**Multiple sources of HSCs exist. Here, Verfaillie discusses the long-term engraftment capabilities of each source and the search for *ex vivo* expansion conditions to allow bulk culture for therapeutic HSC transplantion.**

# Hematopoietic stem cells for transplantation

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Human hematopoietic stem cell (HSC) transplantations have been used to reconstitute hematopoiesis after myeloablation for more than three decades. Studies in animal models and clinical transplantations have taught us that subtle differences in proliferative and differentiation ability exist in HSCs collected from umbilical cord blood, bone marrow or blood, which may affect the ability of HSCs to reconstitute hematopoiesis early or late after engraftment. Although *ex vivo* expansion of long-term repopulating cells has not yet been achieved, it is hoped that the continued quest for mechanisms that govern the proliferation and differentiation of HSCs will lead to the development of culture systems that expand not only committed progenitors but also HSCs.

## HSC characteristics

Hematopoietic recovery after myeloablation is dependent on the number as well as quality of HSCs. Until recently most transplantations were done using HSCs collected from bone marrow (BM). HSCs can also be found in blood collected from patients treated with chemotherapy and/or administration of cytokines, termed "mobilized peripheral blood" (PB) progenitors, and in umbilical cord blood (UCB).

Most human HSCs express the CD34 antigen, which is also expressed on committed progenitors[1] and nonhematopoietic progenitors[2,3]. HSC are lineage-negative ($Lin^-$), do not express CD38, but do express c-Kit and Thy-1[1,4–6]. In the mouse, HSCs do not uniformly express CD34, and only HSCs present during development, after transplantation or during growth factor administration are $CD34^+$ [7–9]. Some human HSCs may also be found in the $CD34^-$ fraction[10–12]. Whether $CD34^-$ HSCs are precursors of $CD34^+$ HSCs and, therefore, a better source of cells for transplantation and/or *ex vivo* expansion is not known. Of note, recipients of $CD34^+$ BM grafts in which multilineage hematopoiesis had been re-established for at least 7 years did not have donor-derived $CD34^-Lin^-$ cells in the BM[13]. If "true" HSCs are indeed $CD34^-Lin^-$ cells, progressive graft failure might be expected to be seen on longer term follow-up of this group of patients. Human HSCs also express CD133[14] and the Bcrp1 (also known as ABCG2) transporter, which effluxes certain molecules including Hoechst-33342. This allows selection of HSCs based on the absence of Hoechst staining, which is seen as the "side population" profile by fluorescence-activated cell sort (FACS) analysis[15–17].

Depending on the cell source, the frequency of human HSCs in FACS-selected $CD34^+Lin^-$, or side population, cells is 1:200–1:500[17–19]. This inability to purify human HSCs to a homogeneous population makes the use of functional HSC assays necessary. In the mouse, short-term repopulating cells (STRCs) are measured as cells that prevent the death of lethally irradiated recipients[20], whereas the numbers of long-term repopulating cells (LTRCs) are estimated by their successful transplantation into a secondary recipient[21]. The most definitive measure of murine LTRCs is their ability to compete for engraftment against other HSCs[22]. If competitive repopulation is done using low numbers of putative HSCs, it is possible to measure the repopulating ability of a single stem cell[23,24].

Because *in vivo* repopulation experiments cannot be done in humans, surrogate assays have been developed to enumerate human HSCs. The number, proliferation ability and myeloid, B-lymphoid and T-lymphoid differentiation ability of progenitors can be assessed with long-term culture assays[4,25–27]. Although the number of murine cobblestone area-forming cells correlates with the number of LTRCs[28], there is no definitive proof that *in vitro*–defined human progenitors correlate with LTRCs. In addition, *in vitro* assays cannot evaluate homing ability, an HSC characteristic that can only be measured by transplantation. Xenogeneic transplants have been used to assess the number and quality of primitive human progenitors, as well as their ability to home, albeit to a xenogeneic environment[1,29–32]. As in mice, short- and long-term reconstitution—or primary, secondary and even tertiary engraftment—measure committed and primitive human progenitors, respectively[29,30,33]. However, xenogeneic transplant models do not all measure engraftment of cells at a similar stage of differentiation[12,33]. In addition, most xenogeneic models do not provide information on the competitive nature of the putative HSC, as can be obtained from the "gold-standard" competitive repopulation assay in mice. To avoid the xenogeneic nature of stem cell assays, several investigators have examined HSC characteristics in dog or nonhuman primate transplantation models[34,35]. These models have shown that *in vitro*–defined primitive progenitors differ from engrafting HSCs and that studies done in the mouse only in part predict the results seen in larger animal models

## Ontogenic differences in HSCs

Results from laboratory studies and clinical trials must be interpreted in light of the ontogeny of the stem cell population studied (**Fig. 1**). During fetal development and early post-natal life, HSCs multiply *in vivo*, which results in expansion of the HSC pool. However, subtle changes in the quality of HSCs occur throughout development and life. HSCs from murine fetal liver have greater proliferation potential than HSCs from post-natal BM from young and older donors[36,37]. The frequency of progenitors that can reconstitute only the myeloid or only the lymphoid lineage, but not both, increases with age[38]. This is accentuated in the setting of sequential transplantation or after *in vitro* manipulation[39], which suggests that in the continuum of stem cells, less primitive "uni-lineage" stem cells may become more frequent with age or hematopoietic stress.

Human UCB cells may have a similar competitive advantage over BM cells[32]. On a per cell basis, UCB cells engraft 10- to 50-fold better in xenogeneic hosts than BM progenitors[19,40]. No systematic comparison has been done between BM progenitors from younger and older human donors to assess whether uni-lineage progenitors become more frequent with age. However, uni-lineage stem cells are more frequent in human grafts after *ex vivo* expansion[33], suggesting that a similar loss in

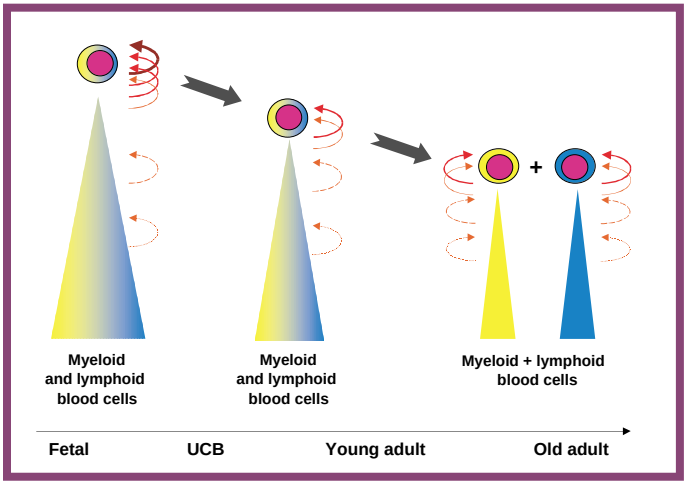


**Figure 1. Subtle changes in the quality of HSCs throughout development and life.** HSCs obtained from fetal liver have greater proliferation and differentiation potential than neonatal HSCs and HSCs from the postnatal BM of young and older donors. In addition, the frequency of HSCs with limited uni-lineage differentiation potential increases with age. Such changes are accentuated with hematopoietic stress. Arrows, the thickness reflects the degree of self-renewal ability. Yellow triangles and HSCs, myeloid differentiation ability; blue triangles and HSCs, lymphoid differentiation ability.

stem cell potential occurs in humans as in mice. Loss of the proliferation and differentiation ability of HSCs with age may reflect the inability of HSCs to undergo true self-renewing cell divisions (see below) or senescence. The effect of stem cell "aging" is discussed in a Review by Van Zant in this issue[41] .

Granulocyte colony-stimulating factor (G-CSF)–mobilized PB grafts have become the preferred cell source for transplantation because of earlier neutrophil and platelet recovery, which is thought to be due to the increased number of STRCs[42]. However, there is evidence that the primitive progenitors in mobilized PB are qualitatively different from those found in BM. Compared to BM $CD34^+Lin^-$ cells, the proliferation and differentiation capacity of PB $CD34^+Lin^-$ cells is inferior[43,44]. More mobilized PB than BM cells are required to reconstitute hematopoiesis in xenogeneic transplant models[12,19], and the long-term repopulation ability of PB progenitors, as assessed by secondary and tertiary repopulation assays, is inferior to that of BM progenitors[12,19]. These studies should be interpreted cautiously, as there is no evidence from clinical allogeneic transplantations that mobilized PB progenitors lead to graft failure. A single study compared hematopoietic senescence in recipients of BM *versus* PB grafts and did not find accelerated telomere loss associated with PB grafting[45]. However, the inferior quality of HSCs in PB may not become obvious early after transplant due to the large cell doses that are administered, and longer term follow-up will be needed to reveal defects in the HSC pool after PB grafting. The addition of early-acting cytokines, such as stem cell factor (SCF), to G-CSF increases the mobilization of long-term–repopulating HSCs in mice[46,47]. In larger mammals—including dogs[48], baboons[49] and humans[50]—the use of PB progenitors obtained after SCF and G-CSF mobilization leads to earlier hematopoietic recovery. Whether SCF increases LTRCs in the grafts of larger mammals has not yet been evaluated.

## *Ex vivo* HSC expansion

One of the holy grails of stem cell research is *ex vivo* expansion of HSCs. The advantages of *ex vivo* HSC expansion are multiple and include the following. (i) Decreased time to hematopoietic recovery after chemotherapy or transplantation; (ii) an increased LTRC component of small grafts, such as UCB grafts, or grafts from patients heavily pretreated with chemotherapy; (iii) the removal of cancer cells from a graft; and (iv) the genetic modification of LTRCs.

To shorten the time to neutrophil and platelet recovery, investigators have focused efforts on expansion of STRCs and precursors. Several clinically suitable culture systems, which use cytokines alone or combined with autologous stroma[51–53], have been developed that support the expansion of colony-forming units (CFUs) and myeloid precursors. Transfusion of such committed cells increases the number of circulating neutrophils in the short-term and shortens, or even eliminates, neutropenia after high-dose chemotherapy[53–55]. For a comprehensive review of these initial clinical trials, see[56]. Other systems under development are aimed at expanding megakaryocytes to decrease the need for platelet transfusions. Studies in nonhuman primates as well as early clinical studies suggest that the infusion of committed cell populations enriched for megakaryocytes generated *in vitro* may hasten platelet recovery[57,58]. Careful evaluation of recipients of *ex vivo*–expanded grafts will be needed to determine whether immune reconstitution—which is already delayed in patients who receive $CD34^+$-enriched grafts[59]—will be further affected by manipulations of $CD34^+$ cells aimed at generating myeloid or megakaryocytic cells.

To generate sufficient cells from small grafts, such as UCB grafts, for reconstitution of adult recipients, expansion of STRCs will not suffice and expansion of LTRCs is needed. Unfortunately, conditions used for expansion of STRCs almost always decrease the number of engrafting LTRCs, thus, potentially jeopardizing the long-term hematopoietic supportive activity of the *ex vivo*–manipulated graft. Few clinical trials to date have used exclusively *ex vivo*–expanded progenitors. Because most trials were done in the autologous setting, without genetic marking and without full myeloablation, long-term repopulation could not be tested[52–55]. One trial in fully myeloablated patients showed a high incidence of long-term graft failure, consistent with the idea that LTRCs may not be preserved in current expansion cultures[60].

To expand HSCs, these cells must undergo repeated symmetrical self-renewing cell divisions, in which the two daughter cells retain the (nearly) exact characteristics of the originating cell (**Fig. 2**). Lack of HSC expansion may be caused by the inability of HSCs to divide under the conditions used. Although most human HSCs are in G0[61], there is little evidence that lack of HSC expansion *in vitro* is due to an inability to recruit HSC from G0. In fact, recruitment from G0 may, in part, be responsible for the defective repopulation ability of expanded grafts[62]. Paramount to engraftment is stem cell homing, followed by *in vivo* proliferation and differentiation. *Ex vivo* culture of progenitors leads to changes in expression of members of the $\beta_1$ integrin family as well as the chemokine receptor CXCR4, which play key roles in homing and engraftment[63–65]. Studies that further characterize the role of these or other receptors underlying cell cycle–mediated alterations in HSC homing, which are critical to engraftment, will be needed to optimize the expansion of engrafting HSCs.

Lack of expansion may also be caused by the cell death of one or both HSC progeny cells. *Ex vivo* culture is associated with increased expression of the Fas ligand CD95[66] and down-regulation of the anti-apoptosis gene *Bcl2*[67] on $CD34^+$ cells, whereas withdrawal activates the caspase pathway in $CD34^+$ cells[68]. Thus, excess apoptosis of HSC daughter cells during or after *ex vivo* expansion may contribute to poor expansion and even loss of HSCs. Apoptosis is a normal mechanism for maintaining tightly control of the HSC pool. Increased numbers of HSCs are present in H-2K Bcl-2 transengic mice, in which Bcl-2 is expressed from the major histocompatibility complex H-2K promoter[67]. H-2K Bcl-2 HSCs have a competitive engraftment advantage over wild-type HSCs, and H-

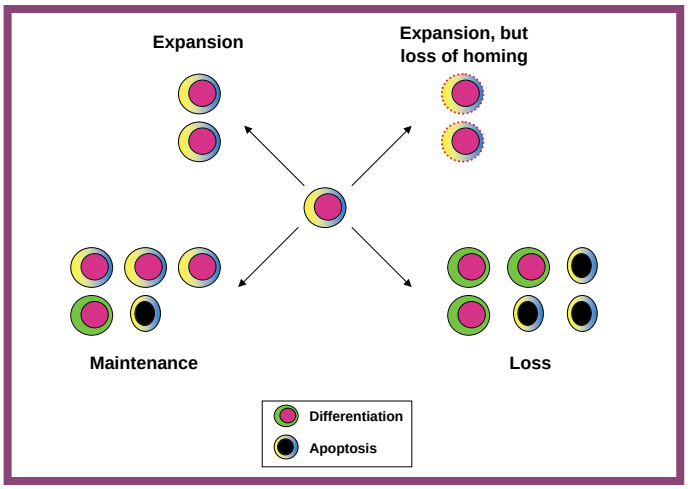


**Figure 2. Stem cell expansion.** To obtain HSC expansion, HSCs must undergo repeated symmetrical self-renewing cell divisions, in which the two daughter cells retain the (nearly) exact characteristics of the originating cell. Even if HSCs undergo self-renewing cell divisions, proliferation itself may silence the ability of HSCs to home, a critical step in engraftment and initiation of marrow reconstitution. The net result of expansion cultures is, however, maintenance of HSCs at best, but more often results in the loss of HSCs. Aside from interfering with homing, the lack of HSC expansion can be caused by the inability of the culture conditions to recruit HSCs from G0 and induce HSC proliferation. Alternatively, one or both HSC daughter cells may undergo programmed cell death of one or both HSC progeny cells *via* mechanisms present *in vivo* to tightly control the HSC pool. Most factors that induce proliferation also induce progressive stem cell commitment, resulting in loss of long-term repopulation ability.

2K Bcl-2 HSCs survive better *in vitro*. The factors or signals that activate anti-apoptotic pathways such as Bcl-2 *in vitro* are not known.

A third possibility is that progeny of HSCs become progressively more committed and lose LTRC capacity. Some have argued that the decision to self-renew without differentiation is stochastic[69]. If this were true, no factor(s) could alter stem cell fate decisions. However, activation of certain genetic programs increases the self-renewal ability of HSCs. For instance, overexpression of the homeobox gene, *Hoxb4*, in murine HSCs leads to expansion of the HSC pool *in vivo*, endows HSCs with the ability to out-compete wild-type HSCs after transplantation and allows expansion of competitive repopulating LTRCs *ex vivo*[70,71]. Signals that activate expression of *Hoxb4* in HSCs are not yet known. However, these studies give credence to the idea that microenvironmental signals may exist that can activate HSC self-renewal and therefore influence stem cell fate. They form the basis for the continued quest for the elusive signals that will support HSC expansion.

In an attempt to recreate the *in vivo* microenvironment, stromal cell lines have been generated from the aorta-gonad mesonephros region[72], the fetal liver[73] and the BM[74,75]. Because some, but not all, feeders support LTRCs, several investigators have initiated studies using functional genomics and proteomics tools to identify molecules present in LTRC-supportive but not nonsupportive cell lines[76,77]. Many soluble growth factors have been identified that play a role in controlling proliferation and differentiation of HSCs. Expansion of STRCs and modest expansion of cells that establish hematopoiesis in secondary recipients are observed when murine or human HSCs are cultured in the presence of combinations of potent hematopoietic cytokines[78–81]. However, defects in engraftment potential are seen when murine or human HSCs cultured with cytokines alone compete against unmanipulated cells[22,82]. This suggests that known cytokines, as the sole stimulus, do not allow cell divisions without the loss of repopulating ability. The effect of cytokines on progenitors is modulated by components of the extracellular matrix. For instance, engagement of $\beta_1$ integrins on HSCs may protect them from apoptotic cell death[82] and from excessive proliferation induced by cytokines[84]. Glycosaminoglycans, which are abundantly present in hematopoietic microenvironments, support adhesion of $CD34^+$ cells[85] and certain growth factors[86]. Selective colocalization of cytokines and progenitors may be crucial for the regulation of proliferation and differentiation[87]. But how to recreate such complex interactions in "defined" cultures is not yet clear. Finally, whether nonclassical hematopoietic cytokines thought to play a role in during hematopoietic development—such as bone morphogenetic proteins[88] or hedgehog proteins[89]—will aid in inducing LTRC expansion remains to be determined.

Factors other than hematopoietic cytokines are expressed differentially in LTRC-supportive and nonsupportive feeders. These include ligands for the Notch family of receptors[77], which play a role in cell-fate decision processes in nonhematopoietic systems[90]. LTRC-supportive feeders express Jagged-1[91,92] and Dlk[93], and addition of Jagged-1 or Dlk to expansion cultures can, at least in part, preserve the competitive repopulating capacity of HSCs associated with LTRC-supportive feeders. It is hoped that further analysis of signals and factors expressed in common between LTRC-supportive feeders for which receptors are identified on HSCs[94,95] will identify factors important for activating self-renewing cell divisions of HSCs without differentiation.

Finally, in the era of "adult stem cell plasticity"—which is discussed in a Commentary by Dorshkind[96] and a Review by Orkin[97] in this issue—one might speculate that other stem cells that are less prone to senescence and differentiation when induced to proliferate[98,99] may in the future be suitable sources of cells for hematopoietic reconstitution. Likewise, as human embryonic stem (ES) cells can be induced to differentiate to hematopoietic cells, they may be a suitable source of cells, provided that, aside from committed progenitors, engraftable HSCs can be generated from human ES cells[16].


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