European Review for Medical and Pharmacological Sciences 2015; 19: 4047-4063

Focus on biological identity of endothelial progenitors cells

V. ZACCONE[1], R. FLORE[1], L. SANTORO[1], G. DE MATTEIS[1], B. GIUPPONI[2], D.D. LI PUMA[3], A. SANTOLIQUIDO[1]

[1]Department of Internal Medicine, Complesso Integrato Columbus, Catholic University of the Sacred Heart, Rome, Italy
[2]Emergency Department, Gemelli Hospital, Catholic University of the Sacred Heart, Rome, Italy
[3]Institute of Human Physiology, Gemelli Hospital, Catholic University of the Sacred Heart, Rome, Italy

Abstract. – Circulating Endothelial Progenitor Cells (EPCs) were discovered by Asahara et al in 1997 and defined as bone marrow CD34+/KDR+ cells endowed with angiogenic potentialities *in vitro* and *in vivo*. The most likely assumption is that EPCs consist of several cell subpopulations with functions targeted at accomplishing the post-natal neovascularization process in a synergic and complementary fashion. Indeed, the subsequent identification of numerous and differentiated hematic populations, characterized by the capacity to develop an endothelial phenotype, has posed a number of questions as to the real identity of EPCs. This concept does not represent a sterile speculation but rather it suggests important implications for the future practice of stem cell therapy.

The aim of this report was to explore through a critical analysis the two main experimental methodologies applied to EPCs, *in vitro* culture and flow cytometry, followed by a brief revaluation of the endothelial progenitors employing a globally functional approach.

Key Words:
Stem cells, Endothelial Progenitor Cells, Endothelium, Angiogenesis, Ischemia, Cellular plasticity.

Abbreviations

CD = Cluster of Differentiation; EPCs = Endothelial Progenitor Cells; CEPCs = Circulating Endothelial Progenitor Cell; VEGFR = Vascular Endothelial Growth Factor Receptors; FGF = Fibroblast Growth Factor; EGF = Epidermal Growth Factor; MNCs = Mononuclear blood Cells; PB-MNCs = Peripheral Blood Mononuclear blood Cells; CB-MNCs = Cord Blood Mononuclear Cells; CFU-ECs = Endothelial-cells Colony Forming Unit; ac-LDL = *Acetylated-Low Density Lipoprotein*; CACs = Circulating Angiogenic Cells; ECFCs = Endothelial Colony Forming Unit Cells; EOCs = Endothelial Outgrowth Cells; IGF 1 = Insulin like Growth Factor; Flt-1 = Fms-related tyrosine kinase 1; eNOS = endothelial Nitric Oxide Syntase; vWF = von Willembrand Factor; HUVEC = Human Umbilical Vein Endothelial Cells; Flk-1 = Fetal Liver Kinase 1; Tie-2 = Tyrosine Protein Kinase Receptor; CEC = Circulating Endothelial Cells; HSC: Hematopoietic Stem Cells; TCSC = Tissue Committed Stem Cells; M-CSF = Monocyte Colony Stimulating Factor; NGF = Nerve Growth Factor; HGF = Hepatocyte growth factor; G-CSF = Granulocyte Colony Stimulating Factor; GM-CSF = Granulocyte Macrophage Colony Stimulating Factor; TNF = Tumor Necrosis Factor; SDF-1 = Stromal cell-derived Factor 1; CXCR4 = C-X-C chemokine receptor type 4; HIF 1 = Hypoxia-Inducible Factors1; c-MET(HGF-R) = Hepatocyte growth factor Receptor; LIF-R Leukemia Inhibitory Factor Receptor; MSC = Mesenchymal Stem Cells; HAECs = Human Aortic Endothelial cells; HPP-ECFC: Huge Proliferative Potential Endothelial Colony Forming Unit Cells; TEPs = Resident Endothelial Precursors; TPC = Tissue resident Progenitor Cells.

Introduction

Until 1997, it was believed that mesodermal cells differentiating in angioblasts and subsequently in endothelial cells took place only during embryo growth. This belief was dismantled by Asahara et al study[1], in which hematopoietic progenitor cells CD34+, able to differentiate *ex vivo* in an endothelial phenotype, were purified in adults. These cells, known as circulating "Endothelial Progenitor Cells" (EPCs), showed characteristics of "staminality" (self-renewal) and "clonogenicity" as well as the possibility to differentiate themselves in an endothelial way. They showed characteristics of expressing various endothelial markers and the ability to incorporate in neo-vessels in the ischemic sites.

In 1998 the Shi's group[2] also reported the presence of bone marrow-derived "Circulating Endothelial Progenitor Cells" (CEPCs) in adults. These cells also constituted a group of hematopoietic CD34+ stem cells able to differentiate in the endothelial lineage and to express typical markers of the endothelial cell, such as

Corresponding Author: Li Puma Domenica Donatella, Ph.D; e-mail: donalipuma2015@libero.it

the vWF; likewise, in a canine model, donor bone marrow cells were found to be able to coat a Dacron graft implanted on the host thoracic aorta. These pioneering researches suggested the presence of circulating hemangioblasts in adults.

The first discoveries defined EPCs or CEPCs as cells positive not only for markers typical of hematopoietic stem cells, such as CD34, but also for endothelial surface proteins, such as VEGFR2/KDR. Since CD34 is expressed both on hematopoietic stem cells and mature endothelial cells (in this last case in lower amounts), its specific characteristics were not sufficient to discriminate EPCs. Further studies used a marker typical of more premature hematopoietic cells, CD133[3], and showed that the purified CD133+ cells can differentiate into endothelial cells *in vitro*. The CD133, also known as prominin or AC133, is a well-conserved antigen expressed on the hematopoietic stem cells, but it is absent on the mature endothelial cells and is characterized by a biological activity not yet clearly defined. Therefore, positivity for CD133 and VEGFR2 allows a more specific identification of the premature endothelial progenitors, while CD34+ VEGFR2+ cells could correspond to vascular wall lost cells.

Cell isolation is technically performed through adherent culture of all mononuclear cells, or by positive pre-selection using magnetic micropearls coated with antibodies against surface markers such as CD133, CD34 or CD31. Subsequently, these cells are cultured on fibronectin plates with specific growth factors such as VEGF, bFGF or EGF, which facilitate the growth of endothelial-like cells.

Materials and Methods

We systematically reviewed the current literature on biology, physiology and physiopathology of EPCs and examined the two main methods of investigation: *in vitro* culture and flow cytometry.

Results

Characterization and Origin of Endothelial Progenitor Cells: a New Tower of Babel?

Methodological and Theoretical Studies

Although Asahara et al[1] and Rafii et al (Nat Rev Cancer 2002; 2: 826-835) clearly defined EPCs as circulating stem cells characterized by endothelial markers, we do not have clear indications about the origin of these cells; this gap can be attributed to the low specificity of the techniques used for isolation and identification of EPCs: *in vitro* culture and flow cytometry.

Circulating EPCs are isolated from mononuclear peripheral blood cells and are subsequently cultured in a medium which favours endothelial differentiation. However, many possible sources of endothelial cells among the mononuclear peripheral blood cells can be identified: (1) the rare hematopoietic stem cells, (2) the circulating mature endothelial cells that are released from the vessel wall and can adhere to culture medium, (3) the myeloid cells, that can differentiate into endothelial cells in a culture adapted to stimulate the differentiation, (4) other circulating progenitor cells (side population cells); therefore it is difficult to establish which of above-mentioned cells is directly involved in the formation of endothelial cell and in neo-angiogenesis. Moreover, there are numerous cultural methods which have led to the recognition of cells able to produce an endothelial-like phenotype, although none of them provide a clear indication as to the real identity of EPCs.

In addition, even flow cytometry is not able to accurately define the precise nature of EPCs. The markers identified by Asahara as characteristic of the circulating EPCs (CD34+, VEGFR+) can be found in various cellular types present in blood and in peripheral tissues (hematopoietic stem cells, circulating endothelial cells, monocytes-macrophages). This immunophenotypic overlap could counteract the identification of the ontological and phylogenetic characteristics of EPC. On the other hand, subsequent flow cytometry studies, which more generally analyze immunophenotype of the blood cell populations able to generate differentiated endothelial cells, have identified among these populations phenotypes other than those classically attributed to EPCs (CD34+, KDR+, CD133+). Moreover, the correspondence between the quantitative results of the culture method and the numerical data of the cytofluorimetric technique is low[4].

The following biomedical research added further confusions to the previously mentioned methodological limits rather than clarifying them. The first EPCs scientific works were conducted without considering two important principles of the biological structures: the plasticity of cellular systems and the complexity of physio-

logical processes. Only by evaluating these factors the true nature of EPCs can be effectively identified.

Cellular Plasticity

In recent years biomedical research has demonstrated that our organism represents a rich source of stem cells. Moreover, these stem cells have a variable level of cellular differentiation (totipotent, pluripotent, multipotent, cell precursors) and a plastic ability to modify their characteristics according to the organism's needs. Schofield[5] in 1983 wrote: "Stem cell properties do not reside in one specific cell type in the population but, when necessary, cells other than those normally playing the stem cell role, can have stem cell function imposed upon them by the appropriate microenvironment. The postulate is offered that there are no cells which are intrinsically stem cells, but a range of cells in a tissue possess stem cells potential to a greater or lesser extent".

From this point of view, the pro-angiogenic microenvironment (present in EPCs cultures) puts cultivated mononuclear cells in a condition of stress, in which the system's plasticity directs the differentiation and the proliferation in endothelial direction. In this culture microenvironment we have identified cells able to duplicate and to differentiate in an endothelial fashion but this fact does not necessarily mean that a population of lineage-committed stem cells (so-called "Endothelial Progenitor Cells") has been characterized. On the contrary, as described below, there could be several confounding factors like: (1) the proliferation of already differentiated cells, (2) the trans-differentiation of specific circulating stem subsets (3) or the possibility of a differential contribution of more subsets to this process.

Nevertheless, it is clear that all these cells can contribute to the neoangiogenesis process *in vivo*, when the most likely microenvironmental conditions of the organism are similar to the cultural ones, for example during a peripheral local ischemia.

Complexity of the Neovascularization Postnatal Process

Different cellular populations contribute to neovessel construction in a specific and complementary way. Numerous studies have demonstrated that EPCs have the ability to enhance the neovascularization process *in vivo*; however, this functional common denominator did not allow to provide EPCs with a precise cytologic label. On the contrary, it highlighted an extreme variability in EPCs populations capable of promoting neoangiogenesis. In this regard, it is likely that each of these cellular populations plays a specific role in the complex process of neovascularization.

In conclusion, at present, the origin and nature of EPCs seems to be anything but defined; in this sense some authors defined EPCs "a new tower of Babel"[6]. We can ask several questions on the functional characteristics of such cells[7] (proliferation and physical incorporation? Paracrine function? Re-modeling of the micro-environment?). The fundamental answer to the latter can be found only after a precise cytological identification of EPCs.

Though this debate begin by evaluating a biological perspective it does not represent a sterile speculative matter and it could reveal fundamental aspects for potential clinical diagnostic and therapeutic applications of EPCs.

One or more EPCs? The Role of Culture

Researchers are more certain than ever that the cell population, defined as EPCs, is composed of heterogeneous subpopulations, in which the common denominator is the ability to differentiate/trans-differentiate into an endothelial phenotype and/or contribute in strengthening the neovascularization process[8]; research in this field aims to distinguish the various populations, to clarify their origin and hierarchy and to define their role within the complex neovascularization process.

Three culture methods to select EPCs *in vitro* by mononuclear blood cells (MNCs) can be used, as described below.

According to the original method, improved by Asahara et al and afterwards modified, the culture of MNCs (Mononuclear Cells), taken from peripheral blood (PB-MNCs, Peripheral Blood Mononuclear Cells) or from cord blood (CB-MNCs, Cord Blood Mononuclear Cells), is made on fibronectin medium. After 48 hours of pre-plating, so as to remove mature macrophages and adherent endothelial cells, the cells that do not adhere to the medium are taken and re-cultured on fibronectin substrata. After 5-9 days of culture, cell colonies formed by central round cells and peripheral spindle-like cells were found; these kinds of colonies were defined as CFU-ECs (Colony Forming Unit-Endothelial

Cells) or CFU-Hill cells. The combination of cultural and flow cytometry data shows that, among the mononuclear cells adhering to fibronectin in the early phase of the culture, 15.7% expressed CD34+, 27% VEGFR+, while 11% showed a double positivity CD34+ VEGFR+. After 7 days of culture, CFU-ECs showed an endothelial phenotype positive for CD34, VEGFR, CD31, Tie-2 and E-selectin, positive for both endothelial and hematopoietic markers.

According to a second culture method, MNCs are put in a medium enriched to help the endothelial growth for 4 days; the behavior of the adherent population is then analyzed while the non-adherent fraction is removed. The cultured cells show a phenotype and some functional characteristics identical to those of endothelial cells (for example the ability to bind the Ulex Europaeus Agglutinin I [UEA-1] and to incorporate ac-LDL), but at the same time they have hematopoietic markers. Such adherent cells were defined as CACs (Circulating Angiogenic Cells) and show phenotypic and functional characteristics which are compatible with those of CFU-EC; for this reason, both cell populations have been named EPCs. It's interesting to notice that, on the one hand, the morphology in CACs culture is not identical to that in the CFU-EC one, but, on the other hand, separate culture of spindle-like cells, taken from CFU-EC, show a similar shape to that of CACs. Moreover, it can be observed that CACs are well represented in the population of MNCs (about 2%).

According to the third culture method, PB-MNCs or CB-MNCs are put on a substratum formed by collagen type 1, enriched for endothelial growth. During the culture, non-adherent cells were repeatedly removed; after 10-21 days we observed the development of cell colonies from PB-MNCs, while for CB-MNCs 5-7 days were sufficient. Such colonies are constituted by adherent cells defined by some authors as ECFCs (Endothelial Colony Forming Unit Cells), by others as EOCs (Endothelial Outgrowth Cells), which show a phenotype identical to that of endothelial cells, do not display hematopoietic markers, present cobblestone morphology (typical of endothelial cells), have a high proliferative potential *in vitro* and the ability to form new vessels *in vivo*.

A first rough differentiation among the subpopulations of EPC can be made on the basis of the replication kinetic. CFU-ECs and CACs emerge too early from the cultures and, for this reason, they have been defined as "early outgrowth" EPCs; ECFCs develop later and so they have been defined "late outgrowth" EPCs. Hur et al[9] analyzed the differences and the links existing between these two EPCs populations, reproducing them in culture and verifying the cytologic and functional characteristics both *in vitro* and *in vivo*. These authors cultured the PB-MNCs on a pro-angiogenic medium, enriched with factors like VEGF, hEGF, hFGF-B, IGF-1, and subsequently considered end-points such as the growth curve, the secretion of cytokines, the ability to develop tube-like structure *in vitro* and the ability to produce neo-vasculogenesis *in vivo*.

Early EPC increase in number within two weeks of culture, reaching 3×10^5 -5×10^5 from 50 ml peripheral blood cells, subsequently slightly proliferate and finally slowly die; the immunophenotypic characterization shows both monocytic and endothelial surface characteristics, with intimal markers like VE-cadherin, KDR, Flt-1, e-NOS, vWF. Late EPCs, on the other hand, proliferate slowly in the early phase of the culture, but subsequently increase their replicative activity rapidly and expand from 20 to 10^{19} cells within six weeks. Late EPCs displays a immunophenotypic profile lacking in CD1a and in CD14, which is identical to the one present on microvascular endothelium, with an expression of endothelial markers which is more marked than that of early EPCs. From a functional point of view, *in vitro* early EPCs show an extension of their cellular structure, forming spindle-like shapes, but without determining the development of tube-like structures. Late EPCs, on the contrary, are able to form tubular capillaries on a matrigel substratum. However, early EPCs, if cultured together with the HUVEC (Human Umbilical Vein Endothelial Cells), are able to form tubular structures at a rate that is slower than that of late EPCs. Conversely, *in vivo* vasculogenic potential of early and late EPCs shows no significant differences. Moreover, late EPCs proved to be more competent in producing nitric oxide (NO), therefore fulfilling the endothelial function.

Hur et al[9] tried to interpret these data, making a comparison with those resulting from previous studies. Firstly, from an immunophenotypic point of view PB-MNCs are positive for CD45, but also for Flt-1, eNOS, vWF and CD31. Early EPCs lose CD45 and CD31 markers progressively, but show high levels of KDR and VE-cadherin expression, which, in turn, disappear after three weeks of cul-

ture; these data agree with those of Asahara et al[1]. At the same time other studies showed conflicting evidences with regard to genic expression of early EPCs, above all to the expression of KDR, CD14 and Flt-1. This suggests that early EPCs represent a very heterogeneous cell group. On the other hand, considering that genic expression of late EPCs is quite constant, this population could be formed by homogeneous elements.

Hur et al[9] assumed the connection between these two populations observing the behavior of early EPCs marked with red fluorescent protein, in order to analyze the culture end of these cells. Although most of them died within two days of re-plating, a small amount formed numerous late EPCs colonies and caused a dilution of the red coloring agent. This data suggested that the heterogeneous population of early EPCs could contain the progenitors of late EPCs, which could have produced final late EPCs; this means late EPCs could derive from a subset of early EPCs.

Evidence of both genic/immunophenotypic expression and functional behavior shows that late EPCs have an endothelial characterization that is more marked than that of other cells. Some of these differences seem to be correlated to a greater expression of KDR by late EPCs, and this condition implies a greater responsivity to VEGF, causing a greater NO production and a greater ability to form tubes *in vitro*. Moreover, a more consistent presence of VE-cadherin, an endothelial adhesion molecule, on late EPCs, helps incorporation of them on HUVEC monolayers.

These data *in vitro* do not agree with those *in vivo*, where we find a vasculogenesis potential which is basically identical in the two groups. However, in the situation *in vivo,* the two populations could act in a different way during the process of neoangiogenesis. Particularly, early EPCs, because of their heterogeneity, could help the construction of new vessels in many ways, but their principal activity would be the secretion of growth factors and pro-angiogenic cytokines; on the contrary, late EPCs would be more specifically associated to a physical incorporation of numerous endothelial elements proliferating in the new vessels[9,10]. Supporting this hypothesis, it was demonstrated that the combined transplant of early and late EPCs in the site of ischemic lesion, allowing the synergic functional collaboration between the subpopulations to the neovascularization process, determines an increase in the number of neo-vessels, against the situation in which a single population is used[11].

One or More EPCs? The Role of Flow Cytometry

Now we have to more precisely define the nature of EPCs by: (1) identifying the possible subpopulations included in the classic EPCs population (CD34+, VEGFR+, CD133+); (2) considering the possible existence of other EPC sub-populations, besides the classic phenotype described by Asahara; (3) finally, verifying which of the sub-populations can be considered real EPCs, to which level and which function they carry out in the complex process of neoangiogenesis.

Since their discovery, researchers ascribed EPCs an anatomic bone-marrow origin and identified, on the basis of the immunophenotypic data, a possible derivation from the hematopoietic lineage; subsequent studies pointed out how such basic assumptions cannot be considered absolute, but must be related to a particular analysis of the origin of the various EPC sub-populations.

At the beginning of this research, Asahara[12] worked on transgenic rats, expressing beta-galactosidase under the action of specific endothelial promoters (Flk-1/LZ or Tie-2/LZ), and used them as bone-marrow donors; transplanted rats showed, in the endothelial cells of physiologic and pathologic neovascularization sites (e.g. corpus luteum, endometrial tissue, tumoral tissue, local ischemia), an evident expression of donors genes, displaying a bone-marrow origin of EPCs and their role in the neovascularization *in vivo*.

Gunsillius et al[13] tried to better analyze the derivation lineage of EPCs. They verified how patients with chronic myeloid leukemia (disease characterized by the presence of chromosomal translocation $t^{9,22}$ in the hematopoietic clone) presented this mutation also on CFU-EC colonies. They concluded that EPCs should derive from hematopoietic lineage.

Strictly related with Asahara et al[12] and Gunsillius et al[13] studies, Yoder et al[14], with the aim of defining the clonal origin of cells derived from CFU-ECs and ECFCs cultures, demonstrated that the cell types expressed in the two different methods would derive from non-related lineages. In particular, the authors analyzed the EPCs of 11 patients with polycythemia vera, a clonal disease of the bone marrow, characterized by the presence of the V617F Janus Kinase 2 (JAK2) mutation. CFU-ECs cultures showed mutation in all their colonies, therefore proving their origin from hematopoietic lineage. *Vice versa*, ECFC showed this mutation only in three of 89 colonies, displaying how ECFC derive from a

cellular lineage which is different from the hematopoietic one and questioning, but not totally denying, also the bone-marrow origin of these cells.

Yoder et al[14] studies do not completely contradict Asahara and Gunsillius' hypothesis, rather they supplement it, better defining the matter in the light of the characteristics pointed out by the different methods of cell culture. Yoder says that CFU-EC/CAC belong to the hematopoietic lineage and, therefore, confirms their bone-marrow origin; this is in line with the studies of Asahara and Gunsillius, who probably analyzed the precursors of this cultural population. Afterwards, Yoder et al[14] show that ECFC population is extremely different from CFU-EC/CAC not only in their cytologic and cultural characteristics but also in their intrinsic clonal derivation, originating from non-hematopoietic lineage.

The fact that a fraction of EPCs can derive from a non-hematopoietic lineage lead us to believe that EPC/ECFC can originate not only from a bone marrow source of non-hematopoietic nature, but also from a non-bone marrow source (peripheral tissue). In this sense, previous studies had already supported this hypothesis: in 2002 Hillembrands et al[15] observed that the endothelial regeneration in a model of transplant of arteriosclerosis was induced by the action of cells of non-bone marrow derivation. In this perspective, Lin et al[16] analyzed circulating endothelial cells (CEC) and EOC/ECFC colonies derived from their culture. This study pointed out how 95% of CEC derived from peripheral tissue, probably of vasal origin; they showed a slight proliferative potential *in vitro*, with a peak value of growth in the early phase and a 27-fold cell expansion after 4 weeks. Remaining 5% of CEC was of bone-marrow origin, displayed maximum proliferative potential in the late phase, and had a 1023-fold expansion after four weeks, therefore greatly helping the expansion of the total volume of cells. The authors clearly show how in peripheral blood two different lineages of ECFC progenitor cells exist, included in CEC; the first, deriving from periphery, with a more represented number, but with a limited proliferative potential; the second, deriving from bone-marrow space with a great proliferative potential.

These studies have a great effect on the below classification, because they point out two ways to follow in the research of the origins of EPCs subpopulations: (1) the bone-marrow hematopoietic lineage for CFU-EC/CAC and also for a part of ECFC (in its ancestral component, as hemangioblast); (2) the non-hematopoietic lineage for ECFC, in which we can identify: (a) cells of bone-marrow origin and (b) cell of non-bone-marrow origin (Figure 1).

Hematopoietic Lineage

Hematopoietic lineage has a great number of cell types assigned to represent EPC subpopulations able to adopt an endothelial phenotype and

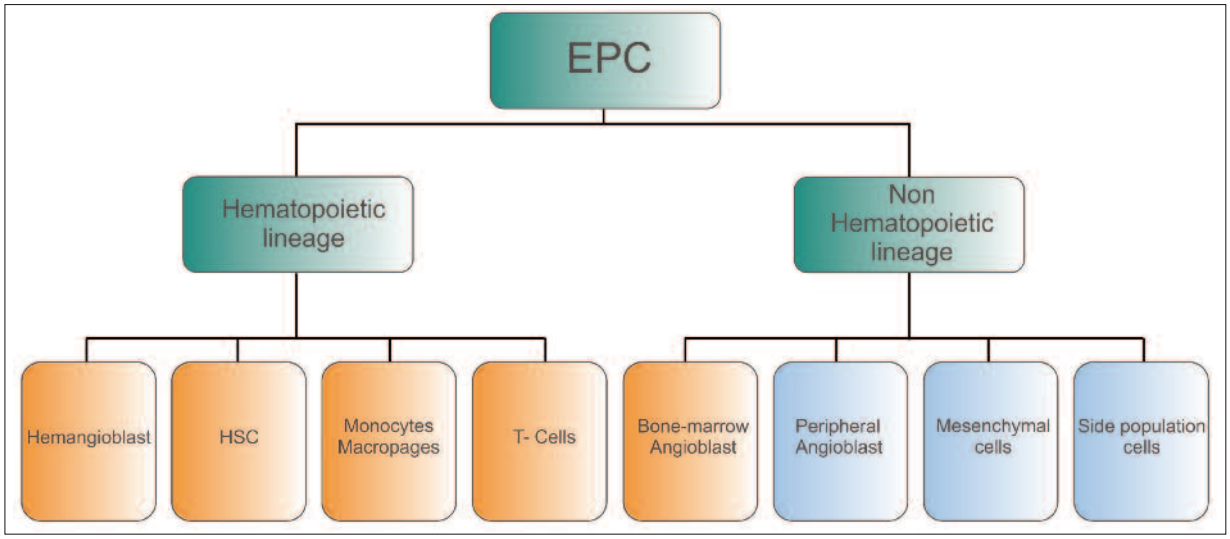

Figure1. Possible EPCs subpopulation and their anatomic derivation (Orange = Bone marrow origin; Blu = Peripheral tissues origin).

to help neovascularization processes; they start from ancestral progenitors, like hemangioblasts and Hematopoietic Stem Cells (HSC), to the most differentiated monocyte population. Even other cell types, generally not considered EPCs, such as the immune system cells, have shown unexpected pro-angiogenic capacity.

Hematopoietic Stem Cells

First of all, with regard to the relationship between EPCs and HSC, even in this particular case the immunophenotype definition, based on clusters of differentiation (CD), caused a lot of problems in the exact identification of the existent relationships between cell types. EPCs experimental data have identified a CD34+, CD133+, KDR+ phenotypic pattern which is identical to the one that characterizes HSC. This biological overlap caused many problems in the exact identification of EPCs. More studies have been taken up to find out if this immunophenotypic concordance between HSC and EPC also implies a cytological and functional concordance. In other words the problem is to find out the relationship between two cellular types whose immunophenotype is identical but with different functions and also if there are differences that allow us to distinguish the two cells.

Bailey et al[17] remark HSC trans-differentiation ability by using *in vivo* tests of bone marrow transplant on irradiated mice; the analysis of peripheral tissues clearly showed an incorporation of bone marrow cells in the intima. For this reason, scientists concluded that HCS plays a role in the constitution of peripheral vessels by multiplying and modifying its own cellular phenotype.

This theory was doubted by a study carried out by Case et al[18] who measured *in vitro* the EPC potential of the hematopoietic progenitors. By cultivating the classic CD34+ CD133+ VEGFR-2+ cells, they showed that these had only a Hematopoietic CFC (Colony-Forming Cell) capacity and no EPCs capacity. As a matter of fact, the problem unresolved by this conclusion can be solved by considering the important role played by CD45 pan-leucocyte markers in characterizing this population. While C34+ and CD45+ cells (> 99% of CD34+ CD133+) continued to express hematopoietic power and did not act as endothelial progenitors, CD34+ CD45- cells formed EPC/ECFC colonies in culture but never CFC. These results are important for EPC experimental practice, considering that many diagnostic and therapeutic tests have only studied CD34+ CD133+ VEGFR-2+ markers not considering CD45 which, if negative, identifies a TCSC (Tissue Committed Stem Cells) stem cell population (EPCs potential) with different characteristics from hematopoietic progenitors (see forward)[19].

However, other studies do not exclude that CD34+ CD45+ hematopoietic phenotype cells can evolve into a sort of endothelial development. Timmermans et al[20] showed how CD34+ CD45+ cells can generate CFU-EC endothelial cell type by producing monocytes progenitors, as demonstrated by the presence in culture of many monocytes/macrophages (even if EOC/ECFC remains typical only of CD34+ CD45- cells).

For this reason, the HSC's endothelial production is probably not direct but linked to the constitution of monocyte progenitors. These cells have different stages of pro-endothelial activity.

Hemangioblast

A second kind of cell population endowed with EPCs potential is represented by hemangioblasts, bipotent embryonal cells which originate from mesoderm line. Their function is to produce both hematopoietic and endothelial progenitors during the ontogenesis. The first identification of the hemangioblast was achieved by analyzing embryonal blood islands in vitelline membrane where it was possible to observe cellular homogeneity accompanied by a double cytogenetic capacity, which is the capacity of producing an external layer of endothelial cells and an internal layer of blood progenitors. Therefore, we concluded that during fetal life the hematic and endothelial cells' progenitor is the same: the hemangioblast[21].

From that time onwards many studies tried to demonstrate the duration of the hemangioblast in full age. Some researchers, like Grant et al[22], tried to prove the existence of adult hemangioblast by using *in vivo* models. In animals suffering from diabetic retinopathy it was possible to show that HSC cells that came from bone marrow had hemangioblast qualities. But we also know that in these cases *in vivo* models are not very specific and this evidence could be due to the angioblast activity of TCSC medullary cells which could show HSC's phenotypic characteristics[19].

Pelosi et al[23] study seems to be more convincing. He demonstrated *in vitro* that 5% of CD34+ KDR+ cells ($5/10^6$ of MNCS) has hemangioblastic qualities. In particular, this cell type, when was

isolated in pure culture of individual cells in pro-hem, pro-end or pro-hem-end micro-environmental stimulations, produced respectively hematopoietic, endothelial or mixed colonies. This proved the bi-potential of these types of cells and their great ability in modifying themselves according to micro-environmental modification.

Therefore, the hemangioblast, as demonstrated for other cell progenitors, remains even later in life, thus representing a source of endothelial cells[24,25]. Although its scarce presence in peripheral blood makes minimum the contribution to the vasculogenesis process in basal conditions, some studies show how, like the other EPC subpopulations, the hemangioblasts undergoes a process of reactivation in case of ischemia[26]. This supports that its neo-angiogenic activity is not slight in conditions of alterations of vascular homeostasis.

Monocytes/Macrophages

Monocytes/macrophages, a blood population highly represented in peripheral blood, in many *in vitro* studies demonstrated the ability to proliferate and differentiate in endothelial sense, thus giving a contribution to *in vivo* neovascularization process.

Monocytes/Macrophages represent the 5-10% of circulating leukocytes. They are made up of an extremely heterogeneous population whose phenotypic features are strongly influenced by stimulations from the surrounding microenvironment. Therefore, circulating monocytes could develop and differentiate themselves according to the body's local needs. This is demonstrated by monocytes' huge phenotypic range in peripheral blood: dendritic cells; Langerhans cells; osteoclasts; alveolar macrophages; NCS's macrophages; splenic macrophages; Kupffer cells; inflammatory monocytes[27]. In this prospective, the chance of a possible differentiation of monocytes/macrophages in endothelial sense cannot be left out. These two cell types seem to share many functional and immunophenotypic qualities and they also seem to play the same role in the formation of tissue structures (we can take the reticulum-endothelial system as an example); some authors[28] also remark on the chance of a cell overlap between these two populations which would lead to the use of more restrictive classification criteria.

In 2000 Fernandez Pujol et al[29] proved that human CD14+ monocytes, in the presence of pro-angiogenic growth factors and hydrocortisone, produce the so called "endothelial-like cells". These cells were generated in culture after one week and exhibited v-WF, CD144 (VE-cadherin), CD105, acLDL receptor, CD36, VEGFR-1 endothelial markers first, and also VEGFR-2 later. The morphological characteristics of the "endothelial-like cells" cultures were similar to those of CFU-EC cultures: at the beginning there are small mononuclear cells which become oval cells; these cells eventually produce a population of "spindle-shaped granulated cells". With this experiment Fernandez Pujol et al[29] demonstrated that pure monocytes in peculiar conditions can produce endothelial cells.

Gulati et al[30] tried instead to find out the role played by monocyte populations in EPC cultures. These authors were able to obtain CFU-EC and EOC-ECFC cultures from PB-MNC. Unlike ECFC which were CD14-, CFU-EC cultures were positive to CD14 (marker typical of monocyte population). This demonstrated the cellular heterogeneity of the various EPC cultures and allowed to better understand the characteristics of CFU-EC monocyte population. Gulati et al[30] hypothesized that, even if monocytes in culture (according to Fernandez Pujol studies[29]) were able to differentiate in endothelial populations; they had neither stem characteristics in proliferative capacity, nor were they able to give a remarkable contribute to the formation of neovessels. The authors thought their function was to produce paracrine secretion during the angiogenic process.

In 2003 moreover, Zaho et al[31] identified a particular subset in the complex monocyte population with all the characteristics of a pluripotent stem cell. Such cells, identified as f-macrophages (f-MΦ because of their resemblance with fibroblasts), have a CD14+, CD34+ and CD45+ immunophenotype. Unlike classical monocyte, they reproduce themselves in the presence of Monocyte Colony Stimulating Factor (M-CSF) and in case of specific environmental stimulations they are capable of forming different types of cell: mature macrophages (under Lipopolysaccharide), T lymphocyte (under Interleukin-2), epithelial cells (under EGF), neuronal cells (under Nerve Growth Factor), hepatic cells (under HGF). VEGF stimulation allowed the production of endothelial cells, with the formation of cobblestone-like structures positive to VEGF-R2, VEGFR-3 and vWF.

An evident heterogeneity of EPCs monocytes characteristics poses some questions on the real function of these cells in the neovascularization

process; more in general, this problem can regard all Endothelial Progenitors Cells. Gulati et al[30] emphasized the paracrine function; this would agree with the most intrinsic cellular qualities of the monocytes/macrophages line and would partially lead to justify the apparent paradox for which *in vivo* they give a huge help in increasing the formation of neovessels, even if there are very few EPC cells in the vessels. Rehman et al[32] studies agree with this thesis. They managed to show that in EPC's cultures originating from monocytes there is a production of many pro-angiogenic cytokines such as VEGF, HGF, G-CSF and GM-CSF. On this basis we think that's appropriate Rabelink et al[33] question, if EPC reaction is actually a stem activity or if it is just an inflammatory reaction to ischemic damage. In support of this hypothesis, recent studies[34,35] show how EPCs represent, compared to other cell types, the privileged target of post-ischemic pro-inflammatory mediators, like TNF-alpha.

Fujiama et al[36] on the other hand, remarked that monocytes can physically help the process of focal re-endothelization; this would find a physiopathological placement to the high differentiation capacity and to the little proliferating capacity of most of these cells. In contrast, Moldovan et al[37] demonstrated that monocytes/macrophages, during neovascularization process, can help the construction of neovessels by forming metalloelastase-dependent tunnels in ischemic myocardium.

More studies emphasize the contribution that myeloid line gives to endothelial regeneration, remarking on its important role in physical neovessels production[38]. On the other hand, Urbich et al[39] tried to remark that CD34-/C14+ myeloid cells can help to produce *in vivo* neovessels on ischemic tissue, only if they are previously put in *ex-vivo* culture in medium with VEGF.

Even in this case, the different results achieved by the authors probably depend on different experimental conditions and on the analysis of different cellular subsets in monocyte population. In 2001, in this way Harraz et al[40] demonstrated that CD34- CD14+ cells, which had differentiated in endothelial sense, subordinated their physical incorporation in damaged vessels to the presence of other CD34+ cells, thus remarking that leukocyte-leukocyte interactions, which are typical of neovascularization process, are necessary for a correct homing of EPCs derived from monocytes.

Macrophages seem to have a wide range of functions in neovascularization process, according to the plasticity and the extreme functional heterogeneity of these cells.

Many authors, on the basis of the particular characteristics of the monocyte derived "endothelial-like cells", does not classify CD45+ CD14+ endotheliocytes as proper endothelial cells; according to Schatteman et al[28]. This concept seems not be justified because it would deny demonstrated EPC properties of monocyte.

Other Types of Cells

Finally, we would like to refer about some experimental data which proved the presence of pro-angiogenic populations among hematopoietic cells that are not generally classified as endothelial progenitors, such as the immune system cells. Rohde et al[41] pointed out that the formation of CFU-EC colonies deriving from mononuclear peripheral blood cells is highly dependent on both monocytes and T cells' action, probably because of the important role they play in the secretion of pro-angiogenic cytokines, like TNF-α. CD34+ cells are not required for CFU-EC production and pure cultures of CD34+ cells would not allow to obtain CFU-EC colonies. Furthermore, it seems that in the immune system neither B cells nor NK cells are necessary for CFU-EC formation.

Non-hematopoietic Lineage

Lin et al[16] data (see above) proved the existence of two subpopulations among non-hematopoietic blood sources of EPC/ECFC. The bone-marrow subpopulation is poorly represented but has a high proliferative activity; whereas the not-bone marrow subpopulation is highly represented but has a much lower proliferative potential. Bone marrow population could be made up of all those stem cells that in the differentiation development process (image 1) come before endothelial cells (stem totipotent cell, mesenchymal cell). Among these, the angioblast lineage-committed progenitor, would surely represent a type of cell specifically directed to play EPCs function. Peripheral cells could be represented instead by a CECs stem subset derived from vessels or even by pluri-multipotent stem cells derived from parenchymal tissues.

Bone Marrow-Derived Cells

Medullary Angioblast

Different studies, directed to enquiry on bone marrow's regeneration capacity, support the hypothesis that endothelial/angioblast progenitor exists even in adults. To understand stem cell balances, it is essential to take into account the medullary heterogeneity theory, supported by Kucia et al[19] on the basis of proven experimental data. This theory is in contrapposition to both hematopoietic transdifferentiation and cellular fusion theories. According to these authors, during embryogenesis, the chemotactic stimulation, which is necessary in order to introduce HSC into the bone marrow, would be linked to signalling SDF-1/CXCR4 axis' action. At the same time this stimulation would not only recall HSCs, but would also play a role in attracting the so-called tissue committed stem-cells (TCSC), which own CXCR-4 receptor. TCSCs own CXCR4+, CD34+, AC133+, CD45- immunophenotype. There is a high concentration of these cells in fetal blood and they are also programmed to form different types of tissues (hepatocytes, myocardiocytes, muscular cells, nervous cells); in adults TCSC peripheral levels approximate to zero, but the existence of a possible reservoir in medullary cavity, as Kucia et al suggest, gives important prospects.

TCSC own qualities that are similar to those that are generally attributed to EPCs and these characteristics are also identical to those that are generally ascribed to HSC, thus causing a lot of confusion. According to many clinical studies on EPCs[42], G-CSF demonstrated that it can increase circulating TCSC levels[43]. TCSC also are mobilized in peripheral blood when stress and tissue damages occur because of hypoxia-HIF1-SDF1-VEGFA axis activation[43,44]. If this is true, likewise in the bone marrow a kind of EPC/TCSC cells exists. These cells are useful to endothelial development, have high proliferative capacities and can be defined as angioblasts. Angioblast theoretical characteristics seem to match with experimental data demonstrated by Lin et al[16] to characterize ECFC endothelial progenitor originating from bone marrow: bone-marrow cell, poorly represented in peripheral blood (5% of CEC) has highly endothelial proliferative capacities.

From a functional point of view angioblast, such as TCSC and HSC, would represent a reaction mechanism to tissue damage, ruled by signalling VEGF-SDF1; in ischemic-hypoxic damage it is important neovessels' rebuilding, which is obtained by EPC/angioblasts mobilization.

TCSC's possible immunophenotype contains CD34, CD133, CXCR-4 and other markers including CD117, c-MET (HGF-R), LIF-R; the expression of specifically endothelial proteins such as VE-cadherin or v-WF, can help in distinguishing the angioblast from other types of TCSC. CD45 negativity is necessary to distinguish angioblast and the other TCSCs from HSC[19].

By using this immunophenotype data, Kucia et al[19] pointed out that TCSC CXCR4+, CD34+, AC133+, CD45- can contaminate preparations of HSC CXCR4+, CD34+, AC133+, CD45+ which, without CD45 dosage, can be wrongly considered as HSC pure cultures. Thus, the presence of endothelial cells in HSC cultures is probably not due to a transdifferentiation of pure HSC's plastic phenotype, whereas it seems likely a possible activation of angioblast TCSC which contaminate medullary sample. Considering Case's tests on HSC CD45+[18], this would be another demonstration of HSCs' improbable EPC activity.

These conclusions seem to be right also for hemangioblasts; blood-endothelial bi-potential of a culture can simply come from hematopoietic HSC and angioblast TCSC coexistence.

These conclusions do not completely leave out hemangioblast existence and HSC's angiogenic power, but they also suggest to be really careful while considering past experimental results and they show that is important to take into account CD45 expression as a marker necessary to guarantee cellular sample's purity.

Mesenchymal Cells and Other Multipotent Cells

Mesenchymal Stem Cells (MSC) are multipotent cells which are not hematopoietic; they are placed in bone marrow and circulate in peripheral blood, they are able to differentiate in many mesoderm-derived cells (and not only). It seems that CD140b is a specific marker for mesenchymal cells[45]. Liu et al[46] studies showed that if cultured in Endothelial Growth Medium (EGM-2), MSC are capable of producing endothelial-like cells (MSCE) which are different from the usual EPCs, but still able to favour the neovascularization process, even *in vivo*. Other researchers have demonstrated how the mesenchymal cells can contribute to the process of tissue reparation, and so of neovascularization, even through the secretion of cytokines[47].

We cannot leave out EPCs' possible activity of other pluri-multipotent stem cells which are placed in our bone marrow but whose characteristics have not been examined yet.

Non-bone Marrow-Derived Cells

Peripheral Angioblast

Lin et al study[16] proved that if peripheral tissue Circulating Endothelial Cells-CECs (95% of CEC whole population) are cultured on a proper medium, they showed some proliferative capacity. So, a source of endothelial progenitors also exists in peripheral vassals beyond the bone marrow space. In this sense, a fraction of EPCs would be constituted by peripheral non-bone marrow cells of vasal origin, being part of a stem subset of CECs.

CECs (Circulating Endothelial Cells) are an extremely small hematic population (0.01%-0.001% of PB-MNC) which has a positive immunophenotype to endothelial markers (vWf+, CD146+, VE-cadherin+), in the absence of hematopoietic markers (CD45-, CD14-) and in the presence of progenitors' markers (CD133+, CD34+). CECs can be isolated by using either anti-CD146 immunobeads or flow cytometry, even if following quantitative results achieved by different work teams are extremely various. It would seem that they have vassal origins. In physiological conditions, even if intima layer, is in a relatively quiescent state, vascular sites submitted to stressful conditions, like branching off or turbulent flow zones, are more likely to be desquamate and would need a larger cellular turnover. Circulating CECs levels tend to have a 10-fold increase (or more) when pathological conditions, which damage vases (such as cardiovascular diseases, connective tissue, infections, cancers, blood diseases) occur[48].

The relationship between CECs and EPCs is not so well defined. Numerous data evidence that between these two cells there is a functional and phenotypic overlap which gives reason to believe that they have common origins. On the one hand, tissue CEC showed a good proliferative potential[16], on the other the increasing evidence shows the presence of progenitors/EPC markers (CD34 and CD133) on CEC[49]. Moreover, CEC and EPC levels seem to be inversely linked (for example when cardiovascular diseases occur, the number of CEC increases and the number of EPC decreases). Some authors wondered whether EPC and CEC represent two sides of the same coin[50]. It is probable that a CECs stem subset represents an EPC subpopulation. Thanks to classical microscopic anatomy, we know there are endothelial stem cells on intimal wall. It is possible to suppose that endothelial desquamation process causes the release of both senescent and apoptotic cells and cells endowed with stem potentials, which enrich circulating endothelial progenitors.

The stem potentials of the resident endothelium have already been demonstrated in 2001 by Alessandri et al[51] and better studied by Ingram et al[52]. Ingram analysed the proliferation characteristics of both "Human Umbilical Vein Endothelial Cells" (HUVECs) and "Human Aortic Endothelial cells" (HAECs)[52]. *In vitro* culture of HUVECs and HAECs single cells demonstrated that 52% and 53%, respectively, divided after 14 days and that 27% and 28% of above-mentioned proliferating cells formed colonies made up of 2000 cells. Other quantitative studies[53] demonstrated that 20% of HUVEC's progeny and 29% of HAEC's progeny had a huge proliferative potential (HPP-ECFC). This proved that even in vascular wall endothelium there are cells endowed with huge stem potential. These cells, desquamating and entering the blood flow, can enrich circulating EPCs' pool.

Grenier's[54] group has demonstrated the existence of "Resident Endothelial Precursors" (TEPs), stem cells which reside in peripheral tissues (muscle, adipose tissue, derma) in correspondence of small tissue vessels, characterized by low proliferative potential and by high ability to differentiate into endothelium. Physiopathologically a tissue damage leads them to proliferate and differentiate, although their angiogenic abilities are also present in basal physiological conditions. So, it is possible that these recent studies characterized a new angioblast resident population with proliferative ability *in situ*.

It seems that actually these cells carry on their activity not only at the origin tissue level, but they are released in the circulating flow in particular conditions to form sites of neovascularization at a distance. In this sense, Aicher et al[55] demonstrated how in a murine model of parabiosis lower limb ischemia can induce the mobilization of tissue stem cells non bone marrow derived, placed in perivascular zone and defined as TPC (Tissue resident Progenitor Cells c-kit+ CD 45-). They have, *in vitro*, the ability to form CFU-EC colonies and *in vivo*

can incorporate in the vessels of an ischemic tissue. In the same experiment, these authors showed that a part of TPC, EPC potential, derives from two precise parenchymal compartments: intestine and liver. Although in the discussion of the study the authors do not exclude that these cells could belong to tissue-specific stem cells (probably side population cells), likewise these populations can also characterize as resident angioblasts, both for their anatomic perivascular localization and their immunophenotypic definition.

Side Population Cells

The so-called "side population cells", that is the population of stem cells located in the parenchymal structure of peripheral organs, demonstrated to be able to represent a further source of endothelial cells. Because these cells are already resident, their role in the vasculogenesis process could have a double functional meaning: first, they would act as in situ EPCs, able to contribute to neovascularization directly on the injured site, without mobilizing; second, they could be released into the circulation by parenchymal organs, so constituting the circulating pool. Such cells are potentially present in every tissue; their existence has been visibly shown in the adipose tissue[56,57], in the cerebral tissue[58], and even in the dental pulp[59].

From a functional point of view, these cells could contribute in different ways to the process of postnatal neovascularization. If the transdifferentiative hypothesis is true, they could assume, in particular conditions, an endothelial phenotype, so physically contributing to the constitution of neovessels[56]. Some study groups, however, do not exclude that these cells could have paracrine functions, as we have already seen, for example, for the monocytes/ macrophages[57] (Table I).

Conclusions

Correlation Between Culture, Cytological Data and Future Directions

In a study, Prater et al[60] tried to interpret the heterogeneity of EPC population evaluating the cytology of PB-MNC.

In the population of PB-MNC CD45+ (99.9% of total PB-MNC), about 5% is composed of mononuclear circulating macrophages: most of them form the CACs and, for this reason, can be defined as EPCs. Especially, the CACs would represent a heterogeneous population of myeloid progenitors, particularly differentiated.

Table I. Summary of the main immunophenotypic and functional characteristics that define the different EPC subpopulations.

	Immunophenotype	Cytologic and functional characteristics
Hematopoietic lineage		
HSC	CD34+, CD133+, KDR+, CD45+	Improbable direct role in the EPCs constitution; possible vasculogenic activity through monocytic progeny
Hemangioblast	CD34+, KDR+,CD45-, PECAM+, FLK-1+ and VE-Cadherin+ (non-specific immunophenotype)	Proliferation and differentiation into endothelial cells; low presence in peripheral blood in basal conditions
Monocytes Macrophages	CD14+, CD64+, CD34 +/-	Eterogeneous population with variable charatheristics of plasticity and staminality
Others (B-lymphocytes)	B-lymphocytes phenotype	Essential collaboration to the development of the CFU-EC colonies
Non-hematopoietic lineage		
Bone-marrow angioblast	CD34+,CD133+, CXCR-4+,CD117+, c-MET+(HGF-R), LIF-R+,CD45-	The expression of specific endothelial proteins like VE-cadherin (CD144), CD146 or vWF, distinguish the angioblast from other TCSC
Peripheral angioblast	CD34+, CD144+, CD146+, CD117+	Stem subset CEC probably of vasal origin; medium proliferative potential
Mesenchymal cells	CD140b+	Develops endothelial-like EMSC
Side population cells	Variable phenotype	Variable proliferative potential

On the other hand, the cells with the function of hematopoietic progenitors (CD45+) are represented by a small number of adult peripheral blood (80/800 cells/ml); we find the same quantity (50/500 cells/ml) of the CFU-EC CD45+ in peripheral blood. According to this data, the CFU-EC population could derive partly from hematopoietic progenitors (CD45+)[61].

Among CD45- (0.01% of total PB-MNC), ECFCs would represent, instead, a subset of CECs, whose blood contents are extremely low: in adult peripheral blood we can find 3 CECs/ml, 0.05-0.2 ECFCs/ml; in umbilical cord blood 2-5 ECFCs/ml. Lin et al data[16] showed that CEC have 95% tissue origin and 5% bone marrow origin, so probably ECFCs CD45- are formed mostly by peripheral angioblasts and also by bone marrow angioblasts.

Future research should be directed toward the characterization of each of these cell populations capable of having neoangiogenesis, establishing the functional role of each one in the post-natal neovascularization process. Experimental practice of regenerative therapy could benefit from this characterization; by clarifying physiological and pathophysiological aspects of the angiogenesis, we will have the opportunity to improve the experimental methods at the base of clinical research.

Inclusion of EPC Subpopulation in the Complex Process of Neovascularization: an Integrative Hypothesis

The MNC subpopulations that would contribute to the constitution of EPCs would be extremely numerous and variegated: mononuclear circulating phagocytes, hematopoietic precursors, hemangioblasts, circulating endothelial cells, circulating angioblasts. Realizing that this "Babel" of populations constitutes those that once were defined EPCs is nothing to be afraid of, if we consider the two principles shown previously: the plasticity of cell systems and the complexity of the neovascularization process.

The conditions determined by the pro-endothelial culture stress stimulate all cell systems potentially angiogenic to address their genetic and cell program towards the realization of the neovascularization process. In this sense, PB-MNC subpopulations, undergone to culture and differentiated in EPC, can be divided, in the light of Hur's studies[9] and complemented with Prater's ones[60], into two principal groups:

1. Lineage-committed group in endothelial sense ("late" EPCs?, ECFCs? EOC?): probably homogeneous it would be intrinsically programmed in order to produce endothelial cells. In pro-endothelial culture it stimulates to realize its cell program through an intense activity of proliferation and differentiation.
2. Non lineage-committed group in endothelial sense ("early" EPCs?, CFU-ECs? CACs?): heterogeneous and numerically predominant. It would be constituted by cells that are not intrinsically endothelial but that in particular condition can assume an endothelial phenotype. The conditions caused by pro-endothelial culture stress would address their cell program towards a endothelial cells differentiation/trans-differentiation.

Now we can suppose that similar mechanisms replicate *in vivo*, following a basic rhythm. In other words, it is possible to have, even in a healthy organism, the environmental conditions reproduced in culture. So, a share of MNC is physiologically subjected to this process of proliferation/differentiation/trans-differentiation, so forming the classic EPCs. On the other hand, this hypothesis is supported by the fact that some emergency situations (such as a peripheral ischemia), that stress the micro-environmental and pro-angiogenic characteristics (i.e., exposition and release in blood of injured tissues, production of VEGF, release of chemiotactic factors), increase the number of circulating EPCs. This happens because the cells of our organism are plastic and try to adapt their characteristics to the needs of the systems, in order to maintain the homeostasis.

Such changes have to be considered from a perspective of complexity. The fact that different types of cell program/reprogram themselves in an endothelial sense shows that the realization of the neovascularization needs the involvement of more cell populations. Each of these, following the principle of the functional specialization typical of pluricellular organisms, contributes in a differentiated and complementary way to the complex process of neoangiogenesis (paracrine secretion, focal reendotelization, neo-tunells' formation, proliferation and constitution of tubiform structure, etc).

The mechanism could be the following: (1) the vascular system, being repeatedly stressed in some points[62], needs continuous processes of repair[63] (condition that is intensified in case of is-

chemic damage); (2) the organism produces signals (release of pro-inflammatory factors, exposition of the extra-cell matrix, release of endothelial growth factors like the VEGF, release of chemiotactic factors) that stimulate the cells to mobilize/proliferate/differentiate/transdifferentiate themselves in order to repair the harm caused through mechanisms of neoangiogenesis; (3) the various cell populations of peripheral blood and of bone marrow respond to the signals programming/reprogramming the cell system in pro-angiogenic way, through the activation of important genetic pathways[64]; (4) we can see the mobilization, the proliferation and the differentiation of the cells functionally assigned to neoangiogenesis, that is to the constitution of EPCs[65,66]; (5) the EPCs, not still completely mature, reach the site of vascular damage, where the interaction with the molecules of tissue adhesion, with the pro-angiogenic paracrine factors[67] and shear-stress[68], finish the process of cell differentiation in EPC sense; (6) we have a functional collaboration among the various subpopulations in pro-angiogenic sense; (7) finally, we have the neoangiogenesis and the reconstruction of the vascular homeostasis (Figure 2).

So, the term "Endothelial Progenitor Cells" assumes in this sense a different meaning from that given by the first authors. It does not represent a monomorphic cytological category but turns into a polymorphic functional category. EPCs would be a very heterogeneous cell population, constituted by entities with various cytological and functional characteristics, activated in order to contribute to the process of neovascularization.

If, on the one hand, this definition is correct, justifying the experimental identification of vari-

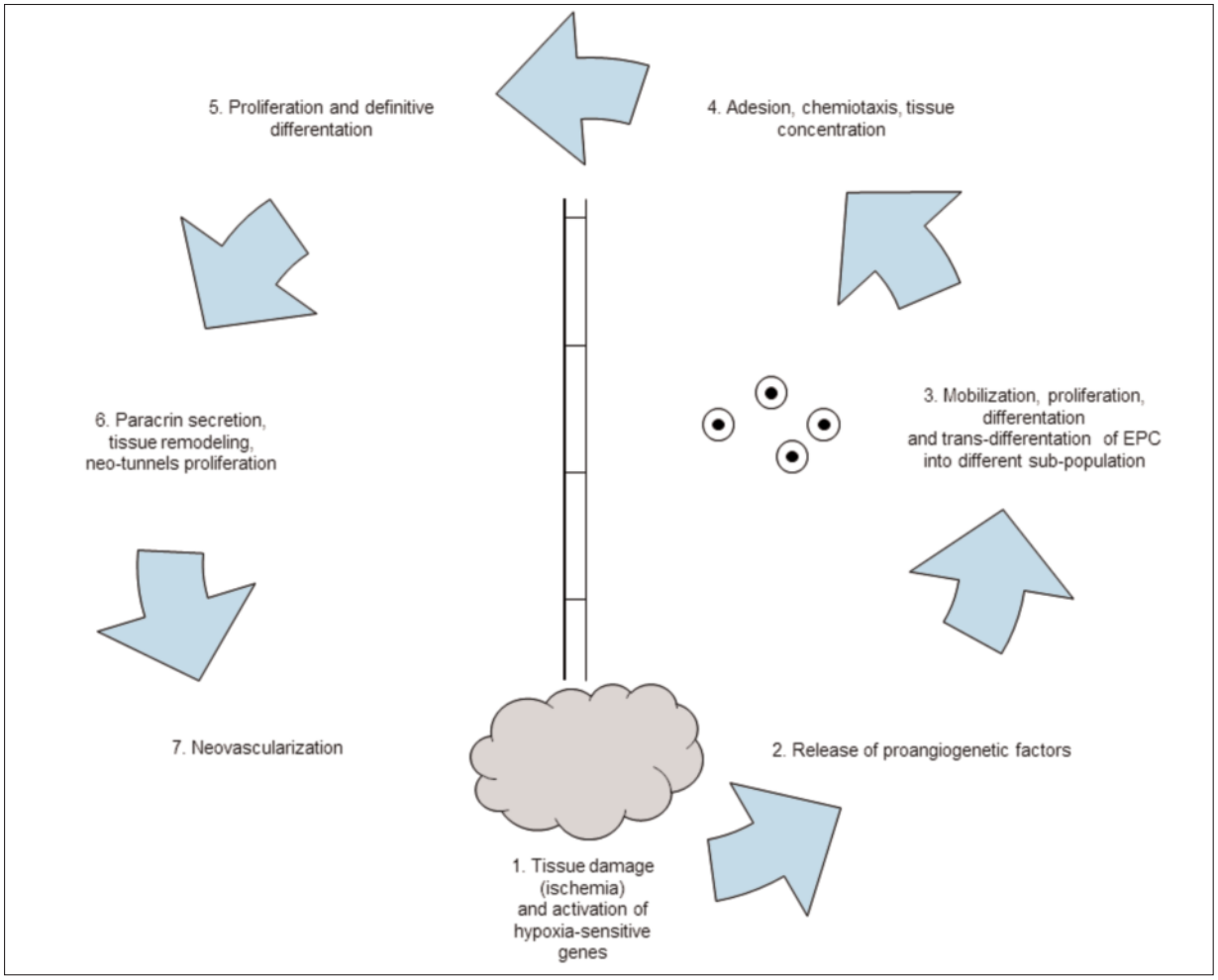

Figure 2. EPC mechanism of neovascularization (see text).

ous cells capable of assuming an endothelial phenotype and repairing the injured vessels, we have to wonder if, after more than fifteen years after the discovery of Asahara et al, the term Endothelial Progenitor Cells is by now obsolete and too "narrow" as regards to our knowledge, and it requires a complete critic revision.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science 2001; 275: 964-967.
2) Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MA, Storb RF, Hammond WP. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998; 92: 362-367.
3) Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, Rafii S. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000; 95: 952-958.
4) Tura O, Barclay GR, Roddie H, Davies J, Turner ML. Absence of a relationship between immunophenotypic and colony enumeration analysis of endothelial progenitor cells in clinical haematopoietic cell sources. J Transl Med 2007; 5: 37.
5) Schoilfield R. The stem cells system. Biomed Farmacother 1983; 37: 375-380.
6) Leor J, Marber M. Endothelial progenitors. a new Tower of Babel? J Am Coll Cardiol 2006; 48: 1588-1590.
7) Heil M, Ziegelhoeffer T, Mees B, Schaper W. A different outlook on the role of bone marrow stem cells in vascular growth: bone marrow delivers software not hardware. Circ Res 2004; 94: 573-574.
8) Miyamoto Y, Suyama T, Yashita T, Akimaru H, Kurata H. Bone marrow contain distinct types of endotelial progenitor cells and angiogenic cytokine producing cells. J Mol Cell Cardiol 2007; 43: 627-635.
9) Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, Oh BH, Lee MM, Park YB. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 2004; 24: 288-293.
10) Sieveking DP, Buckle A, Celermajer DS, Ng MK. Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. J Am Coll Cardiol 2008; 51: 660-668.
11) Yoon CH, Hur J, Park KW, Kim JH, Lee CS, Oh IY, Kim TY, Cho HJ, Kang HJ, Chae IH, Yang HK, Oh BH, Park YB, Kim HS. Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases. Circulation 2005; 112: 1618-1627.
12) Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999; 85: 221-228.
13) Gunsilius E, Duba HC, Petzer AL, Kähler CM, Grünewald K, Stockhammer G, Gabl C, Dirnhofer S, Clausen J, Gastl G. Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. Lancet 2000; 355: 1688-1691.
14) Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT, Ingram DA. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 2007; 109: 1801-1809.
15) Hillebrands JL, Klatter FA, van Dijk WD, Rozing J. Bone marrow does not contribute substantially to endothelial-cell replacement in transplant arteriosclerosis. Nat Med 2002; 8: 194-195.
16) Lin Y, Weisdorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 2000; 105: 71-77.
17) Bailey AS, Jiang S, Afentoulis M, Baumann CI, Schroeder DA, Olson SB, Wong MH, Fleming WH. Transplanted adult hematopoietic stem cells differentiate into functional endothelial cells. Blood 2004; 103: 13-19.
18) Case J, Mead LE, Bessler WK, Prater D, White HA, Saadatzadeh MR, Bhavsar JR, Yoder MC, Haneline LS, Ingram DA. Human CD34+AC133+VEGFR-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. Exp Hematol 2007; 35: 1109-1118.
19) Kucia M, Ratajczak J, Rataiczak MZ. Are bone marrow stem cells plastic or heterogenous-That is the question. Exp Hematol 2005; 33: 613-623.
20) Timmermans F, Van Hauwermeiren F, De Smedt M, Raedt R, Plasschaert F, De Buyzere ML, Gillebert TC, Plum J, Vandekerckhove B. Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors. Arterioscler Thromb Vasc Biol 2007; 27: 1572-1579.
21) Kennedy M, Firpo M, Choi K, Wall C, Robertson S, Kabrun N, Keller G. A common precursor for primitive erythropoiesis and definitive haematopoiesis. Nature 1997; 386: 488-493.

22) Grant MB, May WS, Caballero S, Brown GA, Guthrie SM, Mames RN, Byrne BJ, Vaught T, Spoerri PE, Peck AB, Scott EW. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. Nat Med 2002; 8: 607-612.

23) Pelosi E, Valtieri M, Coppola S, Botta R, Gabbianelli M, Lulli V, Marziali G, Masella B, Müller R, Sgadari C, Testa U, Bonanno G, Peschle C. Identification of the hemangioblast in postnatal life. Blood 2002; 100: 3203-3208.

24) Lacaud G, Robertson S, Palis J, Kennedy M, Keller G. Regulation of hemangioblast development. Ann N Y Acad Sci 2001; 938: 96-107.

25) Wang L, Li L, Shojaei F, Levac K, Cerdan C, Menendez P, Martin T, Rouleau A, Bhatia M. Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. Immunity 2004; 21: 31-41.

26) Dekel B, Metsuyanim S, Garcia AM, Quintero C, Sanchez MJ, Izraeli S. Organ-injury-induced reactivation of hemangioblastic precursor cells. Leukemia 2008; 22: 103-113.

27) Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005; 5: 953-964.

28) Schatteman GC, Dunnwald M, Jiao C. Biology of bone marrow derived endothelial cell precursors. Am J Physiol Heart Circ Physiol 2007; 292: H1-18.

29) Fernandez Pujol B, Lucibello FC, Gehling UM, Lindemann K, Weidner N, Zuzarte ML, Adamkiewicz J, Elsässer HP, Müller R, Havemann K. Endothelial-like cells derived from human CD14 positive monocytes. Differentiation 2000; 65: 287-300.

30) Gulati R, Jevremovic D, Peterson TE, Chatterjee S, Shah V, Vile RG, Simari RD. Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. Circ Res 2003; 93: 1023-1025.

31) Zaho Y, Glesne D, Huberman E. A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. PNAS 2003; 100: 2436-2431.

32) Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocytes/macrophages and secrete angiogenic growth factors. Circulation 2003; 107: 1164-1169.

33) Rabelink TJ, de Boer HC, de Koning EJ, van Zonneveld AJ. Endothelial progenitor cells: more than an inflammatory response? Arterioscler Thromb Vasc Biol 2004; 24: 834-838.

34) Kwon YW, Heo SC, Jeong GO, Yoon JW, Mo WM, Lee MJ, Jang IH, Kwon SM, Lee JS, Kim JH. Tumor necrosis factor-α-activated mesenchymal stem cells promote endothelial progenitor cell homing and angiogenesis. Biochim Biophys Acta 2013; 1832: 2136-2144.

35) Prisco AR, Prisco MR, Carlson BE, Greene AS. TNFα increases endothelial progenitor cell adhesion to the endothelium by increasing bond expression and affinity. Am J Physiol Heart Circ Physiol 2014; 308: H1368-1381.

36) Fujiyama S, Amano K, Uehira K, Yoshida M, Nishiwaki Y, Nozawa Y, Jin D, Takai S, Miyazaki M, Egashira K, Imada T, Iwasaka T, Matsubara H. Bone marrow monocyte lineage cells adhere on injured endothelium in a monocyte chemoattractant protein-1-dependent manner and accelerate reendothelialization as endothelial progenitor cells. Circ Res 2003; 93: 980-989.

37) Moldovan NI, Goldschmidt-Clermount PJ, Paker-Thornburg J, Shapiro SD, Kolattukudy PE. Contribution of monocyte/macrophages to compensatory neovascularization: the drilling of metalloelastase-positive tunnels in ischemic myocardium. Circ Res 2000; 87: 378-384.

38) Schmeisser A, Garlichs CD, Zhang H, Eskafi S, Graffy C, Ludwig J, Strasser RH, Daniel WG. Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. Cardiovasc Res 2001; 49: 671-680.

39) Urbich C, Heeschen C, Aicher A, Dernbach E, Zeiher AM, Dimmeler S. Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. Circulation 2003; 108: 2511-2516.

40) Harraz M, Jiao C, Hanlon HD, Hartley RS, Schatteman GC. CD34- blood-derived human endothelial cell progenitors. Stem Cells 2001; 19: 304-312.

41) Rohde E, Bartmann C, Schallmoser K, Reinisch A, Lanzer G, Linkesch W, Guelly C, Strunk D. Immune cells mimic the morphology of endothelial progenitor colonies in vitro. Stem Cells 2007; 25: 1746-1752.

42) Leone AM, Giannico MB, Bruno I, Giordano A, Zaccone V, Perfetti M, Niccoli G, Porto I, Burzotta F, Rebuzzi AG, Biasucci LM, Crea F. Safety and efficacy of G-CSF in patients with ischemic heart failure: The CORNER (Cell Option for Recovery in the Non-Eligible patients for Revascularization) study. Int J Cardiol 2011; 150: 75-78.

43) Kucia M, Ratajczak J, Reca R, Janowska-Wieczorek A, Ratajczak MZ. Tissue-specific muscle, neural and liver stem/progenitor cells reside in the bone marrow, respond to an SDF-1 gradient and are mobilized into peripheral blood during stress and tissue injury. Blood Cells Mol Dis 2004; 32: 52-57.

44) Kucia M, Dawn B, Hunt G, Guo Y, Wysoczynski M, Majka M, Ratajczak J, Rezzoug F, Ildstad ST, Bolli R, Ratajczak MZ. Cells expressing early cardiac markers reside in the bone marrow and are mobilized into the peripheral blood after myocardial infarction. Circ Res 2004; 95: 1191-1199.

45) He Q, Wan C, Li G. Concise review: multipotent mesenchymal stromal cells in blood. Stem Cells 2007; 25: 69-77.

46) Liu JW, Dunoyer-Geindre S, Serre-Beinier V, Mai G, Lambert JF, Fish RJ, Pernod G, Buehler L, Bounameaux H, Kruithof EK. Characterization of

endothelial-like cells derived from human mesenchymal stem cells. J Thromb Haemost 2007; 5: 826-834.

47) Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One 2008; 3: e1886.

48) Goon PK, Lip GY, Boos CJ, Stonelake PS, Blann AD. Circulating endothelial cells, endothelial progenitor cells, and endothelial microparticles in cancer. Neoplasia 2006; 8: 79-88.

49) Khan SS, Solomon MA, McCoy JP Jr. Detection of circulating endothelial cells and endothelial progenitor cells by flow cytometry. Cytometry B Clin Cytom 2005; 64: 1-8.

50) Blann AD, Pretorius A. Circulating endothelial cells and endothelial progenitor cells: two sides of the same coins or two different coins? Atherosclerosis 2006; 188: 12-18.

51) Alessandri G, Girelli M, Taccagni G, Colombo A, Nicosia R, Caruso A, Baronio M, Pagano S, Cova L, Parati E. Human vasculogenesis ex vivo: embryonal aorta as a tool for isolation of endothelial cell progenitors. Lab Invest 2001; 81: 875-885.

52) Ingram DA, Mead LE, Moore DB, Woodard W, Fenoglio A, Yoder MC. Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy progenitor cells. Blood 2005; 105: 2783-2786.

53) Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, Pollok K, Ferkowicz MJ, Gilley D, Yoder MC. Identification of a novel hierarchy of endothelial progenitor cells utilizing human peripheral and umbilical cord blood. Blood 2004; 104: 2752-2760.

54) Grenier G, Scimè A, Le Grand F, Asakura A, Perez-Iratxeta C, Andrade-Navarro MA, Labosky PA, Rudnicki MA. Resident endothelial precursors in muscle, adipose, and dermis contribute to postnatal vasculogenesis. Stem Cells 2007; 25: 3101-3110.

55) Aicher A, Rentsch M, Sasaki K, Ellwart JW, Fändrich F, Siebert R, Cooke JP, Dimmeler S, Heeschen C. Nonbone marrow-derived circulating progenitor cells contribute to postnatal neovascularization following tissue ischemia. Circ Res 2007; 100: 581-589.

56) Planat-Benard V, Silvestre JS, Cousin B, André M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Pénicaud L, Casteilla L. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 2004; 109: 656-663.

57) Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, Pell CL, Johnstone BH, Considine RV, March KL. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells march. Circulation 2004; 109: 1292-1298.

58) Wurmser AE, Nakashima K, Summers RG, Toni N, D'Amour KA, Lie DC, Gage FH. Cell fusion-indipendent differentiation of neural stem cells to the endothelial lineage. Nature 2004; 430: 350-356.

59) Iohara K, Zheng L, Wake H, Ito M, Nabekura J, Wakita H, Nakamura H, Into T, Matsushita K, Nakashima M. A novel stem cell source for vasculogenesis in ischemia: subfraction of side population cells from dental pulp. Stem Cells 2008; 26: 2408-2418.

60) Prater DN, Case J, Ingram DA, Yoder MC. Working hypothesis to redefine endothelial progenitor cells. Leukemia 2007; 21: 1141-1149.

61) Walenta K, Friedrich EB, Sehner F, Werner N, Nickenig G. In vitro differentiation characteristics of cultured human mononuclear cells-implications for endothelial progenitor cell biology. Biochem Biophys Res Commun 2005; 333: 476-482.

62) Santoro L, D'Onofrio F, Campo S, Ferraro PM, Tondi P, Campo V, Flex A, Gasbarrini A, Santoliquido A. Endothelial dysfunction but not increased carotid intima-media thickness in young European women with endometriosis. Hum Reprod 2012; 27: 1320-1326.

63) Santoro L, D'Onofrio F, Campo S, Ferraro PM, Flex A, Angelini F, Forni F, Nicolardi E, Campo V, Mascilini F, Landolfi R, Tondi P, Santoliquido A. Regression of endothelial dysfunction in patients with endometriosis after surgical treatment: a 2-year follow-up study. Hum Reprod 2014; 29: 1205-1210.

64) Xiao M, Lu X, Li J, Li L, Li Y. Physiologic ischaemic training induces endothelial progenitor cell mobilization and myocardial angiogenesis via endothelial nitric oxide synthase related pathway in rabbits. J Cardiovasc Med (Hagerstown) 2014; 15: 280-287.

65) Leone AM, Rutella S, Giannico MB, Perfetti M, Zaccone V, Brugaletta S, Garramone B, Niccoli G, Porto I, Liuzzo G, Biasucci LM, Bellesi S, Galiuto L, Leone G, Rebuzzi AG, Crea F. Effect of intensive vs standard statin therapy on endothelial progenitor cells and left ventricular function in patients with acute myocardial infarction: Statins for regeneration after acute myocardial infarction and PCI (STRAP) trial. Int J Cardiol 2008; 130: 457-462.

66) Leone AM, Valgimigli M, Giannico MB, Zaccone V, Perfetti M, D'Amario D, Rebuzzi AG, Crea F. From bone marrow to the arterial wall: the ongoing tale of endothelial progenitor cells. Eur Heart J 2009; 3: 890-899.

67) Yu D, Chen W, Ren J, Zhang T, Yang K, Wu G, Liu H. VEGF-PKD1-HDAC7 signaling promotes endothelial progenitor cell migration and tube formation. Microvasc Res 2014; 91: 66-72.

68) Obi S, Masuda H, Shizuno T, Sato A, Yamamoto K, Ando J, Abe Y, Asahara T. Fluid shear stress induces differentiation of circulating phenotype endothelial progenitor cells. Am J Physiol Cell Physiol 2012; 303: C595-606.