of dysregulation of processes that govern proliferation of ECFCs among PAH patients. Intriguingly, comparison of the colony outgrowth within the hyper and normal-proliferative PAH ECFCs donors unraveled that the latter generated more colonies during the isolation procedure than the former group of patients. This indicates that not only hyper-proliferation plays a role in the aberrant local accumulation of ECs in the pulmonary vasculature of PAH, but also the increased clonogenicity and EC-differentiation ability of the reserve pool of EPCs of PAH patients do.

Furthermore, the proliferation status of PAH ECFCs was used in this study to investigate its correlation with clinical parameters indicative for the function of cardiovascular system in PAH patients. Possible correlation of clinical parameters with cell-based functional assays opens the perspective to use in vitro assays as predictive or prognostic biomarkers. Analysis of the proliferation curves of PAH ECFCs revealed that the proliferation status of the cells is significantly correlated with RVEDV and RVESV. The real significance of this correlation and its relevance to clinical practice remains to be investigated in longitudinal-type clinical studies in future. If the observed correlation is strongly indicative for a better PAH status, than it would be possible to use such in vitro assay for patient stratification in clinical settings. In addition, questions like “How hyper-proliferative ECFCs contribute to better RV status in PAH” warrant future research.

Adaptive response of PB-ECFCs to shear stress is well elucidated in healthy volunteers. Healthy ECFCs covering tubules in fibrin constructs react to shear stress with elevated NO production, decreased platelet adhesion to endothelialized tubular fibrin constructs, and with a reduction of VCAM-1 and ICAM-1 in a TNF-α challenge model\textsuperscript{30}. Another study showed that ECFC exhibit a mature response to fluid flow, comparable to that of HUVEC and human umbilical artery endothelial cells (HUAEC)\textsuperscript{31} or human aortic endothelial cells (HAEC)\textsuperscript{32}. In our study, the healthy ECFCs reacted to high shear stress in a similar fashion as already reported by others\textsuperscript{30,31,32}. Surprisingly, we observed two groups of PAH ECFCs that differently reacted to high shear stress. One group (aligning
PAH-ECFCs) reacted in a similar fashion as the healthy, control ECFCs over the course of 120h application of high shear stress. On the other hand, the second group of ECFCs obtained from PAH patients (not aligning PAH ECFCs) failed to orient in flow direction at any of the time points of assessment. Delayed response of PAH MVEC has been well documented and the cells are aligned in the direction of flow only after 120h\textsuperscript{28}. However, although the not aligning PAH ECFCs exhibited, especially after 24h and 72h shear stress application, a similar behavior as PAH MVEC, it remains unclear why the cells failed to respond similarly to the same experimental conditions after 120h as PAH MVEC did.

Additional analysis of not aligning PAH ECFCs demonstrated that there was no relation between HSS maladaptive cell response and the clinical status of the PAH patients. Furthermore, we could not identify any significant relation by plotting the flow data against the proliferative capacity of their ECFCs or against any of the patient’s characteristics. This finding suggests that HSS cellular maladaptation in this group of PAH patients is 1) possibly regulated by a mechanism different than the one identified in lung MVEC of PAH patients and 2) that the phenotype of PAH is unlikely a factor that determines cell response to shear stress.

Our data implicate an intrinsic alteration of angioproliferative features of the cells involved in PAH, which plays a crucial role in the pathogenesis of this disease\textsuperscript{33}. Plexiform lesions express makers of angiogenesis\textsuperscript{33}. However, during tube-formation assay in 3D fibrin matrices, PAH ECFCs displayed reduced angiogenic activity suggesting a global dysfunction of angiogenic program might underlie our observation. It appears that uncoupling of proliferation from angiogenesis initiates a series of events that makes increased proliferation rather than impaired angiogenesis a driving force of the formation of obliterating plexiform lesions in PAH. Of note, reduced \textit{in vitro} angiogenic capacity of PAH ECFCs grants further investigation prior to eventual use of these cells as a therapeutic alternative for treatment of PAH.

In this study, we investigated certain biological features of PB-ECFCs isolated from PAH patients. PAH patients generated more ECFCs colonies than healthy controls indicating either higher presence of circulating EPCs or increased EC-differentiation ability of same progenitors. Heterogeneity of cell responses during proliferation, tube-formation and exposure to HSS warrants further investigation in order to identify the underlying master regulators that are responsible for observed differences. The prospects how to employ the accumulated knowledge regarding PAH ECFCs are 1) to use these cells as therapeutic means, 2) to use them as a screening platforms for identification of new therapeutic targets, 3) or to design ECFC-based assays that can be used in clinical practice for patient stratification and disease or therapy monitoring.
ACKNOWLEDGEMENTS

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Proliferation, shear stress response and angiogenic properties of ECFCs obtained from peripheral blood of pulmonary arterial hypertension patients


Identification of circulating endothelial progenitor cells introduced the paradigm that these cells drive postnatal neovascularization and vasculogenesis\(^1\). At present, accordingly to the accepted consensus, a circulating endothelial progenitor displays an ability to produce endothelial progeny which forms primitive tube-like structures in vitro as well as contributes to the re-establishment of functional vasculature into ischemic tissue via neovascularization or participate in maintenance of endothelial lining of blood vessels. Based on these criteria, only ECFCs fit the definition of true EPCs\(^2\). Due to their unique neovascularization features and high proliferation, the ECFCs are currently considered the most promising endothelial cell source for future clinical application, both as a means for regenerative neovascularization and tissue repair as well as to study pathophysiological mechanisms at cellular level in patients.

Cell-based therapies hold great potential to shift the outcome of debilitating diseases as well as to facilitate the tissue regeneration\(^3\). Systemic treatments with hematopoietic stem cells are part of clinician armamentarium to fight blood cancers such as leukemia and multiple myeloma. Clinical trials of application of modified patients’ T-cells with innovative technologies and approaches (adoptive cell transfer) are proof of principle that we can successfully alter patients’ T-cells or other cells types for therapeutic purposes. Chimeric antigen receptors modified T-cells (CAR-T) attack cancer cells in acute lymphoblastic leukemia (ALL)\(^4\) while ~500 clinical trials demonstrated that mesenchymal stem cells hold great promise for efficient treatment of graft versus host disease, diabetes, diseases in the liver, kidneys, and lungs, as well as cardiovascular, bone and cartilage, neurological diseases\(^5\). Considering the utmost importance of the vasculature in health and disease it is really puzzling why endothelial cell-based therapies are not incorporated in the clinician’s toolbox for disease treatments when an excellent cell substrate with potent regenerative potential is available.

Some of the reasons and pending questions why ECFCs are still at the “bench” and not at the “bedside” are summarized in table 1.

This study aims to provide a conceptual framework for addressing of the most important unknowns regarding the translation of ECFCs in clinical practice. In Chapter 3 we addressed some of the pitfalls as outlined in A and presented an improved protocol for isolation and in vitro manipulation of ECFCs in xenogeneic-free environment based on the use of platelet lysate. The value of the use of platelet lysate was further underpinned by recent studies of Kim et al. and extended in a study of Fortunato et al. in which a platelet lysate gel was used to stimulate the formation of a microvascular network in vitro\(^6\). Our procedure enables amplification of potent angiogenic and phenotypic stable ECFCs up to the numbers required for eventual clinical application.
and reduces the risk of transfer of potentially harmful xenogeneic material or induction of an immune response when cells are isolated and manipulated with animal-derived cell culture materials and supplements (FBS). At present, there is a cacophony of methods for isolation of ECFCs. Assembling of a panel of experts that will assess the current protocols for isolation and manipulation and provide a guidelines as well as standardized protocols will pave the way to implement these cells in clinical practice in safe and control manner. Initial steps to reach consensus on nomenclature of ECFCs have been taken\textsuperscript{2,8} and at least for isolation and culture of human ECFCs time seems appropriate to make a next step.

<table>
<thead>
<tr>
<th>A) ISOLATION, CHARACTERIZATION AND IN VITRO MANIPULATION</th>
<th>A.1</th>
<th>Cell lineage of progenitor that produces ECFCs progeny has not yet been identified.</th>
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<tbody>
<tr>
<td></td>
<td>A.2</td>
<td>Lack of consensus about the source of ECFCs (cord blood vs. peripheral blood vs. other tissues) for clinical application.</td>
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<tr>
<td></td>
<td>A.3</td>
<td>Lack of standardized procedure for isolation ECFCs.</td>
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<td></td>
<td>A.4</td>
<td>Isolation and amplification protocols developed on use of animal-derived products.</td>
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<td></td>
<td>A.5</td>
<td>Absence of unanimity regarding the phenotypical and functional characterization of ECFCs.</td>
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<td></td>
<td>A.6</td>
<td>Heterogeneous terminology in the field of ECFCs research.</td>
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</table>

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<tr>
<th>B) BIOLOGICAL FEATURES OF ECFCs</th>
<th>B.1</th>
<th>Can ECFCs be specified as arterial or venous endothelial cells?</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>B.2</td>
<td>Phenotypic plasticity of ECFCs and the role of EndoMT in vivo still needs further research.</td>
</tr>
<tr>
<td></td>
<td>B.3</td>
<td>Heterogeneity in expression of cell-surface markers (CD34, VEGFR2) or different angiogenic features.</td>
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<td></td>
<td>B.4</td>
<td>Unknown molecular mechanisms by which ECFCs participate in neovascularization, vascular homeostasis, and disease.</td>
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<tr>
<th>C) APPLICATION</th>
<th>C.1</th>
<th>Poorly defined indications for use of ECFCs as therapeutic means in clinics or as a tool for future investigation about involvement of ECFCs in disease as well as therapy assessment.</th>
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<tr>
<td></td>
<td>C.2</td>
<td>Lack of well-defined approaches how to use these cells in clinics (cells or tissue-engineered constructs).</td>
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<tr>
<td></td>
<td>C.3</td>
<td>The routes and means of delivery, number of cells, “go or no-go” to support ECFCs with accessory cells (MSC, pericytes, immune cells) are still in the area of scientific debate.</td>
</tr>
<tr>
<td></td>
<td>C.4</td>
<td>Lack of interest for commercialization of ECFCs by biomedical industry.</td>
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Table 1. Roadblocks to successful transition of ECFCs from bench-to-bedside
Implementation of standards and guidelines will help to solve several of the issues indicated under “A. Isolation, characterization and in vitro manipulation culture” of ECFCs. Provided a reproducible procedure to obtain sufficient ECFCs - as given in chapter 3 - peripheral blood has the advantage as source of ECFCs above cord blood that it is autologous and not from a donor. This is in particular the case if the isolation and culture of ECFCs can be achieved in a fully synthetic or at least animal-product-free way. The procedure described in chapter 3 meets the latter criterion and therewith overcomes point A.2, A.3 and A.4. For answering points A.5 and A.6, a clear picture is emerging and consensus is needed rather than new data. A recent consensus on nomenclature is a first step to a more standardized approach. The only point that remains (A.1) regards in fact the period before the ECFC isolation and regards the nature and origin of the circulating cell that gives rise to expanded ECFCs. This is an important issue as it may elucidate whether the release of ECFCs can be stimulated and whether tissue-specific factors are involved in shaping the expression profile of these endothelial progenitors.

With respect to the biological features, besides investigating how hypoxia determines ECFCs differentiation and cells’ behavior (Chapter 4), we have also gained some further insight, in particular with regard to the existence of specific angiogenic subpopulations including the expression of CD34 in these populations (Chapter 5).

As the application of ECFCs will often occur in a rather hypoxic in vivo environment, it is important to understand the responses of ECFCs in hypoxic conditions. Furthermore, the differentiation of ECFCs from their circulating progenitors occurs at the transition from oxygenated blood to the hypoxic tissue that requires angiogenesis support. The effect of oxygen environment on the differentiation of circulating EPC to ECFCs was addressed in Chapter 4 indicating that reduced outgrowth of ECFCs from MNCs under hypoxic condition (1%O2) can be rescued by exposure to higher than 1%O2 environment. Of note, peripheral blood derived MNCs generated more colonies than the MNC fraction obtained from umbilical cord blood further strengthening the fact that at present PB-ECFCs are more viable therapeutic option than CB-ECFC. Interestingly, addition of MNCs rescued the drop in proliferation of sub-cultured ECFCs which emphasises the necessity to investigate the cell-cell interaction between ECFCs and different types of cells (immune, MSCs, pericytes) during neovascularization in order to design and develop more efficient cell-based treatment.

Generation of multiple clones of ECFCs from blood-derived MNCs during isolation procedure implies of possible existence of phenotypical heterogeneity in cell cultures.
We identified two populations of ECFCs with different expression of CD34 on cell surface (Chapter 5). The CD34+ and CD34- ECFCs identified and characterized in our study are not a separate cell lineages but merely reflect a different but interchangeable phenotype of endothelial cells. Functional assays demonstrated that CD34 characterizes more pro-angiogenic phenotype and can be used for selection of ECFCs with potent angiogenic features or as a marker for endothelial angiogenic activation. Selection of cells with desired biological features that fit to the needs of having an efficient therapeutic tool for treatment of tissue ischemia represent attractive approach for introduction of ECFCs in the clinics. Although the feasibility of this “selective” approach remains to be in vivo investigated it also reflects our and other’s efforts to add a small piece to the mosaic called “Therapeutic and disease-modelling potential of ECFCs”.

The data presented in Chapter 4 contributes to better understanding of the nature of ECFCs (point B4) whilst identified heterogeneity of CD34 expression in ECFCs cultures further underpins the importance of gaining as much as possible knowledge (point B.3). Mechanisms that regulate phenotypical plasticity during EndoMT (point B.2) or arteriovenous differentiation of ECFCs (point B.1) remain still not fully elucidated.

The main advantages as well as the pitfalls of the present concepts for use of ECFCs as therapeutic means were outlined and reviewed in Chapter 2. The general conclusion which to some extent also addresses some of the concerns in C (Application) can be summarized in the urgency to clearly define the experimental outcomes in large animal models that can significantly contribute to translation of collected data in clinical relevant situations. Therefore, the proof-of-concept investigation in the field of basic science ought to be translatable and must mirrors the pathological entity in clinics. Notwithstanding, accumulated in vivo evidence suggests that the pre-vascularization of tissue engineered constructs for bone and skin regeneration, endothelialization of tissue-engineered vascular grafts, cell bolus application for vascular repair and treatment of myocardial infarction are fields where ECFCs can penetrate as new therapeutics in the very near future.

A special category of translational research where ECFCs can greatly contribute to extend the bounders of our medical knowledge is the investigation of the role of these cells in certain pathological conditions that are characterized by vascular dysfunction. Biological features of PB-ECFCs isolated from PAH patients were investigated in Chapter 6. This study helped us to pinpoint the prospects how to employ the accumulated knowledge regarding PAH and other patient-derived ECFCs. In general ECFCs derived from patients
Discussion & future perspectives

can be used as 1) therapeutic means, or 2) screening platforms for identification of new therapeutic targets, or 3) for development of ECFC-based functional assays that can be used in clinical practice for patient stratification, and disease or therapy monitoring.

To conclude, the multiple therapeutic potential of ECFCs made these cells an attractive target for innovative vascular regenerative therapies and tool for investigation of vascular pathologies.

We hope that our work contributes to a better understanding of ECFCs and their roles in postnatal vascularization and disease.
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CHAPTER 8

SUMMARY

NEDERLANDSE SAMENVATTING

ACKNOWLEDGEMENTS

CURRICULUM VITAE
SUMMARY

Identification and characterization of endothelial colony-forming cells as endothelial progenitors introduced the concept of therapeutic vascularization as treatment option for correcting tissue ischemia by re-establishing blood flow through newly formed blood vessels. Proof-of-concept studies have shown that the normal physiological processes that drive the generation of a new vascular bed or the expansion of an existing one - such as de novo blood vessel formation, angiogenesis and arteriogenesis - also depend on incorporation of ECFCs.

The work in this thesis addresses several important aspects regarding the translation of ECFCs from bench to bedside. The main advantages as well as pitfalls of the present concepts for use of ECFCs as therapeutic means were outlined and reviewed with special emphasis on the contribution of large and small animal models in testing new concepts for clinical implementation. Furthermore, the core of this thesis regards experimental data on ECFCs, which expand our knowledge of their cellular responses under various experimental conditions.

Isolation and manipulation of ECFCs prior to clinical application requires standardized technology based on use of products devoid of animal-derived products. We have defined an in vitro system for isolation and manipulation of ECFCs from peripheral blood based on the use of human platelet lysate. Isolated ECFCs displayed all the hallmarks of endothelial lineage as well as in vitro angiogenic capacity in line with previously published reports. Our optimized amplification protocol grants generation of sufficient number of healthy and angiopotent cells in less than a month after blood sampling in entirely human culture conditions.

We also have demonstrated that environmental factors such as hypoxia affect the isolation process by reducing the number of generated ECFCs colonies and impairs the proliferation capacity of isolated cells. Additional experiments indicated that very likely the cell-cell interaction between immune cells and endothelial precursors under hypoxia defines the outcome of isolation procedure. Hence, this work provides a technological framework for isolation and manipulation of peripheral blood-derived ECFCs for therapeutic application and pinpoints how the environmental factors such as hypoxia or immune cells affect the generation of ECFCs from peripheral blood. Knowledge of these conditions is important as application of ECFCs often will involve a hypoxic environment.
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ECFCs belong to the endothelial lineage yet represent a heterogeneous cell population with regard to the expression of stem-cell surface marker CD34. In line with our goal to facilitate the transition of ECFCs in the clinic, we hypothesized that CD34 might mark a subpopulation of ECFCs with potent angiogenic capacity and can be used for selection of angiopotent cells prior clinical application in order to shorten the formation of new blood vessels. We have identified that CD34 indeed is positively involved in capillary-like sprout formation in fibrin matrices and that the destabilization of local barrier function mediated via CD34 might be the process which underlies better angiogenic capacity of CD34⁺ ECFCs. We also observed that the CD34⁺ and CD34⁻ phenotypes of subcultured ECFCs were interchangeable during culture. These in vitro data support selection of more potent angiogenic ECFCs based on CD34 expression prior clinical application, but this concept needs in vivo verification.

Besides being an attractive cell type for therapeutic neovascularization, ECFCs are of great value to study diseases, in which vascular dysfunction is one of the hallmarks of displayed phenotype.

Pulmonary arterial hypertension is a lung disease characterized by right ventricular dysfunction due to structural changes of arterioles in lung vascular bed. Disproportionate proliferation and functional incompetence of the endothelial cells (EC) underlies the obliteration of lung arterioles. The comparison of ECFCs outgrowth between healthy subjects and PAH patients followed by functional characterization of PAH-ECFCs as reported in this thesis indicate that indeed an excessive generation and proliferation of ECFCs might play an important role in pathogenesis of PAH. Data collected from functional assessment of PAH-ECFCs also pinpoint to existence of heterogeneity among PAH patients with respect to the extent of and the manner by which ECFCs might be involved in vascular remodelling during PAH. This work contributes to unravelling of the role that ECFCs very likely have in the mechanisms that make PAH such as devastating disease.

In summary, the studies presented in this paper improve our understanding of PB-ECFCs as well as their roles in postnatal vascularization. We anticipate that they will be helpful for future therapeutic application of ECFCs.
**NEDERLANDSE SAMENVATTING**

Dit proefschrift richt zich op het concept dat menselijke endotheel-progenitorcellen gebruikt kunnen worden als onderdeel van verschillende tissue-engineering toepassingen bij het herstellen van de bloedvoorziening en het opheffen van ischemie in organen en weefsels. Allereerst hebben we in dit project een in vitro procedure gedefinieerd en gevalideerd voor de isolatie, bewerking en amplificatie van endotheel-specifieke voorlopercellen, de zogenaamde endothelial colony forming cells (ECFCs), verkregen uit menselijk perifeer bloed. Ten einde toepassing te vergemakkelijken omvatten alle stappen uitsluitend menselijke materialen. De verkregen procedure levert binnen een maand een groot aantal gezonde en angiopotente (in staat bloedvaatjes te vormen) cellen, in voldoende aantal voor toepassing.

Vervolgens onderzochten we het gedrag van ECFCs bij een lage zuurstofspanning, zoals die voorkomt in weefsels die door ischemie zijn aangedaan. We konden laten zien dat een lage zuurstofspanning de vorming en groei van ECFCs uit perifeer bloed sterk vermindert. Kennis over hoe zuurstofbeschikbaarheid de karakteristieken en het gedrag van de cellen beïnvloedt is van belang, omdat toepassing van ECFCs vaak een hypoxische omgeving plaats vindt. Vervolgens onderzochten we de hypothese dat de stamcelmarker CD34 een subpopulatie ECFCs markeert die een potente capaciteit tot bloedvatvorming (angiopotentie) heeft en die voorafgaand aan klinische toepassing gebruikt kan worden voor selectie van angiopotente cellen ten einde de snelheid van vaatvorming door de toegepaste ECFCs te bevorderen. We hebben gevonden dat CD34-positive cellen een groter vermogen hebben tot het vormen van endotheelbuisjes in een fibrine matrix. Hierbij speelt CD34 een rol bij het losmaken van de contacten tussen de aaneensluitende ECFCs, waardoor het uitgroeien van vaatachtige structuren bevorderd wordt ten opzicht van de CD34-negatieve ECFCs. Deze in vitro data ondersteunen het idee dat selectie van de meer angiopotente ECFCs op basis van hun CD34 expressie voorafgaand aan toepassing in vivo bijdraagt aan een snellere uitgroei. ECFCs bieden ook interessante mogelijkheden bij het bestuderen van ziekten, waarbij vasculair disfunctioneren een rol speelt. Pulmonaire arteriële hypertensie is een ernstige longziekte die gekarakteriseerd wordt door structurele veranderingen en afsluiting van arteriolen in het vaatbed en tot hartfalen leidt. Vergelijk van de uitgroei van ECFCs van PAH patiënten en gezonde personen, gevolgd door functionele karakterisering van PAH-ECFCs geven aan dat een toegenomen beschikbaarheid en excessieve groei van ECFCs een rol kan spelen bij de pathogenese van PAH.
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Samenvattend, de studies die in dit proefschrift werden gepresenteerd dragen bij aan onze kennis over ECFCs en hun rol in postnatale vascularisatie (vascularisatie bij herstelprocessen). Dit en de ontwikkelde methodieken bieden perspectief op toekomstige toepassing.
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Their guidance helped me in all the time of research and writing of this thesis.

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Thank you.

Dimitar Tasev
December 2017
CURRICULUM VITAE

Dimitar Tasev was born on 24th April 1974 in Strumica, Macedonia. He was educated and trained as physician during his study at Faculty of Medicine, University St. Cyril’s and Methodius, Macedonia. After obtaining his medical degree and MD license he worked as a general practitioner in Macedonia until 2009 when he moved to the Netherlands to pursue a MSc degree in Biomedical Sciences. During the two one-year research specializations in Advanced Immunology and Cardiovascular Research, he investigated the immunological aspects of multiple sclerosis and the role of endothelial progenitor cells in angiogenesis. From January 2012, he started his PhD track at Department of Physiology in the Angiogenesis research group of Prof. Dr. V. W. M. Hinsbergh and Dr. P. Koolwijk and this thesis was result of his work.