Review Article

Stem Cell INVESTIGATION

Role of endothelial progenitor cells in vascular development, homestatic maintenance of blood vessels and in injury-mediated reparative response

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Abstract: The blood vasculature is a closed circulatory system formed by arteries, veins and capillaries; the inner layer of these vessels is formed by a single layer of endothelial cells. Endothelial cells are specialized according to the specific needs of the tissues that they supply. The vascular system derives from the differentiation of mesodermal stem cells into angioblasts, embryonic endothelial progenitors. Endothelial progenitor cells (EPCs) are also present in adult life. Two types of EPCs have been reported: one of non-hematopoietic origin, endothelial colony forming cell (ECFC) able to generate endothelial cells, resident in vessel wall and present at low levels in peripheral blood and directly participating to the regeneration of endothelium following injury or ischemic damage; another of hematopoietic origin, called myeloid angiogenic cells (MACs), resident in bone marrow, generating monocytic cells, supporting angiogenesis through paracrine mechanisms. ECFCs play a role in reparative processes. ECFCs display a hierarchy of clonal proliferative potential and display a pronounced postnatal vascularization ability *in vivo*. For these properties, ECFCs represent a promising cell source for revascularization of damaged tissue. The use of ECFC for therapeutic use is still an embryonic field, but the therapeutic use of these cells holds great promise for the future.

Keywords: Stem cells; progenitor cells; angiogenesis; vasculogenesis; endothelial cells; hematopoiesis; vessel regeneration; tissue repair; ischemia

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Introduction

The blood vasculature can be considered as an organ pervading all other organs, transporting oxygen, nutrient, metabolites, and blood cellular elements through all the tissues of organisms. The development and presence of a functional vascular system is required for the function of the various organs and it is required for embryogenesis: thus, it is not surprising that the development of all tissues requires the coordinated development of these tissues and of the vascular system (1). The endothelium first forms in the blood island at the level of the extra-embryonic yolk sac under form of a primitive vascular network derived from endothelial progenitors known as angioblasts and then migrate to the embryo to form initial vascular networks that, progressively, during ontogenetic development acquire organ-specific functions to provide support to different functions of the various organs (2).

For many years, endothelial cells were considered as a biological system creating a monolayer that lines blood vessels with a main function of creating a barrier and of transporting nutrients through the body. However, studies carried out in the last decades have clearly supported a

major and significant role of endothelium in many key biological processes, such as organ growth, regeneration and stem cell niche. Particularly, endothelium is involved in the transfer of oxygen and nutrients, the control of the transit of white blood cells into and out the blood stream (3), the regulation of vasomotor tone and the maintenance of vascular integrity (4), the adhesion and activation of platelets (platelets maintain the integrity of endothelium and the endothelium release nitric oxide and prostacyclin to keep platelets in a resting state) (5), the tissue-specific production of angiocrine factors, growth factors that play a key role in tissue homeostasis and metabolism, in control of tissue stem cell activity under normal and pathological conditions (6).

Even at the level of the same organ, endothelial cells can have different development origins, possess heterogeneous phenotypic and molecular properties and acquire a considerable degree of functional specialization. The heterogeneity of endothelial cells has long been recognized, as supported by the observation that in different anatomical sites endothelial cells may morphologically differentiate to form barrier-continuous endothelial layers, fenestrated endothelial cells or sinusoidal endothelial cells. Studies carried out in the last years have clearly supported the existence of organotypically specialized vasculature in different organs (7). These studies have highlighted the molecular structure, the histological peculiarities and the functions of organotypically differentiated microvasculatures, particularly at the level of the brain, eyes, heart, lungs, liver, kidneys, bone, adipose tissue and endocrine glands (7).

Endothelial and hemopoietic cells are strictly interconnected, not only for their anatomic contiguity, but also for their embryologic origin. The hematopoietic system derives from the differentiation of hematopoietic stem cells (HSCs) emerging from mesoderm during embryogenesis. Particularly, hematopoietic cells are originated from peculiar endothelial cells originated through various stages of embryonic development and called hemogenic endothelium for its peculiar capacity to generate hematopoietic cells through a transdifferentiation process of endothelial to hemopoietic differentiation. Hemogenic endothelium can be defined as a peculiar, specialized subset of developing vascular endothelium that possess a potential of a hematopoietic differentiation and is able to generate multilineage hematopoietic stem and progenitor cells during a restricted developmental period in embryonic tissues, such as extra-embryogenic yolk sac and embryonic aorta-gonad-mesonephros (8). In mammals, three waves of hematopoiesis are observed during embryonic life: a first wave of hematopoiesis occurs at the level of hemogenic endothelium of yolk sac and generates primitive erythroid cells, macrophages and megakaryocytes; the second wave of erythro-myeloid progenitors (EMPs) that transiently seed the fetal liver; the third wave corresponds to the production of HSCs from the hemogenic endothelium of the AGM region (9-11). The endothelial origin of HSCs and hematopoietic progenitors has been firmly established by the emergence from the aortic endothelial layer and by linear tracing *in vivo* through genetic labelling (9-11).

For many years it was believed that vasculogenic processes are limited to the prenatal life, whilst angiogenesis is retained to occur in both prenatal and postnatal periods. However, this conception was challenged by the discovery of endothelial progenitor cells (EPCs), cells capable of generating mature endothelial cells. EPCs are a heterogeneous group of cells and comprise cells of different origins and function (12). Thus, two types of EPCs have been described: hematopoietic EPCs and non-hematopoietic EPCs: hematopoietic EPCs, mainly represented by $CD34^+$/VEGF-$R2^+$/$CD133^+$ cells, unable to generate true endothelial cells, but differentiating into monocytic cells exhibiting some endothelial markers and exerting *in vivo* pro-angiogenic effect through a paracrine mechanism; non-hematopoietic EPCs, termed endothelial forming cells colony (ECFC), originated from $CD34^+$/$CD45^-$ progenitors in part circulating and in large part residing in vessel wall and capable of generating *in vitro* and *in vivo* large amounts of mature and functionally competent endothelial cells (12). ECFCs from capillary-like tubes in the extracellular matrix *in vitro* and are involved in neovascularization under ischemic conditions and on reendothelialization upon endothelial injury (12).

Studies in transplant arteriosclerosis in animal models do not support a mayor role of bone marrow to endothelial replacement *in vivo* (13). For their functional properties, EPCs are currently under evaluation as a promising tool for regenerative cardiovascular medicine to treat endothelial or ischemic injury (14-17).

During the last years several clinical studies have attempted to obtain endothelial generation *in vivo* using hematopoietic EPCs. Basically, these studies have shown that: (I) this therapy is safe for the patients; (II) the EPC-based therapy slightly improved the ischemic condition; (III) no clear clinical benefit was observed, as well as reduction of cardio-vascular events; (IV) human bone marrow cells

are unable to transdifferentiate into new blood vessels or into cardiomyocytes (14-17). No clinical studies have been made using ECFCs. ECFCs are broadly used for the vascularization of tissue-engineered construct and to form functionally active endothelium at polymer surface or cell sheets. The combination of emerging progresses in tissue engineering and stem cell manipulation represents the basis for the development of new strategies for the generation of new vessels, recapitulating mechanical properties and biological functions of native vessels (18). Alternatively to EPCs, human pluripotent stem cell-derived endothelial cells (hPSC-ECs) emerged as a potentially important source of cells to be used to obtain endothelial regenerative. Various systems were developed for differentiating hPSCs into functionally-competent endothelial cells. Stepwise two-dimensional systems have been now developed to obtain endothelial cell differentiation of hPSCs under conditions more and more suitable for clinical applicability, progressively removing undefined components from the differentiation system (19,20). Using biomaterial-mediated delivery, the survival of hPSC-EC was extended up to more than 10 months in ischemic tissues, providing an efficient and safe vascularization (21).

Finally, growing evidence indicates that endothelial cells are specialized according to the specific needs of the tissue that they supply: therefore, it is important to understand the mechanism to endothelial specialization through specific pathways of vessel type and origin specific endothelial differentiation (22,23).

Among the various tissue specializations of endothelial cells, particularly relevant is their key contribution to the bone marrow niches for HSCs, local tissue microenvironments that maintain the survival of HSCs and regulate their function by producing factors that act directly on stem cells. In these structures, endothelial cells support HSC survival, proliferation (self-renewal), differentiation, homeostasis and regeneration through the production of autocrine factors. In the adult bone marrow, the HSCs are located near to the endothelium: about 80% of HSCs are associated with sinusoidal blood vessels (sinusoidal niches), about 10% of HSCs are adjacent to arterioles (perivascular niches) and the remaining 10% is adjacent to transition zone vessels (vessels connecting arterioles to sinusoidal vessels); only few HSCs are located at the level of endosteum (24). The vascular architecture and biological properties of bone marrow endothelial cells are consistently different between arteriolar and sinusoidal vessels: arterioles have low permeability, variable vessel diameter, high blood flow and branch to sinusoids, which have a high permeability (fenestrated basal lamina), high vessel diameter and low blood flow. Thus, the sinusoidal blood vessels represent a fenestrated vascular system specifically observed in bone marrow and through which hematopoietic cells can migrate into and out of circulation. One of the key functions of endothelial bone marrow cells consists in the regulation of HSC homing, engraftment and migration (25). In fact, a large number of studies has clearly shown that endothelial cells play an active and crucial role in HSC engraftment and homing to bone marrow by directing their migration to, and polarization across, the vascular endothelium into the niche (25). In this context, a key role is played by constitutive expression of adhesion molecule receptors, such as selectins, on endothelial bone marrow cells and production of stromal-derived factor-1 (CXCL12), interacting with its receptor CXCR4 expressed on the membrane of HSCs and essential for the homing of these cells (25). The different properties of endothelial cells at the level of the different niches affect the properties of HSCs. Quiescent HSCs have been found either in association with arteriolar or with sinusoidal niches (25,26). Furthermore, recent studies have shown that HSCs are not a homogeneous cell population, but are consistently heterogeneous, with evidence for HSC lineage segregation and the presence of lineage-biased HSCs and lineage-restricted progenitors within the HSC compartment (27,28).

This review analyzes recent acquisitions on the development and homeostasis of EPCs. A careful definition of these cells and the understanding of their biological properties is essential for developing suitable strategies for the development of clinical studies aiming to get a regeneration of the vascular system, a need in various areas of medicine, particularly for cardiovascular diseases representing still the major cause of mortality in humans.

Development of endothelial and hematopoietic cells in mouse

Endothelial cells have a mesodermal origin. In zebrafish it was shown that the basic helix-loop-helix-Per-ANT-Sim (bHLH-PAS) protein neuronal PAS domain-containing protein 4-like protein (NPASL) instructs, together with other transcription factors, multipotent mesodermal stem cells to differentiate into endothelial progenitors know as angioblasts. This transcription factor is a potent inducer of endothelial gene expression and acts as a master regulator of endothelial cell commitment (29). In mouse,

the transcription factor NPAS4, the closest homology of NPAS4L, is not strictly required for endothelial cell commitment, probably due to the presence of numerous other bHLH-PAS transcription factors.

Arterial and venous endothelial cells originate from the differentiation of angioblasts (mesodermal progenitors) present at different locations (30). Migration of angioblasts and their organization into blood islands allow establishment of the primitive vascular plexus. In mice, the first angioblasts acquire endothelial arterial or venous fate and assemble to form the first networks of blood vessels, the dorsal aorta and the cardinal vein; in the extraembryonic tissue yolk-sac, angioblasts assemble into blood island that fuse undergoing the formation of a primary vessels network.

Expansion of the initial vasculature occurs through a process of proliferation of pre-existing endothelial cells. This process of expansion is called angiogenesis, a complex process that generates new vessels and vascular networks from pre-existing ones through vessels sprouting, branching and anastomosis. Various tissue growth factors are required to ensure the survival and the proliferation of endothelial cells during the process of angiogenesis, including fibroblast growth factor 2 (FGF2), vascular endothelial growth factor (VEGF) and bone morphogenetic protein 4 (BMP4).

There is a strict connection between the development of endothelial and hematopoietic cells during embryonic development. The hematopoietic system is of mesodermal origin and in mouse three distinct waves of hematopoiesis are observed, characterized by different sites of development. The first wave of hematopoiesis occurs in the yolk sac at day 7 and corresponds to the development of "primitive hematopoiesis", generating a transient progeny of blood elements, such as primitive erythroid progenitors, embryonic macrophages and primitive megakaryocytes, ensuring immediate needs of the growing embryo (31). Recent studies carried out using transgenic mouse embryos have provided direct evidence that primitive erythroblasts emerge from hemogenic endothelial cells (32).

The second wave of hematopoiesis originates in the yolk sac at E 8.25 and is characterized by the development of EMPs and lymphoid progenitors and corresponds to the development of "definitive hematopoiesis" because EMPs differentiate into blood cells with adult features (26). EMPs give rise to tissue macrophages that persist into adulthood (33,34).

The third wave of hematopoiesis occurs from E 9.5 to E 12.5 in the so-called AGM (Aorta-Genital ridge-Mesonephros) region that extends from the anterior limbs to the posterior limbs. During this phase of hematopoiesis are generated for the first-time HSCs: these cells are generated from the so-called hemogenic endothelium, present within the AGM, through a complex and not yet well-defined process of trans differentiation from the endothelial to the hematopoietic lineage (35-38).

Interestingly, erythroid/myeloid progenitors and HSCs originate from distinct populations of endothelial cells of the hemogenic endothelium (39).

There is a very important difference between the hemogenic endothelium present in yolk sac, compared to that present in the AGM region: in fact, while yolk sac hemogenic endothelial cells give rise to multilineage hemopoietic progenitor cells that cannot repopulate at long-term the hemopoietic tissue (thus, they are multilineage progenitors, but not HSCs) (40), AGM hemogenic endothelium generates true HSCs, able to repopulate the hemopoietic tissue, at long-term (41).

Various studies have in part elucidated the peculiar properties of hemogenic endothelium, compared to normal standard endothelium; however, there are no simple phenotypic markers to distinguish these two types of endothelial cells (8).

AGM derived HSCs and HPCs migrate and colonize to other sites of definitive hematopoiesis, such as the fetal liver by E 11.00–E 12.00 and the bone marrow by E 16.5 and contribute to the adult HSC pool and to the definitive hematopoiesis. HSCs generated from hemogenic endothelium of the dorsal aorta first migrate to the placenta via the umbilical arteries and then return to the fetus via umbilical vein. The umbilical vein delivery oxygenated blood to the fetus via the portal sinus, whose branches generate the complex network of portal vessels in the fetal liver. In fetal liver, HSCs undergo a process of expansion through self-renewal, increasing their number more than 30-fold (42).

In line with this finding, it is not surprising that fetal liver HSCs are highly proliferative in fetal liver in contrast to adult bone marrow HSCs that are largely quiescent. The expansion of HSCs at the level of the liver is in large part related to the peculiar properties of the fetal liver hematopoietic cell niche. Endothelial cells and perivascular cells are essential cellular constituents of the niches for HSC that are strictly required for the maintenance of those cells (43).

The study of murine fetal liver during the liver hematopoietic stage showed that Nestin$^+$ NG2$^+$ pericytes associate with portal vessels, contributing to the formation of niches promoting HSC expansion; during development there is a correlation between NG2 pericytes and the number of HSCs (44). At birth, after closure of the umbilical

vein, portal vessels undergo a change from Neuropilin-1$^+$/EphrinB2$^+$ artery to EphrinB4$^+$ vein phenotype, an event associated with a loss of periportal Nestin$^+$ NG2$^+$ cells and emigration of HSCs away from portal vessels (44).

Other studies have shown the essential role of CCL2/CCR7 signaling pathway in promoting HSC expansion in fetal liver microenvironment (45).

Around E 11, two types of HSCs are observed in mouse fetal liver: pre-HSCs CD45$^+$ and more mature CD45- HSCs. Fetal liver HSCs are characterized by positivity for endothelial protein C receptor (EPCR) (CD201) and are localized at the level of the perisinusoidal niche (8). CD201 is expressed in pre-HSCs in fetal hematopoietic tissues (46).

Endothelial cells play an essential role in sustaining fetal liver hematopoiesis and this function required the production of the cytokine Kit ligand by endothelial cells. In fact, inactivation of the epigenetic regulator EZH2 in endothelial cells resulted in embryonic lethality at day 13.5 with severe anemia, but with normal emergence of functional HSCs (47). EZH2-deficient fetal liver endothelial cells overexpressed the metalloprotease MMP9, which induces a depletion of membrane-bound Kit Ligand, a cytokine essential for normal hematopoiesis (47). Kit Ligand is essential also for yolk sac and AGM hematopoiesis (48) and in adult bone marrow is secreted by arterial endothelial cells (49).

After expansion in the fetal liver, HSCs complete their developmental program by migrating to the bone marrow on E 16.5 to 17.5. Bone marrow environmental signals induced by CXCL12, VEGF, SLIT, Kit Ligand, Collagen C-cadherin, VCAM, selectins and fibronectins mediated the migration of HSCs (50).

In addition to environmental signals, also HSC intracellular signaling mediated by the Wave 2 (Wiskott-Aldrich syndrome Verprolin-homologous protein complex 2) scaffold protein Hem-1 is required for the engraftment of fetal liver HSCs into the bone marrow (51).

The origin of EMPs generated from yolk sac at E 8.5 and their differentiation capacities were the object of intensive studies. EMPs are able to support embryo survival until birth and are able to differentiate into macrophages whit in the yolk sac and colonize the fetal liver at E 9 and undergo multilineage differentiation into erythrocytes, megakaryocytes, macrophages, granulocytes and mast cells (39).

Recent studies have provided direct and clear evidence that EMPs derived from endothelial cells (hemogenic endothelium): interestingly, these progenitors express the majority of endothelial cell markers and contribute to the remodeling of embryonic vascular system (52). A recent study added a new important piece of evidence supporting an unexpected important function of EMPs. In fact, using a genetic-engineering approach to generate mouse embryos in which yolk-sac-derived EMPs and all their cell progeny were labelled with a fluorescent protein; unexpectedly, these cells also contribute to the walls of blood vessels (52). Particularly, these authors found that the percentage of endothelial cells in adult blood vessels that originates from EMPs ranged from about 30% in the brain and 60% in the liver (52). Future studies will explore the relationship between the origin of endothelial cells and their function (52). The results of this study are very important because indicate a dual origin of endothelial cells and blood vessels in mouse. The degree to which these findings apply to humans needs to be tested and, eventually, confirmed.

HSCs are generated from hemogenic endothelial cells through the formation of intra-aortic hematopoietic clusters (IAHCs): the process that generates IAHCs from hemogenic endothelium was called endothelial-to-hematopoietic transition (EHT). IAHCs contain HSCs co-expressing both HSC markers and endothelial markers, thus supporting their presumptive endothelial origin. Single-cell transcriptomic studies have supported the progressive shift from and endothelial gene expression program to a hematopoietic gene expression program during EHT (53).

The EHT is regulated by endogenous and exogenous factors: among the endogenous factors, the most relevant are those related to the induction of expression of various specific transcription factors, such as RUNX1, SCL, GFI1/GFI1b and SOX17, that have a stem cell-autonomous role in EHT; among the exogenous factors, the most relevant are dose deriving from the microenvironment and represented by growth factors and cytokines such as CXCL12, CXCLB, Adenosine, BMP4, NOTCH, WNT, HEDGE HOG that play a role both in the development of hemogenic endothelium and in EHT (54). Based on the evidence that HSCs, are derived from hemogenic endothelium, recent studies have developed procedures allowing to recapitulate hemogenic endothelium differentiation from pluripotent stem cells to generate cells with HSC potential and with the capacity to generate various types of hematopoietic cells (55,56). These studies have highlighted the importance of cell-extrinsic and cell-intrinsic signals acting in cooperation to promote HSC-like self-renewal and differentiation (55,56).

During the process of developmental progression HSCs undergo progressive changes involving their differentiation status and their cycling activity. Particularly, HSCs develops through a complex, multistep process, fundamentally

involving four stages: pro-HSC emerging at day 9.5 (with a phenotype VE-Cadherin$^+$, CD41low, CD43$^-$, CD45$^-$); pre-HSCs type I at day 10.5 (with a phenotype VE-Cadherin$^+$, CD41low, CD43$^+$, CD45$^-$); pre-HSCs type II at day 11.5 (with a phenotype VE-Cadherin$^+$, CD41low, CD43$^+$, CD45$^+$); low dHSC emerging at day 11.5, with a phenotype identical to pre-HSCs type II and repopulate irradiated recipients (57,58). These various developmental stages of HSCs are accompanied by concomitant changes in their proliferative status: thus, HSCs undergo at day 10.5 a marked expansion of their number, sustained by a marked enhancement of their proliferative activity (57,58).

The formation of the bone marrow hemopoietic niche during fetal development is of fundamental importance for the development of definitive adult hematopoiesis. The process of development of fetal bone marrow niche was explored and it was clearly shown that repopulating HSCs were observed in murine fetal bone marrow at day 16.5, in coincidence with the vascularization of this tissue (59). However, the appropriate development of a fully functional bone marrow niche requires the concomitant development of osteoblasts and of other stromal components (59).

The adult HSC niche was very intensively investigated and specific cellular constituents have been extensively studied, including vascular/perivascular cells, osteoblasts, nestin-expressing mesenchymal stem cells, adipocytes and sympathetic nervous system cells, and their function as regulators of HSC survival, proliferation and function was investigated in various experimental models (4,24,60,61).

Endothelial cells are essential and fundamental components of the bone marrow niche. HSCs in the adult bone marrow are closely associated with vascular endothelial cells (44,62) and the sinusoidal vascular niche is thought to support active proliferation of HSCs (63).

Growing evidences indicate that endothelial cell populations present in bone marrow niches are heterogeneous. The study of blood vessels during postnatal development led to the identification of a specialized endothelial cell subset, called type H cells, present in active growing regions of the bone (growth plate and endosteum), where they regulate osteogenesis in a NOTCH-dependent manner (64,65). These endothelial cells are called type H high because they express high levels of blood vessel markers, such as CD31 and endomucin, compared to sinusoid vessels, expressing low levels of these markers and for this reason are termed as type L (low). In actively grooving bone, type H capillaries are located at the level of metaphysis and endosteum regions, while type L capillaries are mainly located in the medullary region (64,65).

Type H capillary are densely packed and are interconnected at the level of the growth plate; type L capillaries form a capillary network in the bone marrow cavity of diaphysis, largely corresponding to the sinusoidal structure of the bone marrow (66).

Arteries and arterioles do not deliver blood directly to type L sinusoidal capillaries, but to type H vessels in the metaphysis and endosteum: thus, blood flows from arteries to type H capillaries and them to L type capillaries and, finally, id drained into the central vein (64,65). As a consequence, the bone of postnatal and adolescent mice is hypoxic at the level of diaphysis and well oxygenated at the level of metaphysis (64,65). The angiogenetic activity of type H capillaries requires an active flow and the function of leading fronts of these vessels, containing bulge-shaped luminised structures; with aging, impaired blood flow determines a reduced angiogenetic and NOTCH activity of these vessels (67).

A subpopulation of endothelial cells within the H endothelium, expressing Ephrin B2 (Efnb2) and endomucin generates arteriolar blood vessels (68). NOTCH signaling in endothelial cells acts as a signal for promoting HSC expansion and this involves increased numbers of type H capillaries and perivascular cells, arteriole formation and elevated levels of Kit Ligand (68).

More recently, a third type of endothelial cells, type E endothelium, was identified (69). This third capillary endothelial cell subpopulation possesses a high capacity to support perivascular osteoprogenitors in embryonic/fetal and postnatal bone (69). Type E endothelial cells predominate in early bone (69). The permeability of different bone blood vessels seems to play a key role in the regulation of quiescence and proliferation of bone marrow HSCs: less permeable arterial blood vessels maintain HSCs in a quiescence state, characterized by low Reactive Oxygen Species (ROS) production, whereas the more permeable sinusoids promote HSC/HPC activation as a result of plasma leakage and are sites for immature and mature leukocyte trafficking (70). The functional consequence of high permeability of sinusoids is that plasma exposure increases ROS production in HSC/HPCs, promoting migration and differentiation (70).

The heterogeneity of bone marrow endothelial cells is also supported by another recent study showing that CD73 labeled a part of sinusoidal endothelial cells within bone marrow (71). This endothelial CD73+ subpopulation displayed a mesenchymal gene signature and seemed to represent

an important constituent of the HSC niche (71). Finally, endothelial cells are important not only for homeostatic hematopoiesis, but also for sustaining bone marrow regeneration through the production of Pleiotrophin (72).

Development of endothelial and hematopoietic cells in humans

In humans, the development of the hematopoietic system is characterized by two different waves: primitive hematopoiesis, allowing the generation of transitory hematopoietic cells, which ensure the physiologic needs during embryonic/fetal life and permanent, definitive hematopoiesis, allowing the generation of multipotent, regenerating HSCs that ensure the hematopoiesis during all adult life.

The first hematopoietic cells are observed in humans at the level of the yolk sac during the third week (between second and third week after conception) of development: this is an extraembryonic tissue, formed by visceral endoderm and mesoderm. There is a significant difference between the yolk-sac in mouse and humans: in mouse the yolk-sac surrounds the whole embryo, while in human yolk sac develops in front of the embryo body, forming a balloon-like structure. Within the yolk sac, mesodermal cells generate cell aggregates that differentiate to endothelial cells and form central cavities, representing the lumen of the first blood vessels; the subsequent differentiation of these endothelial cells to hemangioblastic cells allows their capacity to generate hematopoietic cells by a trans differentiation process (EHT); these blood cells localize at the level of the lumen, thus forming blood island, the most primitive hematopoietic tissue. Mesodermal aggregates are observed in human yolk sacs at 16-days of gestation, while blood islands formation is observed from day 19 onward and leads to the generation essentially of primitive erythrocytes, but also of macrophages and primitive megakaryocytes (73).

In vitro studies have indicated that human embryonic yolk sac contains various types of hematopoietic progenitors, involving multipotent, erythroid, megakaryocytic and granulo-monocytic progenitors (74,75). However, since these assays were performed at 4–5 weeks, at stage when already blood circulation is active, the yolk sac origin of these cells is not certain because these progenitors could derive from circulation.

Primitive erythroid progenitors have peculiar properties, not limited only to their capacity to generate erythroid elements producing embryonic hemoglobins but have also a different growth factor requirement compared to adult ones, erythropoietin alone being sufficient to ensure their proliferation and differentiation (76).

The differentiation capacities of hemopoietic progenitors present in human yolk sac is limited: in fact, *in vitro* studies on stromal-supplemented cultures showed that B-lymphoid and myeloid hematogenous potential was present in *in vitro*-embryonic hematopoietic sites, but not at the level of yolk sac (77).

The onset of IAHCs (3–4 post-conception weeks) is marked by the appearance of IAHCs at the level of the central wall of the human dorsal aorta in the AGM region. At the level of IAHCs cluster of CD34+ cells were detected: interestingly, this cell population in addition to hematopoietic cell markers (CD34, c-Kit, CD45) express also endothelial cell markers, such as VE-Cadherin (78,79).

Long-term repopulating activity on the AGM region is exclusively localized in the ventral region of aorta (80). The phenotype $CD34^+$, VE-$Cadherin^+$, $CD45^+$, c-Kit^+, RUNX-1^+, $Endoglin^+$, $CD38^+$low identifies a highly enriched AGM HSC population (80). AGM HSCs are predominantly located at the level of the portion of AGM around or above the vitelline artery; this region shows a high production of progenitor cells (CFU-Cs) and is enriched in intra-aortic clusters of developing hematopoietic cells (81).

HSCs emerge first during embryonic life in the AGM region, specifically in the dorsal aorta (82). Human AGM region HSCs are low in number but possess and extensive self-renewal capacity: a single HSC derived from the AGM region generates more than 300 daughter HSCs in primary recipient (81). The location of HSCs in the dorsal aorta is also supported by studies on the localization of angiotensin converting enzyme (ACE^+) cells at the level of AGM region: ACE^+ CD34 were identified as scattered cells present beneath the dorsal aorta and those cells represent pre-HSCs (83).

The rudiment of human fetal liver starts to be formed at day 21 post-conception as a diverticulum from the floor of the embryonic gut. At immediately later stages, the liver rudiment is progressively colonized by primitive yolk sac-derived erythrocytes and CD45+ monocytic/macrophagic cells. At later stages, corresponding at 27–29 days post-conception, fetal liver is progressively seeded by progenitor cells $CD34^+$ $CD45^+$ corresponding to yolk sac-derived EMPs (84). These cells contribute to the generation of erythrocytes that progressively replace primitive erythroblasts during in the human fetal blood.

At 30–33 days post-conception starts the colonization of fetal liver by AGM-derived cell. A clear presence of HSCs

in the fetal liver is detected only from week 7–8.

A recent study provided a careful characterization of hematopoiesis in human fetal liver with an analysis at single-cell level, providing evidence that at 7–8 weeks post-conception 27 major cell states were identified; neutrophils, basophils and eosinophils were not detected in fetal liver since these cells emerge during fetal bone marrow hematopoiesis (85). The analysis of transcription factors expression in single cells allowed to infer various trajectories of hematopoietic development along various lineages (erythroid, megakaryocytic, mast cells, B cells, innate or T-lymphoid cells and myeloid cells) (85). The changes in HSC/MPP cell numbers, proliferation and differentiation potential occur during fetal liver development in the first and second developmental trimesters and represent a fundamental adaptation of fetal liver hematopoiesis to physiological needs, first represented by the development of an effective oxygen transport system and subsequently by the development of a complete blood and immune system (86).

Four stages of hepatic hematopoiesis have been distinguished in human embryos/fetuses: stage I observed up to week 9 of gestation and characterized by an hepatic tissue, composed by developing hepatoblasts, without signs of endogenous hematopoiesis and with few $CD34^+$ cells present in the sinusoid lumen; stage II occurring from week 10 to 12, characterized by an initial proliferation of hematopoietic foci, occurring about 20–30% of the liver parenchyma and mainly composed by erythroid elements; stage III observed from week 13 to 22, is characterized by a progressive increase of the hematopoietic elements, arriving to occupy up to 70% of the liver parenchyma, with a cell composition represented by about 75% of erythroid elements and 25% of myeloid elements: stage IV, observed from week 23 to 39 is characterized by the progressive involution of hematopoietic activity (86).

Erythroid precursors are mainly localized among fetal hepatocytes and within liver sinusoids, whereas myeloid precursors were observed in association with vascular elements of the portal triads (87).

The endothelial cell population of the liver is peculiar in that, in addition to endothelial cell types that line the large hepatic vessels, the majority of endothelial cells are liver sinusoidal endothelial cells, displaying some unique properties, such as being the main source of factor VIII (88).

Several recent studies have shown some peculiarities of human fetal liver hematopoietic cell differentiation and HSCs. A peculiar population hematopoietic stem/progenitor cells was identified in human fetal liver, characterized by VE-Cadherin expression: this cell population was characterized by the concomitant expression of endothelial (VE-Cadherin), hematopoietic (CD45) and hemato/endothelial (CD34) markers (89). This population possesses a peculiar self-renewal, proliferation and differentiation capacities, compared to the VE-Cadherin negative counterpart (89).

A recent study provided clear evidence that road maps of cell differentiation greatly change during human development, being different in fetal liver compared to cord blood/adult bone marrow (90). In fact, in human fetal liver, the ratio of cells with multipotent differentiation capacities versus cells with unilineage differentiation capacities remained constant in stem cell and progenitor cell compartments; in contrast, in adult bone marrow almost all MPPs were restricted to the stem cell compartment, while unilineage progenitors represent the large majority of the hematopoietic progenitor cell compartment (90). These studies showed also that the megakaryocytic/erythroid activity predominantly originated from the stem cell compartment at all developmental time points; particularly, in fetal liver an almost pure megakaryocytic/erythroid progenitor was found in the stem cell compartment (90).

Human adult HSCs are enriched by positively selecting for CD34 and CD90 and by negatively selecting for CD38 and CD45RA (91-93). The glycophosphatidylinositol-anchored surface protein GPI-80 defines a subset of hematopoietic stem/progenitor cells with self-renewal capacity (94). The $CD34+CD38low/^-$ $CD90^+$ $GPI\text{-}80^+$ fetal liver population maintained a high proliferative potential and an undifferentiated state when grown on a stromal layer and engrafted immunodeficient mice (94). GPI-80 expression enables to track HSC, when these cells emerge from hemogenic endothelium (94). GPI-80 was found also to label a rare population of CD34- HSCs present in cord blood (95). $CD34^-$ $CD133^+$ $GPI\text{-}80^+$ cord blood display self-renewing capacities and show a potent megakaryocytic/erythroid differentiation potential *in vitro* and *in vivo* (96).

Another recent study showed that EPCR, also known as CD201, is a reliable marker for human fetal liver HSCs (97). EPCR was much more expressed in fetal liver, than in other hematopoietic tissue at later ontogenetic stages of development (cord blood, adult bone marrow); EPCR labelled a subpopulation of $CD34^+$ $CD38^-$ $CD90^+$ fetal liver cells endowed with HSC properties (97). EPCR is expressed only on a minority of $CD34^+$ cord blood cells. However, transcriptome analysis of $CD34^+$-enriched cord blood cells showed induction of EPCR gene expression on

treatment of these cells with UM171, an agonist of HSC self-renewal (98). These findings were confirmed, and it was shown also that EPCR$^+$ cord blood CD34$^+$ cells possess both short-term and long-term repopulating activity (99).

Bone marrow is colonized by HSCs during the late stages of fetal development. HSCs emergence in bone marrow occurs in coincidence with marrow vascularization and ossification. Five stages of human fetal bone marrow hematopoiesis have been described: stage I (6.6 to 8.5 weeks), characterized by the presence of cartilaginous rudiments, with presence of chondrocytes and endothelial cells; stage II (8.5 to 9 weeks), characterized by a process of active chondrolysis; stage III (9 to 10.5 weeks), characterized by the development of the vascular bed, in the absence of hematopoiesis; stage IV (from 10.5 to 15 weeks), characterized by the onset of hematopoiesis that consists in the appearance of hematopoietic cells (mostly CD15$^+$ cells) within logettes formed by chondrocytes; stage V (from 16 weeks onward), characterized by the organization of the bone, with a trabecular calcified structure and areas of hematopoiesis, where logettes were no more detectable (100). Analysis of population dynamic of normal human blood inferred from somatic mutations allowed to estimate the number of HSCs during human life: the size of the HSC population grew steadily during early pre-natal and post-natal life, reaching a stable plateau by adolescence (101). The number of HSC contributing to maintain a normal hematopoietic steady-state condition are estimated in the range of 50,000–200,000 (101).

As above discussed, hematopoietic cells arise first in the third week of human ontogeny inside yolk sac developing blood vessels, then, one week later at the level of the embryos in the AGM region. The amounts of evidences supporting the derivation of blood elements from hemogenic endothelium is strong also in the human system. However, only few studies have directly supported *in vitro* the capacity of hemogenic endothelial cells to generate hematopoietic cells through a process of EHT. Obelin and coworkers have isolated endothelial cells from yolk sac, embryonic aorta, fetal liver and fetal bone marrow; these cells were grown *in vitro* in presence of stromal cells that support human multilineage hematopoiesis: as expected, embryonic endothelial cells isolated from yolk sac and AGM generate a progeny of hematopoietic myelolymphoid cells; surprisingly, a subset of endothelial cells isolated from fetal liver and fetal bone marrow also show a blood-forming capacity (102).

Other studies support the existence of endothelial cells with hemogenic potential in neonatal and in adult hematopoietic tissues. Thus, Pelosi et al provided evidence that a subset (about 5%) of CD34$^+$/VEGF-R2$^+$ cord blood cells are able to generate *in vitro* in appropriate culture conditions large colonies, composed by both hematopoietic and endothelial cells (103). Sibling cells generated by a single CD34$^+$/VEGF-R2$^+$ cell, replated in unicellular culture, generate either hematopoietic or hemato/endothelial cells (101). CD34$^+$/VEGF-R2$^+$ positive cell can be maintained for several months in long-term cultures and maintain their hemato-endothelial differentiation capacities (103).

In another study, it was provided further support to the existence of hemangioblasts in human cord blood showing that CD34$^+$CD45$^+$CD144$^-$ cells, grown in the presence of hepatocyte conditioned medium differentiate into adherent endothelial precursor CD144$^+$CD105$^+$CD146$^+$CD31$^+$CD45$^-$, able to function as hemogenic endothelium (104). These cells are able to generate a functional vasculature *in vivo* and if instructed by hematopoietic growth factors, first switch to transitional CD144$^+$CD45$^+$ and then to hematopoietic cells (104).

Interestingly, cord blood–derived hemogenic endothelium when grown in the presence of hematopoietic growth factors undergoes a three-wave differentiation process, involving a first wave with production of erythroid elements and a minority of granulo-monocytic elements; a second wave corresponding to the generation of megakaryocytic elements and a third wave corresponding to the generation of mast cells (105). A very recent study reported the isolation and characterization of a rare subpopulation of mesodermal-derived bone marrow cells with hemogenic potential. In this study, the G protein-coupled APELIN receptor (APLNR) was used as a marker of a rare subpopulation of bone marrow cells; this receptor was found to be positive in posterior mesoderm and anterior mesoderm populations and positive cells were enriched in hemangioblasts (106). APLNR$^+$ cells in embryonic/fetal tissues co-express Stro1, a marker of endothelial and perivascular cells; APLNR$^+$/Stro-1$^+$ cells express VE-Cadherin and VEGFR2 (107). Stro1+/VE-Cadherin+ cells are present in the fetal bone marrow vessels and their expression decreases during fetal development (107). Interestingly, cells expressing APLNR, Stro1 and VE-Cadherin are present as a very rare subpopulation of bone marrow cells; these cells co-express markers of mesodermal precursors and hemogenic endothelial cells and can be expanded *in vitro* (107). These cells, upon transplantation into a fetal microenvironment, contribute to the vasculature and generate hematopoietic cells of various cell lineages,

with repopulating activity maintained upon serial transplantation (107).

The development of definitive adult-type hematopoiesis during human fetal development involves bone marrow formation, which requires a coordinated interplay between osteogenesis, angiogenesis and hematopoiesis. However, very few studies have characterized the development of human bone marrow microenvironment during fetal life. A recent study reported the identification in human fetal bone marrow of endoglin- expressing endothelial cells, displaying properties similar to those previously reported for type H murine bone marrow endothelial cells (108). These endothelial cells seem to play an important role in bone marrow development promoting hematopoiesis, osteogenesis and angiogenesis through angiocrine mechanisms, in part related to their capacity to produce and release IL-33 (109). Human HSCs have the tendency to preferentially localize at the level of the endosteal regions of the trabecular bone area (TBA); HSCs localizing at the level of TBA have a higher regenerative and self-renewing capacity, compared to those localizing at the level of long bone area (109).

The study of hematopoiesis provides the analysis of one of the most characterized system of differentiation hierarchies based on the obtention of two main biologic objectives: (I) to ensure the maintenance in the time of a HSC population through a process of self-renewal; (II) the continuous differentiation and generation of blood elements of the various cell lineages to provide the physiologic production of these elements and to face an increased production need related to specific physiopathologic conditions. The study of hematopoiesis was facilitated by the development of single cell assays capable of defining the immunophenotypic markers of single hematopoietic cells and single-cell clonal assays in semisolid media to define the differentiation capacities of progenitor/stem cells. In parallel the development of reconstitution of hematopoiesis into lethally irradiated mice, coupled with the cell purification technique, allowed to functionally define and to characterize HSCs. The ensemble of these studies allowed to define hierarchical models of hematopoiesis based on gradual staps of hematopoietic cell differentiation starting from multipotent HSCs, through multipotent progenitor cells (MPPs) and then committed progenitors (CFUs) of the various hematopoietic lineages up to maturing precursor cells, finally generating mature blood elements through their terminal maturation (93).

Thus, the initial hierarchical differentiation model indicated the existence of long-term HSCs differentiating into short-term HSCs which in turn generate a common multipotent progenitor for all myeloid lineages and a multipotent progenitor for B, T and NK lymphocytes; the multipotent progenitors differentiate into committed, unipotent progenitors. A second model of hierarchical hematopoiesis was developed in the years 2005–2015 and considered a major heterogeneity of HSC pool, comprising also intermediate-term HSCs and englobing multipotent progenitors; furthermore, the myeloid and lymphoid lineage remain interlinked at the level of a common multipotential lymphoid progenitor (LMPP) capable of generating not only all lymphoid lineages, but also granulo-monocytic progenitors. The actual model, the continuum differentiation model, was largely based on studies of transcriptomic carried out at single-cell level and suggesting that there is a continuum of differentiation from HSCs along different differentiation trajectories (an erythron-megakaryocytic differentiation pathway, a common lymphoid-granulo-monocytic pathways, subdivided into a lymphoid trajectory and a granulo-monocytic-dendritic trajectory) (110).

To better understand the current continuum model of hematopoiesis is of fundamental importance to understand the basic principles of the advanced single-cell technologies that have been used to study hematopoiesis (111). The single-cell transplantation of purified HSCs is a powerful technique allowing to define the repopulating potential of a single HSC, but do not allow to study the function of these cells under steady-state hematopoiesis. Single-cell whole-transcriptomic analysis was a technique that allowed to fully explore molecular heterogeneity at the level of gene expression in the hematopoietic system, providing fundamental informations not only at the level of HSCs, but also at the level of more differentiated cells (111). *In vivo* barcoding (in this technique, hematopoietic cells are barcoded *in vivo* using Sleeping Beauty-mediated transposition to generate unique DNA insertions that can be specifically identified using ligation-mediated PCR) is a technique allowing to investigate the fate and the contribution in term of differentiated cell progeny of individual stem/progenitor cells within large cell populations; this technique allowed to explore the contribution of single HSCs during hematopoiesis steady-state condition (111).

The numerous studies carried out using single-cell techniques have contributed to a better understanding of human hematopoiesis, particularly under steady-state conditions, but it is evident that all these studies have

generated a big amount of data often of very difficult interpretation. However, some general principles emerged from these studies. A first principle was that even cell populations "strongly" enriched in HSCs, such as human cord blood $CD34^+$, $CD3^-$, $CD19^-$, $CD11b^-$, $CD38^-$, $CD45RA^-$, $CD90^+$, $CD49f^+$ were in fact high heterogeneous, as shown by: (I) when analyzed by mass cytometry for 40 different proteins, the cells revealed extensive heterogeneity in the level of these proteins; (II) these purified cells were lentivirus barcoded and their lineage differentiation potential was explored by injecting transduced cells into irradiated mice and showed a high level of heterogeneity with 8% of cells being able to reconstitute both lymphoid and myeloid lineages, 13% generated B lymphocytes, granulocytes and macrophages, 30% generated only B cells, 36% gave rise to granulocytes and macrophages and 13% engrafted only transiently (112).

Transcriptional lineage priming represents a tool for the evaluation of lineage potential. Thus, multipotent progenitors co-express genes and transcription factors typical of the lineage that they can differentiate into. Thus, non-committed cellular elements can be characterized by co-expression of transcription factors necessary for opposing lineage fates (113). Although HSCs are lineage-biased exhibiting a priming to myeloid and megakaryocytic lineage (28), upon differentiation into LMPPs, the acquisition of lymphoid lineage priming corresponds with loss of the megakaryocytic-erythroid cell lineage potential. The transcriptomic analysis of single HSCs and HPCs showed that during homeostasis, individual HSCs gradually acquire lineage biases along multiple differentiation pathways without passing through hierarchically organized populations of hematopoietic progenitors. On the contrary, unilineage-restricted progenitor cells are generated through a continuum process originated from low-primed HSCs and progenitor cells (114). These observations have suggested the existence of a continuous landscape of hematopoiesis downstream of HSCs, where different strategies and trajectoires of differentiation can be used (114). The transcriptomic analysis of the multiples types of hematopoietic progenitors present in human cord blood or bone marrow of a consistent functional and transcriptomic heterogeneity, suggesting that a continuum of progenitors execute lymphoid myeloid differentiation downstream of stem cells (115,116).

Other studies have contributed to better understand the contribution of HSCs to *in vivo* hematopoiesis under steady-state conditions and after transplantation. The pool of HSCs increases from birth to adolescence, reaching at this age the number of HSCs maintained during adult life: this phase of HSC expansion after birth could be dictated by increased physical spaces in BM niches due to bone size growth during adolescence (117). Data derived from the study of X-chromosome ratio drift with aging in blood cells of 1,226 female humans (118), of telomere shortening in granulocytes of 356 healthy individuals (119) and clonal tracking studies of HSCs in patients (Wiskott-Aldrich syndrome patients) performing gene therapy with lentiviral vector-modified HSCs (120), all allowed to estimate that about 1275 HSC-derived clones contributed to human hematopoiesis and are required to maintain steady-state blood cell generation. Two other recent studies based on the analysis of mutations occurring in normal HSCs during life reached the evaluation of a higher number of HSCs contributing to normal steady-state hematopoiesis (101,121).

Patients treated with genetically-repaired HSCs/HPCs represents a precious source to study *in vivo* hematopoiesis and particularly to explore the role of HSCs and HPCs in hematopoietic reconstitution since engrafting by the vector used for gene therapy at the level of a unique DNA site (117). These studies allowed to show that multipotent progenitors and HSCs have distinct roles during the initial phase of reconstitution after transplantation and during the subsequent steady-state phases: during the initial engraftment, hematopoiesis is mainly sustained by short-lived hematopoietic progenitors; during the early phase of reconstitution, the hematopoiesis in ensured by MPPs and ST-HSCs; finally, during the phase of steady-state hematopoiesis, starting about 1 year after transplant, the LT-HSC compartment first expanded and then reached a steady-state number and maintained hematopoiesis at long-term (120,122).

Circulating EPCs

A great effort was made in the last years in order to identify, purify, characterize and define cells exhibiting phenotypic, biochemical, molecular, morphological and functional properties of EPCs (123-126). These studies have led to the identification in hematopoietic tissues (bone marrow, peripheral blood and cord blood) of a very rare population of EPCs, called ECFCs or late outgrowth endothelial cells, not originated from bone marrow, capable of intensive proliferation and differentiation into mature cells, generating a large number of these cells, functionally competent to sustain *in vitro* and *in vivo* angiogenetic

Table 1 Cellular origin, phenotypic and functional properties of myeloid angiogenic cells (MACs) and endothelial colony forming cells (ECFCs)

Properties of endothelial progenitor cells	MACs	ECFCs
Cellular origin	Bone marrow	Vessel wall
CD45	+	–
CD14	+	–
CD11c	+	–
CD133	+	–
CD115	+	–
CD146	Low	High
CXCR4	+	–
CD162	–	+
vWF	–	+
CD144 (VE-cadherin)	–	+
NRP-1	–	+
CD31	+	+
CD105	+	+
CD34	+/–	+/–
VEGF-R2	+	+
CD117	+	+/–
AcLDL uptake	+	+
Mechanism of angiogenetic activity	Indirect	Direct
Paracrine capacity of sustaining angiogenesis	+	+
In vivo vessel formation	–	+
In vitro tube formation	Low	High
Clonal replating and expansion capacity	–	+
In vivo homing to ischemic tissues	–	+
Stimulation by endothelial injury	–	+
Stimulation by severe endothelial damage	+	+

processes (123-126). These EPC were present in the vessel wall, at the level of intima (123-126). ECFCs can be considered true EPCs and must be distinguished from a population of hematopoietic progenitors able to generate monocytic cells endowed with a peculiar pro-angiogenetic activity, but unable to generate true endothelial cells (*Table 1*); the proangiogenic effect of these cells, called circulating angiogenic cells (CACs), is mediated through the release of numerous cytokines and growth factors stimulating the angiogenetic activity of true endothelial cells (123-126).

Although ECFCs and CACs are different cell types, generating a cell progeny pertaining to different lineages, they cooperate *in vivo* in the context of regenerative angiogenetic processes. The definition of CACs and ECFCs is defined according to different experimental assays. CAC assays were based on initial studies of Asahara and coworkers. These authors have developed an assay based on isolation of $CD34^+$ cells and adhesion to fibronectin-coated dishes and the growth *in vitro* in an endothelial growth supplemented medium; the cells generated in these cultures correspond to hematopoietic cells expressing some endothelial markers (127).

A modified version of this assay involved the growth of single $CD133^+$ cells in semisolid medium, a combination inducing the formation of small and large colonies, composed by hematopoietic cells $CD14^+$ and $CD45^+$, co-expressing some endothelial markers (128).

A more simple assay to evaluate CACs was reported by Ito and coworkers in 1999 and consisted in the plating of peripheral blood mononuclear cells on fibronectin-coated dishes; adherent cells are then grown in a culture medium supplemented with fetal calf serum and endothelial growth factors for 7 days; some adherent cells grown under these conditions assembled into small colonies, composed by hematopoietic, monocytic cells, expressing some endothelial markers, such as VEGF-R2, CD31 and Tie-2 (129).

The methodology to grow ECFCs is different and was derived from a pivotal study of Lin *et al.* reporting the methodology of endothelial outgrowth and showing in a group of patients transplanted with allogenic bone marrow that outgrowth endothelial cells starting 1 month after transplantation are mainly derived from bone marrow donor (130). Ingram and coworkers modified this methodology and defined the standard assay for ECFCs, consisting in the culture of mononuclear cells isolated from either peripheral blood, cord blood or bone marrow in complete EGM2 medium on collagen-coated dishes: after 1 or 2 weeks of culture, an adherent cell population starts to develop, forming colonies composed by true endothelial cells ($VE\text{-}Cadherin^+/CD45^-$) (131,132). Importantly, these cells have a

high proliferative potential, achieve at least 100 population doublings, replate into secondary and tertiary colonies, and retain high level of telomerase activity (131,132).

These ECFC colonies are extremely rare in that their number is estimated in the order of less than 1 colony/20 mL of adult peripheral blood, while their number is clearly higher in cord blood being estimated around 8 ECFCs/20 mL of blood (131,132). Martin-Ramirez *et al.* developed a protocol for the growth of ECFCs from mononuclear cells: in this protocol, mononuclear cells were isolated from peripheral blood and plated on collagen type I at 135,000 cells/cm^2 in EGM2 medium; colonies become detectable after 14–28 days; using this optimized protocol, 1 colony/3 mL of blood was obtained (133).

Joo and coworkers have analyzed the phenotype of endothelial cells generated by ECFCs grown according to the protocol of Martin Ramirez *et al.* and observed that endothelial cells generated by these progenitors, compared to HUVEC cells, displayed higher expression of activated endothelial tip markers, such as DLL4, CXCR4, CD34 and VCAM1 and arterial genes, such as DLL4 and CXC40, but lower expression of venous and lymphatic genes, such as COUP-TFII and PROX1 (134). When co-cultivated with HUVEC cells, ECFCs tented to form vascular structures with ECFCs preferentially located as sprouting tip endothelial cells (134). These observations suggest that ECFCs have a consistent angiogenic potential (134). Au and coworkers have evaluated the *in vivo* angiogenetic potential of ECFCs derived from adult peripheral blood compared to cord blood ECFCs showing that in a model of vasculogenesis the latter ones have a greater potential than the former ones to generate functional vessels (135).

A humanized large-scale expansion procedure of ECFC isolation, culture *in vitro* and expansion was reported by Reinisch and coworkers, thus developing a culture system for the production of functional endothelial cells potentially suitable for various types of clinical applications (136). In this humanized version of the cell culture system for ECFCs, the fetal calf serum present in the EGM2 medium was replaced by 10% pooled human platelet lysate (136). A point of fundamental importance was related to the definition of the origin of ECFCs, particularly to demonstrate whether they originate from bone marrow of from other endothelial cells and they are present in peripheral blood or in cord blood just because they circulate in the body. Timmermans and coworkers have shown that PB, BM and CB CD34+ cells are able to generate ECFCs: the sorting of CD34$^+$ cells in the frequent CD45$^+$ subset and in the very rare CD45$^-$ subset showed that CD34$^+$/CD45$^-$, but not CD34$^+$/CD45$^+$ are able to generate ECFCs (137,138). CD34$^+$/CD45$^+$ cells are able to generate only monocytic cells displaying some endothelial cell markers (137,138).

Studies on the characterization of the phenotype of ECFCs confirmed that CD34$^+$/CD45$^-$, but not CD34$^+$/CD45$^+$ cells are able to generate ECFCs (139). Importantly, the fraction CD34$^+$/CD45$^-$ contained also mature circulating endothelial cells (CECs) and express various markers typically observed in mature endothelial cells (139). CECs represent a rare peripheral blood cell population, characterized by typical mature endothelial properties; these cells detach from vessel wall, as a consequence of physiological tissue turnover or of vascular damage, and through this mechanism become circulating (140). CECs have been considered as a useful biomarker in various pathological conditions implying endothelium homeostasis or to monitor angiogenesis inhibition in cancer therapy.

Other studies supported the conclusion that ECFCs do not originate from bone marrow. In fact, Tura and coworkers, using the standard methodology to grow ECFCs, showed that ECFCs were constantly detected in cord blood, very frequently in adult peripheral blood, but apparently were not detected in bone marrow and in mobilized peripheral blood (141). ECFCs displayed a phenotype comparable to mature endothelial cells and were enriched in the CD34$^+$/CD133$^-$/CD146$^+$ cell fraction (141). However, CD34$^+$/CD133$^-$/CD146$^+$ cell isolated from bone marrow failed to generate ECFCs, thus supporting the conclusion that circulating ECFCs are not issued from bone marrow (141).

The expression of CD146 on the membrane of ECFCs is important because offered the opportunity to stimulate the proliferation and the function of these cells using the soluble ligand of this receptor. CD146 is a cell adhesion molecule belonging to the immunoglobulin superfamily, expressed on endothelial cells and functionally involved in the control of angiogenesis; the shedding of CD146 determines the release of a soluble form of CD146 (sCD146) that, through interaction with its receptor, acts as a stimulating factor for angiogenesis. In an initial study, Stalin and coworkers reported that the priming of ECFCs with sCD146 did not modify the number of these progenitor cells, but enhanced their angiogenetic function in revascularization assays, through an effect that seems related to an increased viability of these cells (141). The effect of sCD146 in the promotion of ECFC survival seems to be related to the mechanism of action of this soluble ligand: in fact, sCD146 binding

to its receptor activates the proteolytic processing of the short isoform of CD146, leading to the generation of on intracellular CD146 form that migrates to the cell nucleus and stimulates target genes involved in cell survival and angiogenesis (142). In a second study, the same authors provided evidence that the addition of sCD146 to ECFC cultures increased their number, accelerated the formation *in vitro* of endothelial colonies promoted by ECFCs, stimulated the regenerative activity of these cells *in vivo* in suitable models, enhanced the "stem cell" phenotype of ECFCs and prevented the senescence of ECFCs (143).

Recent studies have provided evidence that NOTCH signaling seems to play a key role for promoting the survival and the angiogenetic activity of CB-derived ECFCs (144). The same authors provided also evidence that human platelet lysate induces effects on ECFCs comparable to those induced by NOTCH activation, eliciting an improvement of ECFC survival and the angiogenetic activity of these cells as assayed in three-dimensional collagen matrices (145). Nagano and coworkers have reported an alternative methodology to grown ECFCs based on a combination of the negative immunoselection (to remove from cord blood mononuclear cells RBCs, erythroid precursors, T and B lymphocytes, monocytes and granulocytes) and growth of the purified cell in the presence of bFGF (146). After 2–3 weeks of culture, the formation of adherent colonies, composed by endothelial elements, is observed (146). These EPCs can be subdivided into two different subpopulations according to the level of expression of aldehyde dehydrogenase (ALDH): cells with low ALDH activity possess a greater capacity to proliferate and migrate compared with those high ALDH activity (146). ALDH-low progenitors express higher levels of hypoxia-inducible factors and were more active in reducing an ischemic damage in animal models (146). In line with these observations, a subsequent study showed that low ALDH endothelial progenitors have a consistent angiogenetic activity *in vivo* and attenuate acute ischemic brain injury in a rat model (147).

WCFCs express the adrenomedullin (a vasoactive peptide) receptor and, in response to stimulation with this peptide increase their proliferation and enhance their angiogenic activity (148).

The standard EGM2-based cell culture system adopted to growth ECFCs had a limited capacity to generate endothelial cells from purified preparations of $CD34^+$ cells. To bypass these limitations, Castelli and coworkers have developed a cell culture system involving the co-culture of purified cord blood $CD34^+$ cells with HUVEC cells or, alternatively, the addition of conditioned medium from HUVEC cells (149). HUVEC conditioned medium markedly improved the proliferation and differentiation and delayed the senescence of ECFCs (149). The endothelial-promoting effect of HUVEC conditioned medium seem to be mediated by smaller vesicles including exosomes released by HUVEC cells and vehiculating to ECFCs endothelial-promoting molecules (149).

The nomenclature of EPCs remains still ambiguous and there is need of concordance in the careful definition of the various progenitor cell populations grouped under the common term of EPCs. To bypass these current ambiguities, Medina and other experts in the field have proposed a consensus nomenclature based on the standards reached at the level of definition of endothelial phenotype, of functional activity, allowing a clear separation of true endothelial progenitors from hematopoietic angiogenic cells (150). According to this new nomenclature hematopoietic cells supporting angiogenesis are defined as myeloid angiogenic cells (MACs), generating monocytic cells $CD14^+/CD45^+/CD31^+/CD34^-$/CD146–/CD144(VE-cadherin)$^-$; conditioned media of these cells enhance endothelial network formation *in vitro* and *in vivo* and, therefore, their angiogenic activity is indirect, mediated by paracrine mechanisms (150). The true endothelial progenitors are defined as ECFCs, generate true endothelial cells, have an intrinsic tube forming capacity *in vitro* and *in vivo* and are directly involved in vasculogenic processes *in vivo*, participating to new blood vessel formation (150).

A correct understanding of the biology of EPCs is essential for developing therapeutic applications based on the administration of these cells. Thus, Petel and coworkers have proposed that ECFCs must be evaluated for their high proliferative capacity through self-renewal assays and multiple passagings and for their *in vivo* vessel-forming capacity through currently used approaches (hind-limb ischemia assay, retinal neovascularization assay or Matrigel plug assay) (151).

Several recent studies have explored some properties of ECFCs particularly relevant for their potential clinical applications. Thus, Nuzzolo and coworkers have recently shown that CB ECFCs exhibit the same pattern of expression of HLA-ABC and HLA-DR as mesenchymal stroma cells, which are transplanted irrespectively of HLA-matching (152). Furthermore, CB-derived ECFCs have a lower expression of various inflammatory mediators (152). For these peculiar properties, CB ECFCs can be transplanted to immunocompetent animals without

being rejected, as shown in a recent study demonstrating the capacity of these cells to promote post-ischemic angiogenesis in immunocompetent mouse models (153). Very interestingly, Merola and coworkers recently showed that Human ECFC-derived ECs lacking MHC molecule expression, by CRISPR/Cas9-mediteds complete ablation of MHC expression, can be utilized for engineering vascularized grafts that evade allorejection (154).

The immunogenic potential of ECFCs is further reduced by stromal cells that have shown to act as "guardians" for EPCs by reducing their immunogenicity (155). This conclusion was reached through co-transplantation experiments using ECFCs and mesenchymal stem cells (MSCs): ECFC/MSC co-application in immunodeficient mice promoted the development of blood vessel architecture and reduced intra-graft immune cell infiltration and endothelial HLA-DR expression (155). Other recent studies support the positive interaction between ECFCs and MSCs in that these two cell types cooperate in sustaining their respective biological activities. Thus, it was shown that: (I) blood-derived ECFCs can act as paracrine mediators, regulating the regenerative potential of MSCs via PDGF-BB/PDGFR-β signaling (156); (II) ECFCs + MSCs delivery functions better than EPC delivery alone to re-establish blood flow in ischemic tissues and this effect is regulated by coordinated recruitment of host myeloid cells (157); priming of ECFCs in a mesenchymal niche improves engraftment and vasculogenic potential by inducing mesenchymal transition of ECFCs orchestrated by NOTCH signaling (158).

As above mentioned, various studies have shown that ECFCs have robust vasculogenic properties. However, other studies have shown that EC FCs can exert also some trophic, paracrine functions, in support of specific stem cells *in vivo*. Thus, Lin and coworkers in xenograft co-transplantation experiments have shown that CB-ECFCs can act *in vivo* as trophic mediators for mesenchymal stem cell engraftment via paracrine signaling (159). CB-ECFCs, in part through a paracrine mechanism, exert a reparative effect in a model of lung disease characterized by oxygen-induced alveolar damage (159). Interestingly, a similar conclusion was reached by Sakimoto *et al.* in animal models of retinal disease: in these models, intravitreally injected CB-ECFCs rescued vaso-obliteration and neurodegeneration (160). A subset of the CB-ECFCs, characterized by high expression of CD44 and hyaluronic acid receptor, was more effective at anatomically and functionally preventing retinopathy (160). Interestingly, the reparative effect of these ECFCs can be obtained also through addition of conditioned media (160). The idea of a cooperative effect between EPCs and MSCs is also supported by *in vivo* experiments in a model of ischemic skeletal muscle showing that the combined infusion of ECFCs and MSCs resulted in a recovery of blood flow in ischemic tissue better than with ECFC infusion alone (161).

As above mentioned, *in vivo* studies have shown the intrinsic vasculogenic potential of ECFCs. Following transplantation in mice, it was shown that ECFCs self-assemble into long-lasting microvascular networks that form anastomoses with the host vasculature (162,163). These new ECFC-generated microvessels are comparable to normal vessels for that concerns blood flow, non-thrombogenicity, capacity to induce leukocyte-endothelial adhesion and transmigration in response to cytokines and macromolecular permeability (162,163).

ECFCs are very rare in adult peripheral blood. Therefore, it would be very important to develop simple procedures enriching for the cell population ($CD34^+$ cells) containing $CD34^+$ cells in a clinically-compatible setting. In this context, a recent study reported the development of a microfluidic system for capture of circulating $CD34^+$ cells from unprocessed blood, fully compatible with the subsequent differentiation of these cells into ECFCs (164).

Other studies have explored the stemness properties of ECFCs showing that, unlike mature endothelial cells, CB-derived ECFCs express genes involved in the maintenance of embryonic stem cell properties such as SOX2, DNMT3A or GDF3 (165). Furthermore, ECFCs can be instructed *in vitro* to develop a peculiar endothelial phenotype: ECFCs co-cultured with astrocytes acquired some blood brain barrier features, while their growth in the presence of high VEGF resulted, through activation of NOTCH signaling, in an increased expression of arterial endothelial markers (166).

Interestingly, ECFCs cultures display significant heterogeneity and a hierarchy among progenitors able to give rise to high proliferative colonies (157). Based on CD34 expression $CD34^+$ and $CD34^-$ ECFCs were distinguished: only $CD34^+$ ECFCs had the capacity to generate high proliferative potential colonies, have self-renewal capacity and were superior at restoring perfusion in ischemic animal models (167). Transcriptomic analysis showed that cyclin-dependent kinase cell cycle inhibiting genes, the NOTCH signaling pathway and the endothelial cytokine IL33 are highly expressed in $CD34^+$ ECFCs (167).

Another study addressed the interindividual heterogeneity of ECFCs in cord blood samples, with some samples

generating a high number of colonies; the capacity of generating endothelial colonies was associated with a higher proliferative potential and with a higher angiogenic activity (168).

Studies carried out in experimental models of hind-limb ischemia in mice have shown that stimulation of sonic hedgehog (SHH) signaling promoted the reparative angiogenetic response through an indirect effect mediated by up-modulation of the expression of VEGF and angiopoietin-1 and -2 in interstitial mesenchymal cells (169). Transcriptomic and proteomic analyses of ECFCs showed a pronounced expression of the Hedgehog-interacting protein (HIP) (170). Inhibition of HIP expression in ECFCs activated canonical SHH signaling, while it activated non-canonical SHH signaling in mature endothelial cells (170). In ECFCs, HIP knockdown markedly enhanced tube formation and resistance to apoptosis. Although HIP is highly expressed in ECFCs, its expression is markedly downmodulated during angiogenesis (HIP expression is reduced in response to VEGF stimulation) (170). Activation of SHH signaling into ECFCs is a promising therapeutic strategy (171).

Flow cytometric identification of ECFCs remains difficult for the rarity of these cells and for their immunophenotypic similarity with mature endothelial cells. Mund and coworkers showed that ECFCs, as well as mature endothelial cells, were characterized by positivity for CD34, CD31 and CD146, but not for CD45 and CD133 (139). Cells purified according to this immunophenotype failed to form hematopoietic colonies but generated highly proliferative endothelial colonies (139). Another study confirmed that ECFCs do not express CD133 on their membrane (172). Hulzer and coworkers have confirmed that ECFCs are comprised in the cell fraction immunophenotypically corresponding to CECs $CD34^+/CD45^-$ (173). A subset of CECs expressing c-kit could correspond to ECFCs (173).

A recent study provided evidence that NOX4 NADPH oxidase, the major ROS-producing enzyme, is a major regulator of cord blood-derived ECFCs. NOX4-overexpressing CB-ECFCs have an increased *in vivo* reparative capacity in the mouse ischemic hind limbs assay, while NOX4-knockdown CB-ECFCs have a reduced capacity to induce *in vivo* blood flow recovery of ischemic limbs (174). Interestingly, NOX4-overexpressing CB-ECFCs have an enhanced *in vivo* reparative and survival capacity at the level of ischemic tissues (158). A study carried out on human ECFCs isolated from vessel walls confirmed that NOX4-type NADPH oxidase is important for proliferation and migration functions of EPCs and protects against cytokine-induced cell death. The functional capacities induced by NOX4 facilitate the role of EPCs in the processes of revascularization and tissue repair (175).

Other studies have shown a role of osteoprotegerin (OPG), an important regulator of bone metabolism, as an important modulator of ECFCs. Thus, these studies showed that OPG contributes to the process of ischemic tissue revascularization, tumor growth *in vivo* and potentiates the proangiogenic properties of ECFCs through the secretion of stromal derived factor 1 (176,177). OPG increased the number of ECFCs generated from cord blood $CD34^+$ cells and accelerated the time of the initial appearance of ECFC colonies (178).

EPCs resident in the vessel wall

Human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (HAECs), both derived from the vessel wall, are commonly considered as differentiated mature endothelial cells. However, similarly to ECFCs, HUVEC and HAEC, can be passaged for at least 40 population doublings (179). The analysis of individual HUVECs and HAECs showed that about 30% of these cells have clonogenic activity, being capable of generating colonies of different size, corresponding to a hierarchy of EPCs (179). According to these findings it was proposed the hypothesis that a population of cells with properties of endothelial progenitors is present in the vessel walls and can respond to different types of physiological or pathological stimuli undergoing proliferation and differentiation, required for neovascularization.

Many other evidences support the existence of endothelial progenitors, resident in vessel walls. The need for loco-regional EPCs is also related to the peculiar specialization of the endothelium at the level of various tissues. Thus, recent studies support the existence of EPCs in the pulmonary circulation. Pulmonary endothelial cells are heterogeneous and display some peculiar features: particularly, microvascular pulmonary endothelial cells possess highly impermeable barrier with respect to pulmonary artery or vein endothelial cells and express calcium channels not found in extra-alveolar endothelial cells (180). Another peculiarity of microvascular pulmonary endothelium is that this endothelium possesses a larger surface area than any other vascular bed and a high proliferative capacity. Alvarez and coworkers have isolated endothelial cells from rat pulmonary artery (PAECs) and from lung microcirculation (PMV ECs): while the majority

of PAECs are well differentiated and do not have clonogenic activity, the majority of PMVECs are clonogenic and form large colonies in *in vitro* unicellular assays (181). PMVECs express progenitor markers (CD34 and CD309) and show vasculogenic activity, thus supporting their progenitor nature (181).

Another study carried out in mouse lung showed that a fraction of $CD31^+$ cells are dividing: these dividing endothelial cells form large colonies of endothelial cells and are able to integrate *in vivo* into various types of vessels, including blood and lymphatic vessels, thus supporting their function to act as bipotent EPCs capable of forming functional blood and lymph vessels (182). Mouse EPCs could express c-kit, as suggested by the observation that a part of mouse endothelial cells express c-kit: transplantation of these cells into injured mouse lung do not regenerate epithelial cells and maintain a vascular endothelial cell fate (183). According to these observations, it was proposed a protocol for the isolation and characterization of ECFCs from rat and human lungs, based on the isolation of CD31-positive cells on enzyme-dispersed cellular suspensions of lung tissue, followed by culture of $CD31^+$ cells in endothelial-specific growth conditions (184).

A recent fundamental study provided evidence about the existence of a hierarchy of EPCs residing in vessel wall and involved in tissue regeneration (185). To identify these progenitors, Patel and coworkers have explored an *in vivo* wound healing assay. Based on expression levels of common endothelial markers three subpopulations of endothelial cells could be identified among VE-$Cadherin^+$/$CD45^-$ cells: endovascular progenitor (EVP, $CD31^-$/low, VEGF-R2low), transit amplifying (TA, CD31int/VEGF-R2low) and definitive differentiated (D, CD31high/VEGF-R2high) (185). In the context of the sequential stages of wound healing, these three populations appeared sequentially in time and in a quantitative ratio, phenomena highly suggestive of a hierarchy, confirmed by lineage-tracing experiments (185). Transplantation experiments showed that EVPs, but not TAs or Ds, are able to form neovessels (185). In line with this finding, EVPs, but not TAs or Ds, form colonies of endothelial cells *in vitro* (185). The exact location of EVPs in the vessel was at the moment unknown. The same authors confirmed through single-cell sequencing the existence of two distinct endothelial cell populations in the aorta (186). Gene co-expression studies showed crucial regulatory networks underlying the EVP and the mature endothelial cell population, including peculiar metabolic gene networks in EVPs (186). Mitochondrial activity assays and phenotypic analyses provided evidence that EVPs display higher mitochondrial content compared to mature endothelial cells (186).

Other studies support the existence of some remarkable differences between ECFCs and mature endothelial cells. Interestingly, a recent study showed that ECFCs possess a high sprouting activity, a property related to angiogenesis (187). In fact, Sturtzel and coworkers showed that the forkhead box transcription factor FOXF1 is selectively expressed in ECFCs compared to mature endothelial cells; FOXF1 expression had a strong impact on the sprouting capabilities of EPCs, as supported by the observation that FOXF1 overexpression in endothelial cells induces the expression of NOTCH2 receptors and induces sprouting (187). Thus, this study supports a key role for endothelial progenitors as the mediators of both vasculogenic and angiogenic processes. A second recent study further supports that capacity of ECFCs to promoting angiogenic sprouting for their capacity to synthesize and to release Cytokine-Like 1 (CYTL1): this proangiogenic factor is induced by hypoxia and promotes in both ECFCs and mature endothelial cells angiogenic sprouting (188). Furthermore, CYTL1 promotes vessel formation in animal models comparable to VEGF-A (188).

Recently, Green and coworkers have reported the presence of EPCs among endothelial cells issued from saphenous veins removed for venous insufficiency (189). Endothelial cells grown in complete EGM2 medium generated endothelial colonies exhibiting a proliferative activity comparable to that observed for CB ECFCs (189). Furthermore, these cells injected into immunodeficient mice in fibronectin/collagen gels promoted the formation of functional blood vessels (189).

Other studies have reported the presence of cells with properties of endothelial progenitors in the vessel wall. Thus, Zengin *et al.* have observed the existence of $CD34^+$/$CD31^-$/VEGF-$R2^+$ and Tie-2^+ in a zone of vessel wall localized between smooth muscle and adventitial layer (190). These cells are capable of capillary sprouting and were observed in large and middle-sized arteries and veins (190).

Invernici and coworkers reported the identification of vascular progenitors in human fetal aorta: upon stimulation with VEGF-A these progenitors generate endothelial and mural cells (191).

Endothelial progenitors were observed in the chorionic villi of term human placenta: these placental ECFCs have properties similar to ECFCs isolated from cord blood but have a higher vasculogenic activity than CB ECFCs (192). Human placenta contains also meso-endothelial bipotent progenitors, called mesangioblasts: these cells, directly

issued from embryonic stem cells, have been shown in dorsal aorta during embryonic life, have extensive self-renewal capacity and have the capacity to differentiate both into mesenchymal cells and endothelial cells (193).

Fang and coworkers have provided evidence that c-kit (CD117)-expressing endothelial cells with the phenotype $CD31^+/CD105^+/Sca1^+/CD117^+$, resident in vessel wall have the properties of endothelial progenitors and are able *in vivo* to generate functional blood vessels that connect to the host circulation (194). However, a recent study based on genetic tracing analysis challenged the previous study on c-kit+ EPCs and showed that c-kit^+ stem/progenitor cells are not a main source for endothelial regeneration, but contribute only to the generation of inflammatory cells that participate to the reparative process of vascular injury-induced neointimal lesions (195). Therefore, the endogenous c-kit^+ cells do not seem to be resident vascular progenitors and do not have the potential to generate new blood vessels after injury.

Recent studies have provided a strong support to the existence and to the functional role of resident endothelial progenitors. McDonald and coworkers have explored the cellular processes underlying injury-induced endothelial regeneration (196). Thus, using an *in vivo* aortic endothelial injury model, the cellular dynamics underlying the regenerative process through multicolor lineage tracing, parabiosis and single-cell transcriptomics was analyzed (193). First, their results, through lineage-tracing experiments, allowed to exclude a contribution of circulating cells to endothelial regeneration (196). Second, through multicolor clonal tracing it was provided evidence that two cellular populations with different proliferative activities participate to the endothelial reparative process: these cells are originated from injury-adjacent endothelial cells (196). These observations indicate that there exists a hierarchy of cells with greater or lesser proliferative activity within the aortic endothelial lining; furthermore, a large part of proliferating endothelial cells within the regenerating endothelium is represented by transient-amplifying cell populations (196). Endothelial regeneration requires activation of stress response genes, including ATF3: this gene is responsible for the activation of quiescent endothelial cells, enabling their expansion within the aortic lumen (196). This important study can be interpreted assuming that heterogeneous populations of EPCs resident in the vessel wall are activated by vessel injury and orchestrate endothelial regeneration according to a biphasic kinetics.

These observations were completed by the transcriptomic analysis of the regenerating endothelium, showing the occurrence of an initial upregulation of genes like JUN, FOS, MYC and of genes involved in cell adhesion; this process was quickly followed by a transient wave of proliferation-related genes; after completion of endothelial healing, a sustained array of extracellular matrix transcripts was upregulated (196).

Singhal and coworkers have studied several irradiation-based myeloablative and non-myeloablative mouse models to explore the cellular sources responsible for the regeneration of liver vasculature. In animals with the intact endothelium (animals were irradiated with a radioprotective shield over the upper abdomen to avoid liver endothelial cell damage) the neo-angiogenetic reparative response was solely mediated by proliferation of resident endothelial cells (197). However, following irradiation-induced endothelial cell damage, bone marrow-derived cells were incorporated into vasculature (198). Additional experiments with direct infusions of bone marrow cells showed that these cells do not contribute to the regeneration of liver vasculature after two-thirds partial hepatectomy (198). In order to provide a plausible interpretation to these intriguing observations the authors concluded that the source of regenerating endothelium depends on the fitness of the residual vasculature (198). In this context, it is important to point out that a recent randomized, controlled phase II clinical trial involving 81 patients with compensated liver cirrhosis, administration of G-CSF alone or in combination with HSCs failed to induce a significative improvement of liver function or to ameliorate liver fibrosis (199).

Other recent studies have shown the contribution of bone marrow cells to reparative processes in conditions associated with radiation-induced endothelial damage in the bone marrow stroma (200) or in the central nervous system (201).

Another set of data were based on the study of a liver injury model and provided evidence about the existence of an endothelial progenitor located in vessel wall. Naito and coworkers initially provided evidence about a population of liver endothelial side population cells (VE-$cadherin^+$/$CD31^+/CD45^-$/Hoechst low) that possesses colony-forming ability, produces large numbers of endothelial cells and when transplanted into ischemic lesions restores blood flow and reconstitutes blood vessels (202). Using genetic recombination and bone marrow transplant models, it was provided evidence that these cells phenotypically pertain to the endothelial lineage and do not originate from hematopoietic cells (203). In a more recent study, the same authors have characterized this cell population showing

that is capable of self-renewing and generates functional blood vessels in a liver injury model; furthermore, by microarray analysis it was shown the high expression of CD157 (bone marrow stromal antigen-1) and CD200 on these cells (204). $CD157^+/CD200^+$ liver cells express only endothelial markers, but not hematopoietic or mesenchymal markers (204). CD157 expression in vessels is restricted to large arteries and veins, but apparently is not observed in capillaries (187). The characterization *in vitro* of the properties of $CD157^+/CD200^+$ cells showed that these cells show a high proliferative potential and are able to generate a robust vessel reconstitution (generating endothelium in great liver vessels, sinusoids and capillaries), resulting in the production of a progeny $CD157^+/CD200^+$, $CD157^-/CD200^+$ and $CD157^-/CD200^-$ cells (204). Another study suggested that protein C receptor (ProCR) is a marker of tissue vascular stem cells. $ProCR^+$ endothelial cells display robust clonogenetic activity in culture, high vessel reconstitution capacity in transplantation studies, long-term clonal expansion in lineage-tracing experiments, thus suggesting their functional properties of vascular stem cells (205). Interestingly, these cells appear to be bipotent progenitors for their capacity to generate both an endothelial cell progeny and perivascular pericytes (205). The most common origin of pericytes is represented by mesenchymal stem cells, but in conditions of neoangiogenesis, pericytes could be regenerated also through the differentiation of $ProCR^+$ vascular progenitors (205).

These observations strongly support the existence of stem/progenitor endothelial cells in the liver vessel walls and suggest hierarchy of endothelial cell types within blood vessels. These studies imply also the existence of a consistent heterogeneity of endothelial cells, as supported by functional (206) and single-cell transcriptomic studies (207).

Since endothelial cells may be derived from local progenitors in different organs and tissues, as above discussed, this represents an important biological factor increasing the heterogeneity and complexity of endothelial cells for that concerns their capacity of adaptation to the specific needs of the various tissues and of response to injury and regenerative capacity. The study of the organ-specific features of endothelial cells is particularly difficult because these cells have the tendency to lose their specific gene expression patterns during passaging. A recent study addressed the problem of organ-specific heterogeneity of human endothelium through the study of: (I) basal protein expression in *ex vivo* microvascular tissue beds; (II) organ-specific endothelial cell population in tissue culture; (III) morphology, structure, protein expression, transcriptional profiling, and vascular function of endothelial cells after isolation, culture and passaging; (IV) transcriptional validation signature, in freshly isolated endothelial cells obtained from four organs, the heart, kidney, liver and lung, from human fetuses (208). This fundamental study provided some key information's: (I) human fetal endothelial cells isolated from the four organs display organ-specific cell population; (II) human fetal endothelial cells maintain *ex vivo* heterogeneity upon *in vitro* expansion; (III) global RNA sequencing shows heterogeneous gene expression profiles in cultured organ-specific endothelial cells; (IV) human fetal endothelial cells isolated from the four organs display organ-specific functional and metabolic properties: heart endothelial cells have the highest trans-endothelial electrical resistance, angiogenic potential and metabolic rates, while human fetal liver endothelial cells support hepatocyte function (208). This study provided evidence about a linkage between human endothelial heterogeneity and organ development, with the contribution of intrinsic and microenvironment to this heterogeneity (208).

Future studies will show whether the organ-specific endothelial cell heterogeneity is reflected and orchestrated by a correspondent heterogeneity at the level of the stem/progenitor endothelial compartment and the contribution of microenvironmental signals to drive the endothelial cell specialization.

Conclusions

The studies carried out in the last years have greatly contributed to better understand the basic biology of endothelial cells and of EPCs. These studies have clearly shown that endothelial cells are heterogeneous and exhibit a clear organ-related specialization to better adapt to the specific needs of the various organs. The generation of endothelial cells during embryonic/fetal development is ensured by progenitor cells, the angioblasts, that though their differentiation and vasculogenic activities generate the first vessel networks, that become more and more complex and specialized, giving rise to the vasculature of the various developing organs.

However, the contribution of EPCs is not limited only to the embryonic/fetal life in that these cells are present in adult life and contribute to vessel homeostasis and to regenerative processes. Two types of EPCs have been identified: a type, called MACs are not true EPCs in that do not generate endothelial but monocytic cells, are originated from bone

marrow and contribute to the promotion of angiogenetic processes through paracrine mechanisms, mediated by the release of proangiogenic cytokines/growth factors; a second type, called ECFCs, are true EPCs because generate endothelial cells, do not are originated from bone marrow but are resident in vessel wall and directly contribute to neo-angiogenetic processes with the building of new vessels.

The identification, characterization, purification and *in vitro* expansion of these cells offers the opportunity for their therapeutic use in all the pathological conditions in which the patients may benefit from a neo-angiogenetic regenerative response. The clinical studies until now performed were based on the use of MACs and were basically associated with a very limited clinical benefit. However, these studies have great limitations related to the heterogeneity of patients and of the cell types used as a source of MACs. Careful assessment of the real therapeutic impact of MACs in pathological conditions associated with vascular diseases will require a standardization procedure for MAC preparation and controlled clinical trials.

Future studies involving the use of ECFCs are greatly expected and should involve: (I) the first evaluations of ECFCs at clinical level in some vascular diseases; (II) the improvement of the methodology used for the amplification of ECFCs and their optimization in function of specific clinical applications; (III) a better understanding of the biology of ECFCs, particularly in view of the definition of organ-specialized ECFCs.

A very promising area under investigation already at the clinical level is represented by the use of bioengineered human acellular vessels (HAVs) which are currently being investigated as a hemodyalisis conduct in patients with end-stage renal disease (209). In few cases, small pieces of HAVs were recovered during surgical interventions and were analyzed to explore the pattern of the host cell response to the HAVs after transplantation. This analysis showed that the implanted HAVs progressively transform from acellular vessels to functional multilayered living and functional vessels, colonized by both myoepithelial and endothelial cells (209).

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/sci.2020.03.02). UT serves as an unpaid Editorial Board Member of Stem Cell Investigation. The other authors have no conflicts of interest to declare.

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