Chapter 3
Hematopoietic Stem and Progenitor Cells (HSPCs)

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Abstract Hematopoietic stem/progenitor cells (HSPCs) isolated from bone marrow have been successfully employed for 50 years in hematological transplantations. Currently, these cells are more frequently isolated from mobilized peripheral blood or umbilical cord blood. In this chapter, we overview several topics related to these cells including their phenotype, methods for isolation, and in vitro and in vivo assays to evaluate their proliferative potential. The successful clinical application of HSPCs is widely understood to have helped establish the rationale for the development of stem cell therapies and regenerative medicine.

Keywords Hematopoiesis · Hematopoietic stem cells · Hematopoietic progenitors · Stem cell purification · Hematopoietic stem cell markers · In vitro assays · In vivo assays · Expansion of hematopoietic stem cells · Very small embryonic-like stem cells · Primordial germ cells

3.1 Introduction

Hematopoiesis is a process that involves the production of all mature blood cells from hematopoietic stem cells (HSCs) by maintaining a fine balance between enormous production and need to supply and regulate the number of erythrocytes, granulocytes, monocytes, lymphocytes, and blood platelets throughout the lifetime of an individual. It has been calculated that the average adult person produces more

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M. Z. Ratajczak (ed.), *Stem Cells*, Advances in Experimental Medicine and Biology 1201, https://doi.org/10.1007/978-3-030-31206-0_3

than 500 billion blood cells every day, and it reflects on the highly dynamic feature of HSC compartment. In fact, HSCs and their progeny hematopoietic progenitor cells (HPCs) represent the best characterized stem cells in mammals. Several well-established experimental and diagnostic in vitro assays as well as appropriate in vivo animal models are available to study these cells [1–10].

HSCs have been identified and can be isolated from embryonic and adult tissues including the yolk sac (YS), aorta-gonad-mesonephros (AGM) region, fetal liver (FL), bone marrow (BM), peripheral blood (PB), and umbilical cord blood (UCB) [9, 11, 12]. This chapter summarizes the following: (i) properties of HSC, (ii) their developmental origin, (iii) hierarchy of the stem cell compartment in hematopoietic organs, (iv) assays to study these cells, and (v) phenotypic markers that allow isolation and identification of HSCs. Recent research has shed new light on better understanding of the embryonic development of hematopoiesis from mesoderm and HSC migration during embryogenesis, which involves multiple anatomical sites in an embryo proper before adult hematopoiesis is established in BM microenvironment [13–16].

Evidence accumulated describes that during early stages of embryogenesis, proximal epiblast-derived pluripotent stem cells (PSCs) give rise to hemangioblasts that are precursors of both HSCs and endothelial progenitor cells (EPCs). Moreover, adult BM most likely still contains a population of development early PSCs that gives rise to the long-term repopulating of HSC (LT-HSC), mesenchymal stromal cells (MSCs), and endothelial progenitors (EPCs) [17–20]. This challenging concept is discussed later on in this chapter. New strategies to isolate/purify HSC have been developed based on a presence of cell surface markers and metabolic properties of these cells [21–23]. It is noteworthy that expression of some of these surface markers depends on the developmental status of HSCs and may change during their activation and isolation. This chapter deals with both murine and human HSCs and discusses their developmental heterogeneity, phenotype, and the experimental strategies that allow to identify these rare cells in embryonic tissues (YS, AGM, FL) as well as in adult BM, PB, and UCB.

According to widely accepted stem cell definition, HSCs possess the ability for self-renewal and may differentiate into HPCs giving rise to any blood cell type of the myeloid and lymphoid lineages. In order to fulfill this mission, HSC must undergo asymmetric divisions where one of the daughter cells retains HSC potential and the other becomes HPCs (Fig. 3.1). HPCs lose the ability to self-renew; however, they are able to expand into clones of functional hematopoietic cells. This mechanism of asymmetric cell division is required to keep constant number of HSCs in hematopoietic organs and prevent their depletion. Therefore, HSCs are endowed with the unique ability to balance self-renewal and differentiation into HPCs in such a way that mature cells necessary for tissue function can be quickly generated and replaced at the same time without exhausting the HSCs pool [5, 7, 16]. Evidence gathered indicates that with age, cells expressing HSC markers increase in the hematopoietic organs, despite the fact that they are less robust as compared to those isolated from the young individuals. Moreover, HSC from old BM are also biased toward myeloid differentiation on expense of other hemato−/lymphopoietic lineages [24–26].

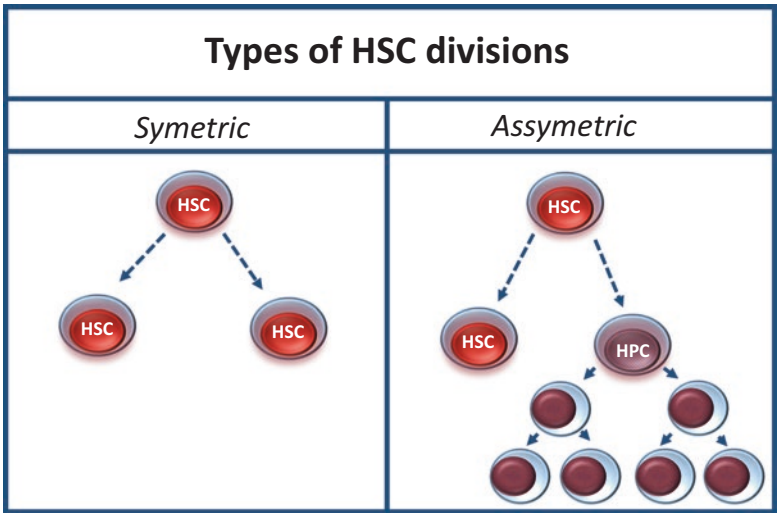

Fig. 3.1 Symmetric and asymmetric division of HSC. The most physiologically relevant is asymmetric cell division that gives rise to one HSC and one HPC. While new HSC secures constant number of HSC in BM, HPC differentiates and gives rise to clone of mature hematopoietic cells

It is well known that HSC are untired travelers and during embryogenesis migrate from one site to another anatomical site where hematopoiesis is active in a well-regulated manner. This developmental journey of the HSCs begins with their first appearance in the YS and subsequently in aorta-gonad-mesonephros (AGM) region or para-aortic splanchnopleure and continues through the FL stage to BM microenvironment. In mammals, FL is a main hematopoietic organ in second trimester of gestation before HSCs could migrate to BM in the third trimester of gestation and also to the spleen in case of rodents [13–16, 27, 28]. In postnatal life, HSCs circulate at low level in the PB, maintaining a balanced number of stem cells located in BM microenvironment in remote locations of skeleton ensuring maintenance of blood homeostasis under normal conditions as well as during state of severe hematopoietic injury. On average, BM constitutes 4% of the total body mass in humans. Based on this calculation, in an adult having 65 kilograms BM typically accounts for approximately 2.6 kilograms. Active hematopoietic BM is called "red" marrow. This color is due to the erythropoietic lineage that is prominent in this organ. In humans with aging, hematopoietic "red" BM is gradually replaced in long bones by "yellow" marrow containing mostly fat tissue also called as marrow adipose tissue (MAT). As a result of this constraint with aging, active hematopoiesis is limited to the "red" marrow present in central skeleton, such as the pelvis, sternum, cranium, ribs, vertebrae, and scapulae, and variably found in the proximal epiphyseal ends of long bones such as the femur and humerus [29, 30]. In contrast in mice, active hematopoiesis in long bones as well as the spleen is maintained during their entire life [31]. In frog, active hematopoiesis occurs in the liver [32], and in fish, the major hematopoietic organ is the kidney [33].

The circulating blood and lymph are highways for trafficking of HSCs and HPCs, and a number of circulating hematopoietic precursor cells in PB follow circadian rhythm where twice as many HSC are detected in early morning hours as compared to late night [34–36]. The number of HSCs increases in PB during strenuous exercise, inflammation, tissue/organ injury (e.g., heart infarct, stroke) [37, 38], and pharmacological mobilization (e.g., after administration of granulocyte-colony stimulating factor; G-CSF [39, 40] or CXCR4 receptor antagonist AMD3100 [41, 42]). Interestingly, these pharmacological agents increase the number of circulating HSCs and HPCs in PB up to 100 times. Pharmacological mobilization is an indispensable mean to harvest HSCs for hematopoietic transplants in clinical setting [39–42].

HSC development has been well characterized in mouse models, which serves as a surrogate model for better understanding of hematopoiesis. As it is shown in Fig. 3.2, HSC gives rise to HPC for myeloid and lymphoid lineage. The HPC for the myeloid lineage is called as the common myeloid progenitor (CMP), and for the lymphoid lineage, it is called as the common lymphoid progenitor (CLP). It has been postulated that HSC, before proceeding into the myeloid vs lymphoid pathway, may form bipotent myelo-erythroid and myelo-lymphoid progenitors. CMP gives rise to the mature cells of myeloid lineages (e.g., erythroblasts, megakaryocytes, monocytes, neutrophilic-, eosinophilic-, and basophilic granulocytes),

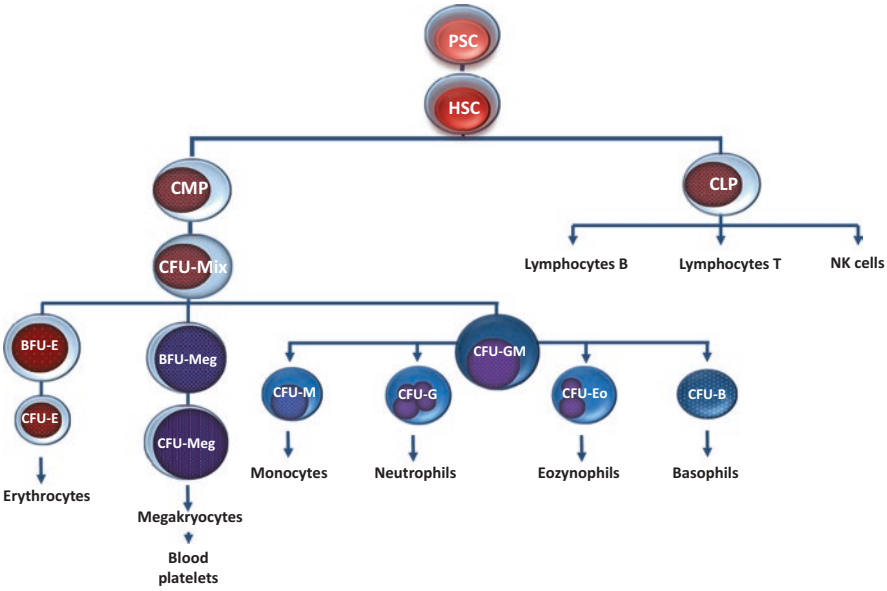

Fig. 3.2 Hierarchy of stem cell compartment in adult BM. The most primitive stem cell in adult BM is proposed as PGC-related PSC (VSEL?). PSC gives rise to HSC that leads to generation of common myeloid progenitor (CMP) and/or common lymphocytic progenitor (CLP). These cells give rise to clonogenic hematopoietic and lymphopoietic HPC

whereas CLP differentiates into lymphoid lineages (e.g., T and B lymphocytes, natural killer (NK) cells) (Fig. 3.2) [9, 10, 43].

However, this chapter discusses features of both murine and human HSCs and HPCs; it is obvious that there are considerable differences in expression of some surface markers between murine and human counterparts. As a consequence, it is sometimes difficult to identify human HSCs based solely on murine phenotype (e.g., the human analog of murine Sca-1 antigen has not been identified yet). Moreover, murine HSCs in contrast to human HSCs loose expression of cell surface marker CD34 antigen very early in development and are predominantly regarded as marker of human HSCs, and in contrast murine HSCs are $CD38^+$ [44, 45]. Due to obvious ethical reasons, functional assays to evaluate human HSCs function are very limited, in contrast to the functional assays available to study murine HSCs. Therefore, human HSC is studied in vivo by using surrogate heterotransplantation models into immunodeficient mice that do not reject human cells or in intrauterine transplants in sheep [46–50]. Development of hematopoietic system is also widely studied with obvious limitations in surrogate zebra fish model [2, 51, 52].

3.2 Developmental Origin of HSCs

The developmental origin of hematopoietic cells during embryogenesis is biphasic, starting from primitive hematopoiesis and culminating in definitive hematopoiesis, and two separate anatomical sites have been identified where these processes occur. Accordingly, the first primitive hematopoietic precursors are identified outside the embryo proper in the so-called hematopoietic islands located at the bottom of the YS that is a part of the extraembryonic ectoderm. The blood islands where primitive hematopoiesis is initiated are believed to develop from Flk-1 $(Kdr)^+$ hemangioblasts, which give rise to both HSCs and endothelial progenitors (EPCs). These cells initiate primitive hematopoiesis in blood islands at 7.0–8.0 days post coitum (dpc) giving rise to endothelial cells and primitive nucleated erythrocytes. Next, at 8.25 dpc. Mixed lineage and myelo-erythroid progenitors as well as primitive pre-HSCs become detectable in this anatomical location [12, 16–20, 53].

The developmental origin of hemangioblasts at day 6.25 dpc in an extraembryonic location at bottom of YS in mice is connected with the migration of a population of primordial germ cells (PGCs) from proximal epiblast of embryo proper (embryonic ectoderm) into extraembryonic ectoderm at the bottom of YS. Interestingly, PGCs are the first stem cells specified in proximal epiblast of developing embryo. These precursors of gametes enter at 6.0 dpc to the extraembryonic ectoderm, and the developmental migration of these cells through this region correlates with appearance of first hemangioblasts at the bottom of YS [54–56]. This may indicate that PGCs or some subpopulation of these cells give rise to hemangioblasts. Moreover, later in embryonic development, definitive hematopoiesis is observed when the first HSC becomes identified in the dorsal aorta wall in the so-called hematogenic endothelium. This area is part of a wider anatomical area of the

embryo known as the aorta-gonad-mesonephros (AGM) region [54–56]. In mice, the origin of definitive HSCs in AGM occurs at the time when PGC after entering through primitive streak of embryo proper migrate on 11 dpc through this region to genital ridges where ultimately, between 12.5 and 14.5 dpc, they will settle down and give rise to gametes [53–56]. Based on above information, initiation of both primitive and definitive hematopoiesis is tightly connected with the migration of primordial germ cells (PGCs), and we cannot exclude the possibility that a subpopulation of cells related to migrating PGCs may be responsible for establishing hematopoiesis at both places.

This challenging concept postulates that potential developmental origin of HSCs could be from PGC-related cells. A potential intermediate developmental link between PGCs and HSCs could be a population of very small embryonic-like stem cells (VSELs) identified in embryonic and adult hematopoietic organs (Fig. 3.3) [57–60]. This is somehow supported by expression of functional sex hormone receptors on PGCs, VSELs, and HSCs. Attesting this statement like VSELs, normal human, and murine HSCs expresses several functional pituitary and gonadal sex hormone receptors and responds by proliferation to follicle-stimulating hormone

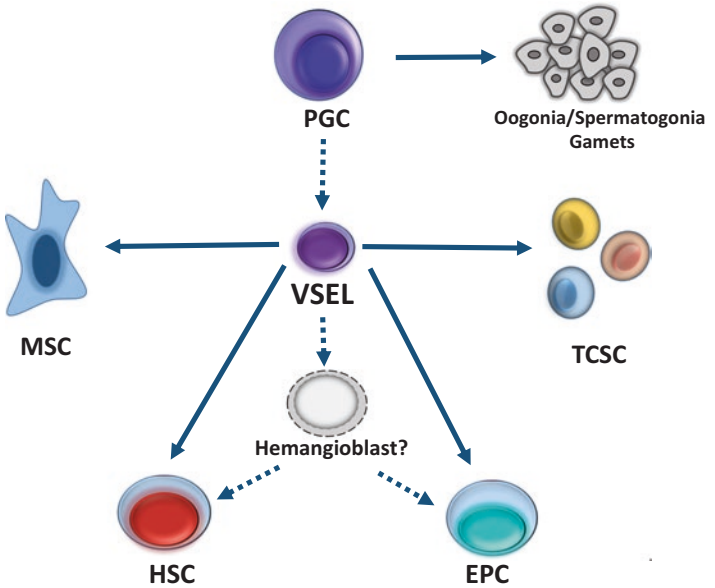

Fig. 3.3 Proposed developmental interrelationship between primordial germ cells (PGCs), very small embryonic-like stem cells (VSELs), hemangioblasts, hematopoietic stem cells (HSCs), and endothelial progenitor cells (EPCs). We propose that migratory PGCs, aside from their major role in establishing gametogenesis, may be a source of certain developmentally primitive stem cells (e.g., VSEL) that in bone marrow give rise to HSCs and EPCs and are a source of mesenchymal stem cells (MSCs) and in other tissues a source of tissue-committed stem cells (TCSCs). Specification of VSEL into HSC and EPC may involve putative hemangioblast as an intermediate precursor cell. Dotted line pathways are still under investigation

(FSH), luteinizing hormone (LH), prolactin, estrogens, and androgens [61, 62]. Since VSELs are also present in adult BM, it is possible that this rare population of PGC descendants (?) residing in adult BM could be developmental precursor of potential long-term repopulating HSCs (LT-HSCs) and perhaps a reserve source of these LT-HSCs. This may change our view on stem cell compartment in adult BM where on the top of stem cell hierarchy are VSELs that can give rise to both HSCs and other stem/progenitor cells including mesenchymal stromal cells (MSCs) and endothelial progenitor cells (EPCs) (Fig. 3.3).

Nevertheless, a current view postulates that hemangioblasts are specified in extraembryonic ectoderm from unknown precursors and definitive hematopoiesis in AGM region occurs due to the blood stream transfer of pre-HSCs from YS blood islands to AGM. This transfer requires initiation of blood circulation in embryo and emergence of a beating heart that pumps blood. In support of this, murine Ncx1-KO embryos, which do not develop a beating heart by 8.25 dpc but still continue to develop until 10 dpc, do not initiate definitive hematopoiesis in AGM region. As reported, while there was no observed significant difference in the number of hematopoietic precursors in the YS from wild-type and Ncx1-KO embryos, the embryo proper, including the AGM region, was nearly devoid of HSC in mutant embryos [63, 64]. However this concept, which pre-HSC from the YS may specify into definitive HSC and colonize aortic endothelium, does not preclude the possibility that the aortic endothelium could be colonized by other PGC-derived cells (e.g., VSELs) migrating through the AGM region by 11 dpc in mice [53, 56]. Moreover, the initiation of definitive hematopoiesis in the aortic "hemogenic" endothelium as suggested is also dependent on an effect induced by shear forces generated due to active blood flow [63, 64]. Again, we cannot preclude that these shear forces act on migrating PGC-derived VSELs that infiltrate the AGM region. What is not clear in support of hemogenic endothelium as source of HSCs is that since shear forces occur in all large vessels in the embryo, why AGM region still seems to be a preferable anatomic location to initiate definitive hematopoiesis. To support this statement and taking into consideration that at this time PGCs migrate through AGM region, it is pertinent to state that derivatives of these cells could be the origin of definitive HSCs [59, 61, 65].

The proposed developmental link between PGCs, VSELs, and HSCs is supported by the presence of germ line precursor cells in adult BM. It has been demonstrated, for example, that SSEA-1$^+$Oct-4$^+$Stella$^+$Mvh$^+$ cells, isolated from murine BM, may differentiate in the presence of BMP-4 (bone morphogenetic factor 4) into gamete precursors [66]. On the other hand, BM-derived Oct-4$^+$Mvh$^+$Dazl$^+$Stella$^+$ putative germ cells supported and restored oogenesis in mice sterilized by chemotherapy [67, 68]. Similarly, Oct-4$^+$Mvh$^+$ Stella$^+$ cells isolated from the BM of male Stra8-GFP mice expressed several molecular markers of spermatogonial stem cells and spermatogonia indicating transdifferentiation of BM cells to male germ cells [69]. Interestingly, in direct mutagenesis studies, it has also been demonstrated that BM cells exposed to methylchloranthrene may give rise to germ line tumors [70]. It is most likely that all these cells employed in the abovementioned studies are related to VSELs. Moreover, in support of the intriguing concept that HSCs may be derived

from PGCs, it has been demonstrated that PGCs isolated from murine embryos, murine testes [71], and certain teratocarcinoma cell lines [72–74] can be specified into HSCs. Similarly, there are reports on the clonal origin of both teratomas and germ line leukemia tumors [75, 76]. Interestingly, it has been demonstrated that VSELs isolated from adult BM has potential to differentiate in vitro into HSCs and HPCs. Also, VSEL isolated from ovaries has been demonstrated to differentiate into precursors of oocytes [77]. Nevertheless, despite accumulating evidence, more studies are warranted to strengthen and better understanding of the developmental link between PGCs, VSELs, and HSC (Fig. 3.3).

3.3 Phenotype of HSC: From YS to Adult BM

As mentioned above, first primitive pre-HSCs are identified in murine YS and AGM at 8.5–10.5 dpc. These cells express CD41 and CD34 antigens but lack CD45 and Sca-1 expression (Table 3.1). Similar populations of pre-HSCs can be also detected in the murine placenta. These pre-HSCs finally give rise to definitive HSCs (Table 3.1) that express CD34, CD45, and Sca-1 antigens at 11.0–12.5 dpc. These

Table 3.1 Phenotype of murine and human HSC at different stages of embryonic and postnatal development. These markers are usually used in combination in order to isolate cells highly enriched for HSC

HSC	Mouse	Human
Hemangioblast *(yolk sac)*	Flk-1 $(Kdr)^+$	?
Pre-HSC *(AGM)*	$CD34^+$ $CD41^+$ $Sca\text{-}1^-$ $CD45^-$	?
HSC *(AGM, placenta)*	$CD34^+$ $CD45^+$ $CD41^{+/-}$ $Sca\text{-}1^{+/-}$	$CD34^+$
HSC *(fetal liver)*	$Sca\text{-}1^+$ $CD34^+$ $CD45^+$ $Mac1^+$ $CXCR4^+$	$CD34^+$ $CD133^+$ $CXCR4^+$ Lin^-
HSC **Long-term engrafting HSC (LT-HSC)** **Short-term engrafting HSC (ST-HSC)** *(bone marrow)*	$CD34^{lo/-}$ $Sca\text{-}1^+$ $Thy1.1^{+/lo}$ $CD38^+$ $c\text{-}kit^+$ lin^- $c\text{-}kit^+$ $Thy1.1(CD90)^{lo}$ Lin^- Sca^{hi} (***KTLS***) $CD150^+$ $CD48^-$ $CD244^-$ (***SLAM***) $Rh123^{low}$, $Hoe3342^{low}$, $pyronin Y^{low}$ 5-FU resistant, $ALDH^{hi}$ Fr25 (small cells) Lin^- Side population (SP) – Cells **LT-HSC**: $CD34^-$ $CD38^-$ $Sca\text{-}1^+$ $Thy1.1^{+/lo}$ $c\text{-}kit^+$ lin^- $CD135^-$ Slamf1/$CD150^+$ **ST-HSC**: $CD34^+$ $CD38^+$ $Sca\text{-}1^+$ $Thy1.1^{+/lo}$ $c\text{-}kit^+$ lin^- $CD135^-$ Slamf1/$CD150^+$ Mac-1 $(CD11b)^{lo}$	$CD34^+$ $CD59^+$ Thy1/$CD90^+$ $CD38^{lo/-}$ c-kit/$CD117^+$ lin^- $CD34^+CD38^-Lin^-$ $CD34^+$ $CD133^+$ $CXCR4^+$ Lin^- $Rh123^{low}$, $Hoe3342^{low}$, $pyronin Y^{low}$ $ALDH^{high}$ $(ALDH^{hi}CD133^+Lin^-)$ $CD150^+$ $CD48^-$ $CD244^-$ (***SLAM***) Side population (SP) – Cells

definitive HSCs begin to colonize FL at 11.5 dpc, and FL becomes a major hematopoietic organ during the second trimester of gestation, and hematopoietic activity remains detectable in murine FL several weeks postnatal [53–56]. As mentioned, FL is also major hematopoietic organ in humans during this period of gestation, and AGM-derived definitive HSCs migrate to FL through the umbilical artery, placenta, and umbilical vein. This migratory route of HSCs may explain why the placenta is also enriched for HSCs [12, 16–20, 53]. HSCs present in second trimester of gestation in murine FL express CD34, CD45, Sca-1, Mac-1, and CD150 antigen (Table 3.1) [78].

It is apparent that HSCs in their developmental migration in embryo must follow gradients of chemoattractants. So far, very few chemoattractants have been identified for murine and human HSCs that include (i) α-chemokine stromal-derived factor-1 (SDF-1) [79, 80]; (ii) two bioactive phosphosphingolipids, namely, sphingosine-1 phosphate (PLc) [81, 82] and ceramide-1 phosphate (C1P) [83, 84]; and (iii) extracellular adenosine triphosphate (ATP) [85, 86].

Interestingly, the migration of embryonic HSCs from AGM to FL does not depend on SDF-1. Evidence to support this notion is that mice with SDF-1 or its corresponding receptor CXCR4 knockout possesses a normal number of HSCs in FL [87, 88]. This suggests the existence of an alternative homing factor/chemoattractant during early embryogenesis that may compensate for SDF-1 deficiency and allows accumulation of embryonic HSCs in FL. This could be S1P, C1P, ATP, or another not yet identified chemoattractant.

In the third trimester of gestation in mice (18.0 dpc.), HSCs begin to seed fetal BM and the spleen [53–56]. Here the colonization of fetal BM by HSCs depends on the expression of SDF-1 that becomes expressed in the developing BM microenvironment [87, 88]. In response to an SDF-1 gradient, HSCs that express the corresponding receptor, CXCR4, move from the FL to BM and establish adult-type hematopoiesis. The HSCs in neonatal murine BM are $CXCR4^+$, $Sca\text{-}1^+$, and $CD45^+$, but as mentioned earlier, murine HSC gradually loses the expression of CD34 antigen (Table 3.1). Human HSCs are $CD34^+$ $CD38^-$, and some of them express CD133 [45].

The colonization of BM by HSCs does not terminate the developmental and postnatal journey of HSCs and HPCs as these cells continuously circulate in PB and lymph. As discussed in introduction, symmetric division results in two niche bound HSCs, and asymmetric division results in one daughter HSC, and one daughter cell enabled with differentiation program has to leave the BM niche and released into the circulation to find a new niche [89]. This mechanism may be responsible for maintaining homeostasis between HSC niches in different areas of BM distributed across various bones. It has been postulated that circulating HSCs and HPCs are also patrolling peripheral tissues for the presence of potential pathogens or damages [90]. This phenomenon allows them to react rapidly to fight infections by providing clones of granulocytes, monocytes, or dendritic cells. Moreover, since not only HSCs and HPCs but also MSCs, EPCs, and VSELs circulate at low level in PB and lymph, all these circulating cells may potentially mend small organ damages.

3.4 Novel View on Possible Hierarchy of Stem Cell Compartment in Adult BM

Stem cells residing in the BM demonstrate developmental hierarchy. It has been proposed that at the top resides the most primitive PGC-derived pluripotent stem cell (VSEL?) that can give rise to LT-HSCs, short-term repopulating HSCs, MSCs, and EPCs (Fig. 3.3).

In fact, the presence of VSELs which can differentiate into germ line cells has been confirmed in adult tissues including BM, by at least 20 independent laboratories. VSELs are small cells, corresponding in size to the cells in the inner cell mass of the blastocyst, and depending on the measurement conditions (in suspension or after adhesion to slides), they measure ~3–5 μm in mice and ~5–7 μm in humans [58–60]. Thus, they are slightly smaller than red blood cells and require a special gating strategy during FACS sorting. Transmission electron microscopy analysis revealed that VSELs have large nuclei containing euchromatin and a thin rim of the cytoplasm enriched in spherical mitochondria, which are characteristic of early-development cells, for example, PGCs. They also express several genes characteristic for pluripotent/multipotent stem cells such as stage-specific embryonic antigen (SSEA), Oct-4, Nanog and Rex-1, and highly expressed Rif-1 telomerase protein. Studies performed on highly purified double-sorted VSELs isolated from murine BM revealed that these cells express high mRNA and/or protein levels of (e.g., *Stella, Fragilis, Blimp1, Nanos3, Prdm14,* and *Dnd1*) and late migratory PGCs (e.g., *Dppa2, Dppa4,* and *Mvh*) [91].

Murine VSELs are isolated as a population by multiparameter sorting as Sca-1$^+$ Lin$^-$ CD45$^-$ cells and human VSELs as CD34$^+$ CD133$^+$ Lin$^-$ CD45$^-$ cells [57, 59]. As mentioned earlier, these cells have potential to differentiate into functional HSCs and HPCs. Accordingly, VSELs that are CD45$^-$ if expanded toward hematopoietic lineage give rise to CD45$^+$ hematopoietic cells that transplanted into experimental animals and protect them from lethal irradiation by differentiating in vivo into all the major hematopoietic lineages (e.g., as demonstrated Gr-1$^+$, B220$^+$, and CD3$^+$ cells) [92]. In parallel, the hematopoietic specification of murine VSELs is accompanied by the upregulation of mRNA for several genes regulating hematopoiesis (e.g., PU-1, c-myb, LMO2, Ikaros). Additionally, functional VSELs have also been purified from human UCB and BM [59, 60]. Despite this exciting data, the proposed hierarchy of stem cell compartment in adult murine and human BM requires further studies.

Figure 3.2 shows widely accepted hierarchy of HSCs and HPCs in adult BM. HSC gives rise both to stem cell for hematopoiesis (CMP) and lymphopoiesis (CLP). Hematopoietic stem cells differentiate into HPCs for common and separate hematopoietic lineages. At the top of CMP hierarchy is the so-called colony-forming unit of mix lineages (CFU-Mix) – a HPC that is able to grow in vitro colonies containing cells from myeloid, erythroid, and megakaryocytic lineage. Therefore, CFU-Mix is often called colony-forming unit–granulocyte-erythrocyte-monocyte-megakaryocyte (CFU-GEMM). Downstream in this hierarchy, we can distinguish more differentiated HPCs specified to erythroid lineage forming big or small

colonies of red blood cells called burst-forming unit of erythrocytes (BFU-E) and colony-forming unit of erythrocytes (CFU-E), respectively. Common progenitor for myeloid cells, colony-forming unit of granulocyte and monocytes (CFU-GM), gives rise to more restricted progenitors such as colony-forming unit of granulocytes (CFU-G) and colony-forming unit of monocytes (CFU-M). In myeloid lineage, we distinguish also progenitor for eosinophils – colony-forming unit of eosinophils (CFU-Eo) and basophils – colony-forming unit of basophils (CFU-Baso). Megakaryocytes that give rise to blood platelets develop from more primitive burst-forming unit of megakaryocytes (BFU-Meg) and further into more differentiated colony-forming unit of megakaryocytes (CFU-Meg). In contrast, CLP for lymphoid lineage gives rise to T cells, B cells, or natural killer (NK) cells [9, 10].

The specification and differentiation of HPCs of both myeloid lymphoid progenitor cells are regulated by several hematopoietic growth factors and cytokines. Figure 3.4 shows the most important players involved in this process. These factors regulate different levels of hematopoiesis and lymphopoiesis in vivo and could also be employed for in vitro assays to grow colonies composed of cells from different lineages [93, 94]. Figure 3.5 shows some examples of such hematopoietic colonies growing in clonogenic assays in vitro. These assays have both experimental and diagnostic meanings and implications.

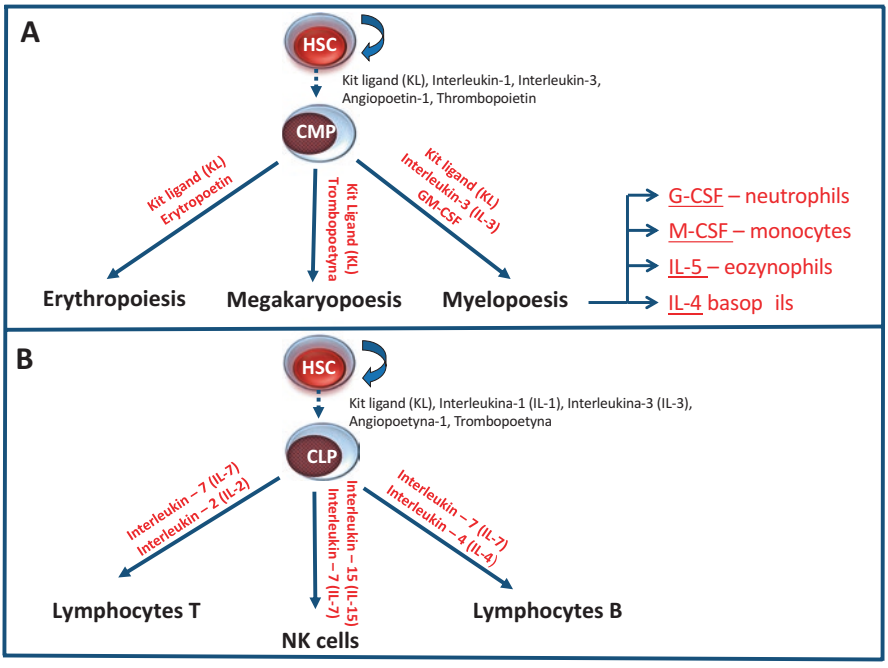

Fig. 3.4 Specification of HSC into CMP and hematopoietic lineages (Panel **a**) and specification of HSC into CLP and lymphopoiesis (Panel **b**). On the scheme, there are indicated growth factors and cytokines that regulate the development of various hematopoietic and lymphopoietic lineages

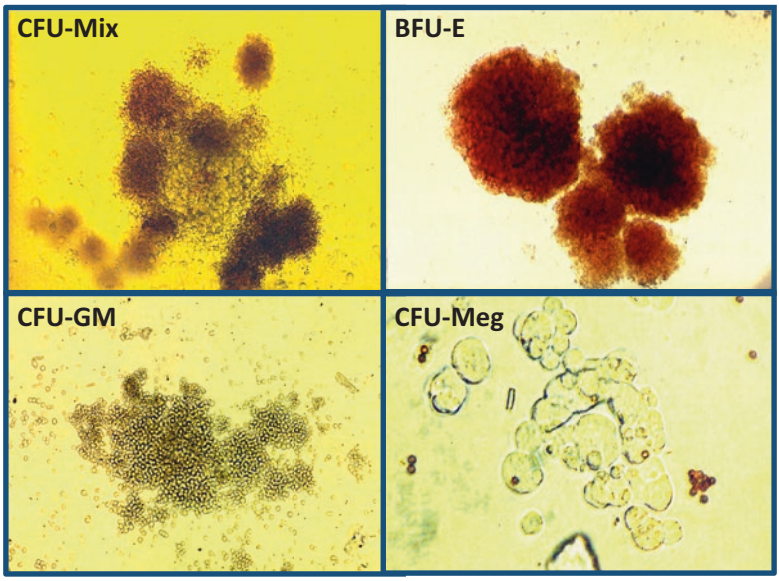

Fig. 3.5 Examples of clonogenic growth of CFU-Mix, BFU-E, CFU-GM, and CFU-Meg. There are shown representative HPC colonies growing in semisolid cloning media

3.5 The Hematopoietic Niche and Retention of HSPCs in BM Microenvironment

HSCs reside in bone marrow niches, where they are anchored due to the interaction between the ligands α-chemokine stromal-derived factor 1 (SDF-1) and vascular cell adhesion molecule-1 (VCAM-1), expressed in cells forming stem cell niches interacting, respectively, with corresponding receptors CXCR4 and α4β1 integrin, very late antigen-4 (VLA-4) expressed on HSCs [89]. Of note, both CXCR4 and VLA-4 receptors are membrane lipid raft-associated receptors, and their incorporation into cell surface membrane lipid rafts is essential to their optimal biological function. Nevertheless, the BM stem cell niche remains incompletely defined and has been described by competing models. Recent research indicates that this niche is perivascular [SDF-1^{+} and kit ligand (KL^{+})], created partially by mesenchymal stromal cells and endothelial cells and, often but not always, located near trabecular bone [95]. While HSCs are located around perivascular cells, early lymphoid progenitors are associated with the osteoblastic niche. The existence of distinct niches, including quiescent $nestin^{bright}$ $NG2^{+}$ arteriolar and proliferative $nestin^{dim}Lepr^{+}$ sinusoidal niches as a source of factors required for the maintenance of distinct subpopulations of HSCs, has been proposed [96]. On the other hand, evidence also indicates the involvement of an osteoblastic niche that is located along trabecular bone in the BM microenvironment, where osteoblasts provide cellular support for HSCs [89]. Within the BM niche, several factors affect the fate of HSCs, including

interactions with adhesion molecules and ligands. In particular, the SDF-1–CXCR4 and VCAM-1–VLA4 axes play important roles in retention of HSCs in BM niches [97]. HSCs are also under the influence of several hematopoietic growth factors, cytokines, and chemokines secreted by niche cells or delivered to the niche via the circulating blood and lymph [98]. In addition, an important role is also played by sympathetic nervous system via innervating HSC niches with neural β-adrenergic fibers and local oxygen tension. Under steady-state conditions, HSPCs are actively retained in BM niches, and these retention mechanisms counteract the continuous sphingosine-1-phosphate (S1P) gradient originating in blood plasma, as recently demonstrated by our research work [99]. This gradient is playing an important role in egress or mobilization of HSCs from BM into PB.

3.6 HSC Egress from BM into PB (Mobilization)

A crucial role in HSC mobilization process is played by the induction of a proteolytic microenvironment in BM due to the release of proteolytic enzymes from granulocytes and monocytes. This, for example, occurs, after administration of pro-mobilizing agent such as G-CSF [39, 40]. It has been demonstrated that several proteolytic enzymes released by cells in BM digest proteins involved in retention of HSCs in the bone marrow microenvironment (e.g., SDF-1-CXCR4 and VCAM-1-VLA-4 axes) [97, 100]. Interestingly, the crucial proteases involved in this process have not yet been identified, since mice with knockout of multiple proteolytic enzymes, such as MMP-9, MMP-2, cathepsin G, and elastase, mobilize HSCs in a similar manner as control wild-type mice [101, 102]. This finding may indicate that their deficiency is compensated by other proteolytic enzymes.

Another previously unrecognized possibility is the involvement of other types of non-proteolytic enzymes. Our recent results indicate the involvement of the lipolytic enzyme hematopoietic cell-specific phospholipase Cβ2 (PLC-β2) [82]. PLC-β2 is an enzyme that targets VCAM-1 expressed in stem cell niches and the glycolipid glycosylphosphatidylinositol anchor (GPI-A), which is important lipid raft component for optimal function of CXCR4 and VLA-4 receptors to support their role in retention of HSCs in BM niches [82].

3.7 Chemoattractants for HSC and Stem Cell Homing

In contrast to differentiated hematopoietic cells and lymphocytes, the complete list of chemoattractants for HSC is quite short. As previously mentioned, HSC responds robustly to SDF-1 gradients [79, 80] as well as to gradients of two phosphosphingolipids, S1P [81, 99] and ceramide-1 [83, 84]. Importantly, extracellular adenosine triphosphate (ATP) [85, 86] has emerged as an important chemoattractant for

HSPCs in addition to SDF-1, S1P, and C1P. These factors, however, would be crucial for homing and engraftment of HSCs after hematopoietic transplant.

Homing is a process where transplanted HSCs circulating in PB migrate to their niches in BM and is followed by engraftment in the bone marrow microenvironment and is a process opposite to mobilization. HSCs infused into PB after myeloablative conditioning by radio- or chemotherapy respond to the chemotactic gradients originating from BM, attach to the BM endothelium, transmigrate through the basal membrane in a metalloproteinase (MMP)-dependent manner, and finally home to the niche where they subsequently survive, expand, and proliferate. Homing of HSCs to BM niches is the first step in the engraftment process before HSCs self-renew and differentiate into hematopoietic lineages. *Short-term engraftment* can be assessed in mice by evaluating the hematopoietic recovery from lethal irradiation of PB parameters after transplant or the formation of spleen colonies in irradiated recipients (colony-forming units-spleen [CFU-S] assay). *Long-term engraftment*, which is most important for long-term survival after transplantation, is achieved by the most primitive HSCs, which are endowed with long-term repopulating capacity to maintain hematopoiesis for months after transplantation [103].

3.8 Markers and Strategies to Isolate HSC and HPC

Neither a single specific marker nor a biological property exists which, if employed alone, allows for isolation of pure murine or human HSCs. Hence, combinations of several markers must be used to enrich murine BM- or human BM-, PB-, or UCB-derived mononuclear cells for HSCs isolation identification and characterization.

3.8.1 Markers of HSCs

To purify HSCs, several markers/parameters have to be employed in combination. Figure 3.6 shows the most important cell surface expressed antigens (CD34, CD133, CXCR4, and c-kit) and metabolic fluorochromes (Hoechst 33342, pyronin Y, and rhodamine 123) that are useful to identify and potentially isolate HSCs. However, there are some differences between markers for murine and human HSCs [104, 105].

The most relevant cell surface markers associated for murine HSCs include Sca-1, c-kit, and Thy1.1 (CD90) and for human HSCs: CD34, CD133, CXCR4, and CD150 [45]. Both murine and human HSCs lack lineage differentiation markers (Lin^-). They also show low accumulation/staining with metabolic fluorochromes such as Hoe33342 (DNA marker), Rh123 (efflux pump substrate), and pyronin Y (mRNA marker). HSCs are also in vivo resistance to fluorouracil (5-FU), express high activity of aldehyde dehydrogenase enzyme (ALDH) that conveys resistance to cyclophosphamide, and are characterized by small size (e.g., on FACS cytograms, HSCs are included in the so-called lymph-gate and during elutriation are recovered

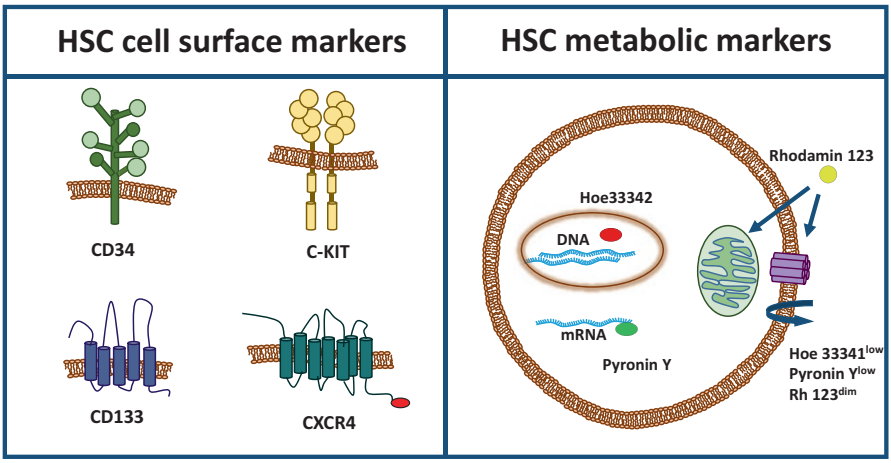

Fig. 3.6 Cell surface antigens (Panel **a**) and metabolic fluorochromes (Panel **b**) that are employed for isolation of HSC. These markers are usually employed in combination to purify HSC

with a fraction FR25 containing small lymphocytes). The majority of HSCs are quiescent. Interestingly, a combination of signaling lymphocyte activation molecule (SLAM) markers ($CD150^{+}CD48^{-}CD244^{-}$) has been recently proposed to define and sort LT-HSCs [106–108]. The SLAM is a group of more than ten molecules whose genes are located mostly tandemly in a single locus on chromosome 1 (mouse). SLAM molecules belong to a subset of the immunoglobulin gene superfamily, and originally thought to be involved in T-cell stimulation include CD150, CD48, CD244, and CD150. The marker CD150 is the founding member of SLAM family and is known as slamF1, that is, SLAM family member 1 [106, 108].

Unfortunately, cell surface markers employed for isolation of HSC such as CD34 antigen are also expressed on more differentiated hematopoietic cells and EPC [109]. Similarly, CXCR4, a receptor for α-chemokine SDF-1, which as mentioned is a major chemoattractant of HSCs, is also expressed by more differentiated hematopoietic cells, for example, by megakaryocytes, monocytes, platelets, and lymphocytes [110]. This requires multiparameter FACS sorting targeting different cell surface and metabolic markers.

3.8.2 Strategies to Purify and Isolate HSCs

HSC is purified and enriched from BM, PB, or UCB mononuclear cells (MNCs). The MNCs in first step may be depleted of erythrocytes and granulocytes using Ficoll gradient centrifugation or hypotonic erythrocyte lysis. MNCs may be also isolated from PB, BM cell suspension, or UCB by leukapheresis [111].

Different methods are employed for HSC isolation, for example, (i) high speed FACS sorters, (ii) elutriators, (iii) paramagnetic beads, or (iv) avidin columns. Additionally, recent development of microfluidic strategy to develop cell-based assays looks very attractive and have provided promising means to monitor and study cellular phenotype and behavior in real time and in a well-controlled microenvironment simulating the in vivo settings [112]. In FACS isolation MNCs are (i) labeled with fluorochrome-conjugated antibodies against above-listed HSC markers [23, 112] or (ii) are exposed to metabolic fluorochromes (Rh123, Hoe33342, pyronin Y) [105] or the fluorescent substrate of ALDH that is Aldefluor. After labeling, HSCs are subsequently sorted from the lymphocyte gate as (i) cells showing the HSC-specific phenotype (e.g., $CD34^+CD38^-Lin^-$ or $CD34^+CD133^+Lin$); (ii) a population that is characterized by low accumulation of Hoe33342, Rh123, and/or pyronin Y ($Rh123^{dim}Hoe33342^{dim}$ pyronin Y^{low} cells); or (iii) the so-called side population (SP) of cells showing low accumulation of Hoe33342. A convenient tool to pre-enrich for HSC is elutriation where HSCs are enriched in the so-called FR25 fraction containing small cells. These cells could be subsequently employed as sorting material to isolate, for example, $Sca\text{-}1^+Lin^-$ cells or as reported recently the $CD133^+Lin^-ALDH^{hi}$ cells [113]. Moreover, cell surface markers and metabolic fluorochromes may also be combined together to sort, for example, $CD34^+c\text{-}kit^+Rh123^{dull}$ cells [114].

For isolation and enrichment of HSCs, magnetic cell separation or biotin columns are used for both experimental and clinical applications. In this approach, HSCs are labeled with antibodies conjugated with paramagnetic beads or biotin. Cells labeled with paramagnetic beads are isolated through columns using magnetic field. In contrast, cells labeled with antibodies conjugated with biotin (biotinylated antibodies) are isolated on streptavidin columns. Paramagnetic beads of biotinylated antibodies may be employed for both positive selection of HSCs (e.g., $CD34^+$ or $CD133^+$ paramagnetic beads or biotinylated antibodies) or to deplete MNCs of more differentiated lineage-positive cells (e.g., Lin^+ beads or biotinylated antibodies) [115].

As it is shown in Table 3.1**,** HSCs are most commonly isolated from the adult mouse BM by FACS as $c\text{-}kit^+$ $Thy1.1^{lo}$ Lin^- Sca^{hi} (KTLS) cells or as $Sca\text{-}1^+c\text{-}kit^+Lin^-$ (SKL) cells [116]. The phenotype of murine long-term engrafting HSCs is described as Thy 1.1^{lo} Lin^- Sca^{hi} $Mac1^-$ $CD4^-$ or $Thy1.1^{lo}$ $Flk\text{-}2^-$ cells [112, 117, 118]. The short-term engrafting HSCs were identified among a KTLS population as $Mac\text{-}1^{lo}$ $CD4^-$ or $Mac\text{-}1^{lo}$ $CD4^+$ $Thy\text{-}1.1^+$ $Flk\text{-}2^-$ cells [103]. Murine HSCs can be enriched in vivo before isolation by depletion of cycling cells in vivo by exposing to 5-fluorouracil (5-FU) [119]. Murine HSCs are also isolated according to signaling lymphocyte activation molecule family (SLAM) phenotype as $CD150^+$ $CD48^-$ $CD244^-$ cells. In contrast human HSCs are purified as a population of $CD34^+CD38^-Lin^-$, $CD34^+$ $CD133^+$ $CXCR4^+$ Lin^-, or $CD150^+$ $CD48^-$ $CD244^-$ (*SLAM*) cells [106–108].

3.8.3 Markers of HPCs

In addition to identification of HSCs, SLAM family of markers has been proposed to identify HPCs. Accordingly, the multipotent progenitor cells (MPP) are identified as $CD150^{-}CD48^{-}CD244^{+}$, the lineage-restricted progenitor cells (LRP) as $CD150^{-}CD48^{+}CD244^{+}$, the common myeloid progenitor (CMP) as $lin^{-}Sca\text{-}1^{-}c\text{-}kit^{+}CD34^{+}CD16/32^{mid}$, the granulocyte-macrophage progenitor (GMP) as $lin^{-}Sca\text{-}1^{-}c\text{-}kit^{+}CD34^{+}CD16/32^{hi}$, and finally the megakaryocyte-erythroid progenitor (MEP) as $lin^{-}sca\text{-}1^{-}c\text{-}kit^{+}CD34^{-}CD16/32^{low}$ [1, 120].

Murine multipotent progenitor (MMP) cells could also be isolated by FACS as early MPP: $CD34^{+}$, SCA-1^{+}, Thy1.1^{-}, c-kit^{+}, lin^{-}, $CD135^{+}$, Slamf1/$CD150^{-}$, Mac-1 $(CD11b)^{lo}$, and $CD4^{lo}$ and as late MPP according to phenotype: $CD34^{+}$, SCA-1^{+}, Thy1.1^{-}, c-kit^{+}, lin^{-}, $CD135^{high}$, Slamf1/$CD150^{-}$, Mac-1 $(CD11b)^{lo}$, and $CD4^{lo}$ [1, 78].

3.9 Expansion of HSC

A common problem in hematopoietic transplants in clinical settings is lack of sufficient number of HSCs. This occurs when the harvest of HSCs from BM or PB is poor or UCB is used as a source of HSCs for transplant, in particular for patients with bigger body mass that require higher number of graft cells. To eliminate this problem, several ex vivo expansion protocols have been proposed for these cells [120–123]. Nevertheless, almost all available HSC expansion protocols result in an increase in the number of HPCs and more differentiated hematopoietic progenitor cells at the expense and loss of HSCs. To explain the failure of ex vivo expansion procedures of HSCs, it has been postulated that we still have not identified all factors operating in vivo in HSC niches that are crucial for the self-renewal and maintenance of these cells. Another explanation is that HSCs employed for expansion claimed to be the most primitive in hematopoietic lineage and have in fact already entered a pathway of differentiation at the expense of their true self-renewing potential [165].

Recent studies point toward significant progress that has been made in this direction both in experimental and clinical settings by employing prostaglandin E2 or small molecular compounds such as aryl hydrocarbon receptor antagonist – SR-1 [124, 125]. Nevertheless, it is clear that in order to proceed with most optimal expansion, we should begin this process with the most primitive quiescent stem cells likely able to give rise to all LT-HSC. Taking into consideration that VSELs are on the top of hierarchy of stem cell compartment in adult BM, expansion protocols should be initiated using these cells (Fig. 3.7) [65]. In fact the promising strategy to expand HSCs in the presence of some small-molecule inhibitors UM171 or histone deacetylase-3 (HDAC) inhibitor nicotinamide [126–129] most likely initiates expansion of HSCs from population of quiescent VSELs.

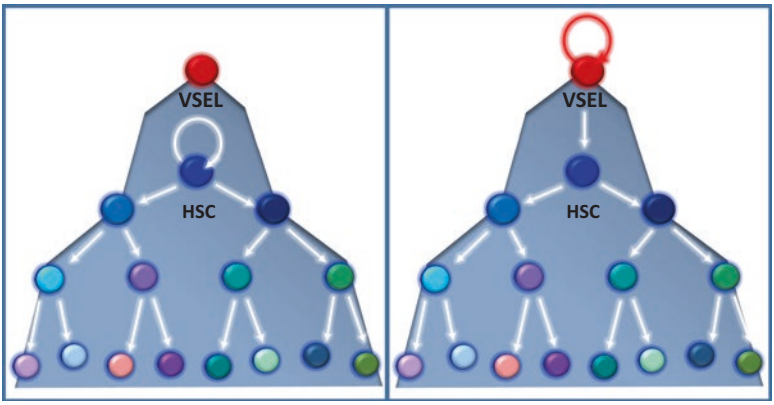

Fig. 3.7 A possible reason for the current poor results for clinical expansion of HSCs. It is most likely that the most of the current clinical expansion procedures employ HSCs that are already "rolling downhill" in following a differentiation pathway (left panel). The expansion strategy should be better initiated at the level of VSELs, which are highly quiescent and positioned at the top of the stem cell compartment hierarchy (right panel)

3.10 Experimental Approaches to Identify and Study HSC

Depending on their developmental stage and hematopoietic organ origin, HSCs are studied using different in vitro and in vivo assays that are listed in Table 3.2. The most primitive pre-HSCs in YS are evaluated by their ability to grow blast colonies (BL-CFC) [130]. YS-derived cells studied in this particular assay are Flk-1 $(Kdr)^+$ hemangioblasts that may give rise in vitro under appropriate culture conditions to both hematopoietic and endothelial cells [19, 20, 130]. Molecular analysis of cells isolated from these colonies has revealed that they express genes associated with both hematopoietic and vascular developments. The most important genes are Flk-1, Scl/Tal1, VE-cadherin, GATA1, fetal βH1 globin, and β-major globin [20, 130]. Moreover, cells isolated from solubilized BL-CFC, after plating in methylcellulose, grow into primitive erythroid and macrophage colonies in the presence of appropriate growth factors [131–133].

In contrast to YS-derived murine HSCs, more differentiated HSCs can be assayed for their ability to establish long-term (>4–6 months) hematopoiesis after transplantation into recipient mice. Cells from these mice can be further assayed for their ability to reconstitute secondary recipients [134, 135]. Unfortunately, as reported murine pre-HSCs display defective homing in adult animals [136–138]. There are several possible explanations for this defect including (i) lack of expression of MHC-I antigens which makes these cells susceptible to recipient NK cell attack, (ii) poor responsiveness of pre-HSCs to chemoattractants that are required for colonization of adult BM (e.g., defective SDF-1-CXCR4 signaling), (iii) lack of a proper repertoire of adhesion molecules, or (iv) lack of production/secretion of matrix metalloproteinases crucial to cross blood-BM barrier on the way to hematopoietic microenvironment [139–141].

Table 3.2 Assays to evaluate murine and human pre-HSC and HSC

Source of HSC	Mouse	Human
Hemangioblast *(yolk sac)*	BL-CFC in vitro assay	Not available
Pre-HSC *(AGM, YS)*	Expansion on OP9 stroma support Expansion on AGM-derived endothelial cells Transplantation "in utero" into embryos transplantation into NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice Transplantation into newborn liver Intra-bone injection	Not available
HSC *(fetal liver, BM, UCB, PB)*	Transplantation into syngeneic mice followed by transplantation into secondary recipients	Clinical results of hematopoietic transplants Engraftment in NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice (*SRC assay*), humanized SCID mice (hu-SCID), or engraftment in large animals (into sheep or goat fetuses)

Therefore, pre-HSCs isolated from embryonic tissues are assayed in vitro for their hematopoietic potential in special conditions that favor development/maturation of classical embryonic stem cells (ESCs). This includes co-culturing with cell lines derived from murine embryonic tissues such as the AGM-derived endothelial cell line AGM-S3 [142]. Since the stromal cell line OP9 (derived from the skull bones of osteopetrotic op/op mice) may also support the expansion/maturation of pre-HSCs, OP9 cells are employed to stimulate proliferation of pre-HSCs [27, 143, 144]. Important to mention that the hematopoietic cells isolated from cultures of pre-HSCs over AGM-S3 or OP9 cell lines acquire the ability to grow hematopoietic colonies in vitro and most importantly engraft in lethally irradiated mice. Furthermore, pre-HSC derived from the YS or AGM regions could also be transplanted in utero into recipient E.18.0 embryos. In order to facilitate this approach, pregnant mice are exposed to sub-myeloablative doses of busulfan [28].

Murine pre-HSCs that have homing defects and do not express high MHC-I antigens and thus are susceptible to NK attack could also be assayed by employing (i) direct intra-bone injection, (ii) transplantation into newborn livers, or (iii) transplantation into Rag2$^{-/-}$ mice that lack lymphocytes or Rag2$^{-/-}$γc$^{-/-}$ mice that lack both lymphocytes and more importantly NK cells [145]. Another popular immunodeficient mouse model employed as recipients of HSCs is NOD/Shi-scid/IL-2Rγ^{null} (NOG) animals [47].

Murine FL-derived HSCs are able to engraft long term and establish hematopoiesis in adult mice. Interestingly, similar evidence does not exist for human HSCs isolated from FL. In the past, a number of studies were performed in attempt to employ these cells for hematopoietic transplants, but results were disappointing. It is not clear at this point whether human FL cells have defective engraftment in adult BM as hematopoietic transplants with HLA-matched human FL cells have not yet been performed in the clinic.

The most valuable and conclusive preclinical assay for studying human HSCs is one that demonstrates that they engraft long term in immunodeficient mice. In this surrogate transplant assay, human hematopoietic cells are tested as xenotransplants to evaluate the number of hematopoietic–repopulating cells that differentiate into multilineage mature cells and self-renew in mouse, and these cells are defined as SCID mouse-repopulating cells (SRCs) [145]. SCID mice lack T and B lymphocytes but possess NK cells; therefore it is more ideal to use NOD/SCID mice with some defects of NK cells. To enhance engraftment of human cells in NOD/SCID, these mice could be pretreated with intraperitoneal injections of TM-β1, a monoclonal antibody against murine IL-2Rβ, to eliminate any remaining NK cell activity [146]. Alternatively, human HSCs could be tested in $Rag2^{-/-}$ or even $Rag2^{-/-}$ $\gamma c^{-/-}$ mice that, as mentioned above, are deficient in both lymphoid and NK cells [145]. Recently, another suitable recipients employed for human HSCs are highly immunodeficient NOD/Shi-scid/IL-2Rγ^{null} (NOG) animals [47]. A useful modification of xenotransplant is transplantation of human HSCs into the so-called humanized mice, immunodeficient animals that carry human tissue implants (e.g., fragments of bones) [48, 49]. This provides a more suitable physiological microenvironment for human cells [50]. Xenotransplants in NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice that give rise to all hematopoietic/lymphoid lineages are currently the best surrogate assay for human HSCs. Nevertheless, because of very low posttransplant human-murine chimerism, the question remains how much real engraftment occurs in this model as opposed to a simple "lodging" and survival of HSC due to the immunodeficient state of the recipient mouse. Human HSCs could also be tested after intrauterine injection into developing fetuses in sheep or goat [46]. Nevertheless, it is an expensive procedure.

In clinical settings, evidence of hematopoietic reconstitution with BM, UCB, or mobilized PB-derived cells indicates involvement of transplanted HSCs. Hematopoietic transplants with a more purified population of HSC ($CD34^+$ or $CD133^+$) are not performed on routine basis in humans owing to the risk of losing engraftment potential. It is speculated that purification of HSCs may deplete transplant from engraftment facilitating cells.

Finally, evidences are accumulating, suggesting human UCB contains more primitive HSCs that do not engraft optimally after intravenous injection ($CD34^-flt^-Lin^-$ cells) [147]. These cells show poor responsiveness to SDF-1; in contrast, these cells engraft much better after introducing cells directly to the mouse bone marrow or intra-bone marrow (iBM) and minimize the factors that interfere with homing of HSCs during intravenous injections [147–150].

3.11 Conclusion

The compartment of HSCs and HPCs has been intensively investigated over the past years. The progress in this area is marked by the development of new experimental tools such as omics strategies. These strategies allow better understanding

of the mechanisms that govern self-renewal and differentiation of HSCs. Furthermore, proteomic approach and progress in phage display libraries lead to the discovery of new markers that are helpful for isolating HSCs. In parallel, there is advancement in better developed imaging in vivo systems, microfluidic devices, and new generations of cell sorters. In addition to mammals, there are other low vertebrates such as zebra fish that are employed as models in studying hematopoiesis [97, 98]. Moreover, progress in techniques of cell labeling, for example, using iron oxide nanoparticles, which allows tracking of HSCs in vivo by magnetic resonance imaging (MRI) and application of in vivo imaging strategies, opens new possibilities for studying HSC homing and migration in living animals. Finally, further studies are needed to assess the biological and physiological significance of VSELs' presence in BM and their potential supply as a pool of long-term repopulating HSCs. In particular, hematopoietic specification of these cells will provide a new source of HSC for transplantation.

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