

REVIEW ARTICLE

## The Biology of Hematopoietic Stem Cells

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Received for publication June 20, 2003; accepted June 23, 2003 (03/124).

Rarely has so much interest from the lay public, government, biotechnology industry, and special interest groups been focused on the biology and clinical applications of a single type of human cell as is today on stem cells, the founder cells that sustain many, if not all, tissues and organs in the body. Granting organizations have increasingly targeted stem cells as high priority for funding, and it appears clear that the evolving field of tissue engineering and regenerative medicine will require as its underpinning a thorough understanding of the molecular regulation of stem cell proliferation, differentiation, self-renewal, and aging. Despite evidence suggesting that embryonic stem (ES) cells might represent a more potent regenerative reservoir than stem cells collected from adult tissues, ethical considerations have redirected attention upon primitive cells residing in the bone marrow, blood, brain, liver, muscle, and skin, from where they can be harvested with relative sociological impunity. Among these, it is arguably the stem and progenitor cells of the mammalian hematopoietic system that we know most about today, and their intense study in rodents and humans over the past 50 years has culminated in the identification of phenotypic and molecular genetic markers of lineage commitment and the development of functional assays that facilitate their quantitation and prospective isolation. This review focuses exclusively on the biology of hematopoietic stem cells (HSCs) and their immediate progeny. Nevertheless, many of the concepts established from their study can be considered fundamental tenets of an evolving stem cell paradigm applicable to many regenerating cellular systems. © 2004 IMSS. Published by Elsevier Inc.

*Key Words:* Stem cell, Progenitor, Hematopoiesis, Bone marrow transplantation.

### Introduction

Like all stem cells, HSCs are defined operationally by their functional attributes, in this case by the potential to regenerate and maintain all of the terminally differentiated lymphoid and myeloid cells that comprise the blood, bone marrow (BM), spleen, and thymus (1–5). Accumulating evidence discussed by Horwitz in this issue also suggests that HSCs may be capable of differentiating into cells of nonhematopoietic tissues including those in the liver (6,7), pancreas (8), heart (9,10), brain (11), and kidney (12) when exposed to appropriate stimuli, although this issue remains equivocal (13–15). This capacity for “transdifferentiation” as it has come to be known, if real, is nevertheless not yet

considered an absolute criterion for cells to be included within the most primitive HSC compartment. It is generally considered that hematopoietic cells must satisfy three basic criteria to be considered stem cells. First, HSCs are pluripotent, characterized by the potential to generate progeny representing (at least) the eight major hematopoietic lineages: B and T lymphocytes; erythrocytes; megakaryocytes/platelets; basophils/mast cells; eosinophils; neutrophils/granulocytes, and monocytes/macrophages (1–5). Second, HSCs possess an extremely high proliferation potential. It is estimated that in normal humans there are approximately 50 million HSCs, some of which can generate up to  $10^{13}$  mature blood cells over a normal lifespan. In mice, it has been shown that a single stem cell can regenerate and maintain the entire lymphohematopoietic system following transplantation into an irradiated or immunocompromised host (16–18). Proliferation and differentiation are not necessarily strictly coupled, and in the most primitive HSCs this renders a capacity for self-renewal, the third cardinal property of all stem cell

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types. Self-regeneration is critical for HSCs because they are constantly subjected to physiological stresses that stimulate their recruitment along maturational pathways that ultimately result in their depletion, for example under conditions of hypoxia to increase red blood cell numbers or during infections to amplify granulocytes and macrophages. Self-renewal, at least at the population level, thus ensures that sufficient numbers of stem cells are available to meet the demands of hematopoiesis over a normal adult lifespan.

The hematopoietic system of mice and humans is organized as a hierarchy of clonogenic cell types with differing capacities for self-renewal, proliferation, and differentiation (Figure 1). Although previously thought to be predominantly quiescent, HSCs at the apex of this hierarchy actually do cycle, albeit very slowly, with an average turnover time of 30 days in steady-state mouse BM (19,20). In response to hematological stress, HSCs are recruited into active hematopoiesis and undergo a series of maturational cell divisions during which time progeny of self-renewing divisions can coexist with various classes of progenitor cells with progressively restricted proliferation and differentiation potentials. Hematopoietic progenitor cells (HPCs) ultimately differentiate into precursor cells and end cells of only one or two lymphoid or myeloid cell lineages (Figure 1). Mature blood cells have a finite lifespan, ranging from 1 (neutrophils) to 120 (erythrocytes) days in humans; thus, maintenance of their numbers under steady-state conditions or amplification in response to hematological stress requires the lifelong sequential activation of successive stem cell clones (21). This process is tightly regulated by a complex network of

glycoprotein growth factors and proliferation inhibitors whose opposing activities on different cell types can be further modulated in a concentration-dependent manner and depending on the context in which they are presented to the target cell, i.e., depending on what other cytokines are present and whether these molecules are presented in soluble form or bound to the extracellular matrix of mesenchymal cells that comprise the BM microenvironment or “stroma” (22,23). Detailed reviews of these regulatory aspects of hematopoiesis abound (24–26) and will not be discussed here. Rather, with these basic concepts in mind the remainder of this review will focus on the functional properties of HSCs *in vitro* and *in vivo*, distinctions among stem cells from different hematopoietic tissues and stages of ontogeny, and recent advances concerning their phenotypic characterization and molecular regulation.

### Functional Properties of Hematopoietic Stem Cells

Less than 0.1% of the hematopoietic cells in BM are pluripotent stem cells capable of long-term proliferation and self-renewal. Therefore, assays designed to identify this rare population must incorporate an assessment of these functional properties into their end-points and ideally, enable these characteristics to be measured independently. The first quantitative *in vivo* assay for “stem” cells was introduced in 1961 by Till and McCulloch (27). In this assay, hematopoietic cells obtained from the marrow or spleen of donor mice are transplanted intravenously (IV) into syngeneic recipients

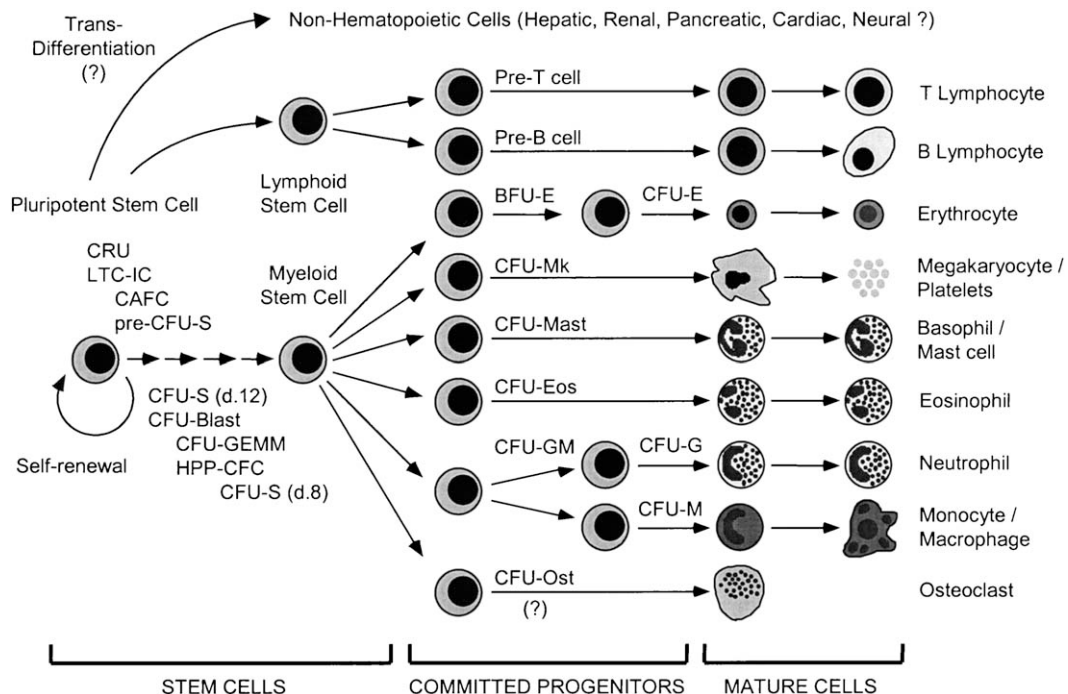


Figure 1. Schematic representation of hematopoietic cell development.

that were previously exposed to a lethal dose of irradiation in order to destroy endogenous hematopoiesis and prevent its regeneration by host HSCs. Approximately 10% of the injected cells become lodged in the spleen (28), where they proliferate and generate clonal macroscopic colonies comprised of predominantly erythroid or mixed myeloid/erythroid cells when observed either 8–9 or 12–13 days later, respectively (29,30). Such colonies can be readily counted; thus, the frequency of spleen colony-forming units (CFU-S) in the initial cell suspension can be accurately measured. The number and types of cells comprising each colony can also be determined to assess the proliferation and differentiation potential of the founder cell. Self-renewal potential can be evaluated by re-transplanting cells from individual excised colonies into secondary irradiated hosts to determine whether CFU-S were regenerated during the formation of the primary colony. Several studies have shown that although some days 12/13 CFU-S are capable of self-renewal (31), they are not able to sustain hematopoiesis long-term and indeed can be physically separated from true HSCs (32). Nevertheless, the CFU-S assay underpinned all contemporary methods for the functional identification and quantitation of HSCs *in vivo*. It is now widely appreciated that the only valid test that defines a HSC is one that demonstrates its capacity for complete and sustained (>6 months) regeneration of the lymphohematopoietic system following transplantation. State-of-the-art assays incorporate competitive repopulation strategies in which the cells being evaluated are co-injected with genetically or phenotypically distinguishable reference cells that serve the dual purpose of exerting a selective pressure to identify HSCs in the test population that possess superior proliferation potential, and enable stem cells to be identified among small grafts (e.g., <math>10^3</math> enriched HSCs) that may not on their own be sufficiently radioprotective as to allow the host animals to survive long enough to allow their detection at later times (33–35). Two different types of competitive repopulation assays have found broad application in experimental hematology. The first of these measures HSC activity relative to a standard competitor, usually  $10^6$  normal BM cells whose repopulating potential corresponds to 1 repopulating unit (RU). When test and competitor stem cells with equivalent activities are co-injected into lethally irradiated mice, 50% of the hematopoietic cells will be regenerated by each population (35). Analysis of covariance in the degree of donor-derived lymphocytes and granulocytes among mice injected with a defined mixture of test and competitor cells can be used to provide an absolute measure of HSC concentration (36). Generally speaking, skewing of chimerism in favor of either donor is considered evidence of its increased RU content. However, despite widespread confusion regarding this issue in the literature, this does not necessarily mean that such a population contains more HSCs. A similar outcome can be observed when the average proliferative output of individual HSCs is increased without any change in their absolute

numbers. For example, HSCs from murine and human fetal liver (FL) or human umbilical cord blood (CB) generate clones that are three- to fivefold larger than those produced by BM or peripheral blood (PB) stem cells *in vitro* and *in vivo* (37–40). In contrast, clone size and competitive repopulating ability can be decreased as a result of *ex vivo* culture (41) or proliferation induced by prior transplantation (33,42,43) or exposure to chemotherapy (44). To address this potential problem, the competitive repopulation assay was modified to incorporate a limiting-dilution design in which groups of recipient mice are co-injected with graded numbers of test stem cells together with a constant excess number of genetically distinguishable competitor cells (34,45). Several months later, the proportion of animals in each cell dose group that are engrafted with test stem cells (irrespective of the level of engraftment, as long as it is above a predefined threshold) is determined, and the frequency of competitive repopulating units (CRU) in the initial cell suspension can be calculated by applying the method of maximum likelihood and Poisson statistics (46). Using this assay, it was shown that murine CRU able to regenerate and maintain both lymphoid and myeloid cells for at least 1 year after transplantation represent ~1 per 15,000 adult BM or day-14 FL cells (34,38,40,41). Similar values have been obtained for human CRU and comparable SCID-repopulating cells (SRC) (47) from different tissues when quantitated by transplantation at limiting-dilution into sublethally irradiated non-obese diabetic severe combined immunodeficient *scid/scid* (NOD/SCID) mice together with irradiated human BM carrier cells to promote engraftment (39,48–50). It is important to note, however, that all repopulation assays of IV-transplanted cells underestimate the true number of HSCs with long-term repopulating (LTR) potential because they will fail to detect cells that do not home to a site *in vivo* where their regenerative potential can be activated. Recently, the seeding fraction of several related populations of murine stem and progenitor cells (*in vitro* colony-forming cells [CFCs], CFU-S, day-35 cobblestone area-forming cells [CAFC], and CRU) was measured at ~5 to 20% (51–53). Similar values have been reported for human CAFCs (54) and CRU able to home to the BM of sublethally irradiated NOD/SCID mice within the first 24 h after transplantation (50). Thus, the true frequency of both human and murine LTR HSCs in hematopoietic tissues is likely closer to at least 1 in 2,000 cells, much higher than predicted by classical studies that did not take into account inefficiencies in the seeding of IV-injected cells. Support for this concept is provided by novel HSC assays in which the requirement for IV-transplanted cells to circulate through the blood, recognize and extravasate through the BM vascular endothelium, and migrate to a supportive microenvironment is circumvented by their direct delivery into the BM via intrafemoral injection. Using this approach, it was recently shown that the frequency of SRC in CB is 15-fold higher (i.e., 1 per 44 CD34<sup>+</sup>CD38<sup>-</sup> cells) than suggested by conventional

IV transplantation into NOD/SCID mice (55). Moreover, CD34<sup>-</sup>Lin<sup>-</sup> CB cells, which do not show SRC activity in tail-vein injected mice, possibly due to their low expression of homing receptors such as CXCR4, efficiently engraft intra-BM-injected mice, albeit with slower reconstitution kinetics than CD34<sup>+</sup> cells (56).

Engraftment efficiency of IV-injected HSCs also varies with cycling status (57). Hematopoietic reconstitution potential of adult stem cells is associated with G<sub>0</sub> phase of the cell cycle and is reversibly diminished or lost with transition to G<sub>1</sub> (58,59). This defect is thought to reflect differences in the ability of mitotically active cells to home to supportive niches in the BM (60). Expression of several adhesion molecules such as very late antigen (VLA)-4 ( $\alpha$ 4 $\beta$ 1 integrin) that are critical for stem/progenitor cell homing is regulated in a cell cycle-dependent manner (61,62). Moreover, Sca-1<sup>+</sup>Lin<sup>-</sup> murine BM cells that express CD43 (leukosialin), CD49e ( $\alpha$ 5 integrin chain), and CD49d ( $\alpha$ 4 integrin chain), a phenotype characteristic of LTR HSCs (60), home predominantly to the BM within 20 h after IV transplantation (63).

Recently,  $\beta$ 2 microglobulin knockout NOD/SCID mice have been developed that, in addition to the B and T cell defect imparted by the *scid/scid* mutation and defects in the complement pathway and macrophage function in NOD mice, also lack natural killer (NK) cells and thus facilitate multilineage differentiation of 10-fold fewer human CB cells than are required to achieve comparable levels of engraftment in NOD/SCID hosts (64). NOD/SCID- $\beta$ 2 mice have not yet been used as recipients in limiting-dilution experiments to re-evaluate the frequency of HSCs among various human tissues, but it is likely that these would yield higher values than those provided by older models that were not optimized for engraftment of xenogeneic cells. Interestingly, human CD34<sup>+</sup>CD38<sup>+</sup> stem/progenitor cells that mediate short-term hematopoietic recovery preferentially engraft NOD/SCID- $\beta$ 2 mice and are undetectable in NOD/SCID hosts, suggesting an enhanced sensitivity of early-engrafting cells to NK cell-mediated rejection (65). The number of transplantable human HSCs that can be recovered from the BM of NOD/SCID mice can also be expanded 10-fold by their treatment with a short course of stem cell factor (SCF), interleukin (IL)-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and erythropoietin (EPO) immediately prior to sacrifice (66). These cytokines appear to increase the probability of self-renewal of human HSCs stimulated to proliferate *in vivo*. Thus, subtle variations in *in vivo* models used to identify unique populations of HSCs can have dramatic effects on their measured frequency and functional properties and are important to consider when evaluating data from different studies.

HSCs can be enriched from hematopoietic tissues on the basis of cell surface antigen expression (67–74), intracellular enzyme markers (75), dye efflux properties (76–81), sensitivity to cycle-active cytotoxic agents (45,82) and/or cell cycling properties (60) to yield suspensions in which up

to one in three to five cells is able to sustain long-term hematopoiesis after transplantation (70,83,84). Taking into account the inherent inefficiency of *in vivo* repopulation assays discussed previously, it is highly likely that an enriched HSC population from which single cells are able to engraft up to 30% of transplanted mice must be nearly pure (83,84), a remarkable achievement that should finally facilitate their detailed molecular analysis (83,85,86). Analysis of engraftment kinetics in mice injected with limiting numbers of “purified” HSCs has revealed significant heterogeneity among cells with transplantation potential. Early (up to ~12 weeks) after transplantation, hematopoiesis is characterized by the simultaneous activity of both lineage-restricted progenitor cells, such as the various classes of CFCs, as well as multipotent and high proliferation-potential (HPP-) CFCs and stem cells with short-term engraftment potential (36,87,88). Due to intrinsic limitations in the self-renewal and expansion potential of most cells that function at this time, and/or the ability of otherwise equivalent stem cells to compete more favorably for appropriate niches in the BM microenvironment (36), this polyclonal phase of engraftment ultimately wanes and long-term hematopoiesis is maintained by relatively few stem cells, some of which can remain active for >2 years and after up to four successive rounds of serial transplantation (89–92). LTR HSCs can be prospectively separated from short-term engrafting stem/progenitor cells (70,80), and if progenitors are sufficiently depleted suspensions of highly enriched HSCs are poorly radioprotective (17,45,93). Their detection thus requires the use of competitive repopulation assays discussed previously. Interestingly, clear genotypic differences can account for the differential contribution of functionally distinct murine stem cell populations to the early and late phases of engraftment. In allophenic C57BL/6 $\leftrightarrow$ DBA/2 mice, DBA/2 cells initially dominate the PB (94). However, as these chimeras age cells from the C57BL/6 partner strain become predominant. DBA/2 stem cells are still present but quiescent and can be reactivated to once again dominate the first temporal phase of engraftment by serial transplantation (95). This difference is attributed in part to a higher cycling activity of DBA/2 cells that decreases with age (96,97), and which confers a proliferative advantage after transplantation and results in faster recovery of CFCs and PB cells following chemotherapy (98). Quantitative trait loci (QTL) that regulate stem/progenitor cell numbers have been mapped to chromosomes X, 2, and 14 (99), while QTLs associated with their cycling have been mapped on chromosomes 7 and 11 (100). Future identification of the genes that control stem cell behavior should help explain the tremendous functional heterogeneity that exists among phenotypically identical subpopulations of human HSCs, as has been revealed in a clinical setting.

### HSCs from Different Tissues and Stages of Ontogeny

In adults, the majority of HSCs reside in the BM, where they are intimately associated with mesenchymal cells that

constitute the hematopoietic microenvironment and which produce a plethora of stimulatory and inhibitory factors that regulate stem cell development. However, low numbers of LTR HSCs constitutively migrate through the circulation (101), and this basal level of trafficking likely accounts for their broad distribution throughout the body, including within non-hematopoietic tissues such as skeletal muscle, kidney, lung, liver, heart, brain, and small intestine in mice (102). Hematopoietic stem and progenitor cells can be mobilized into the circulation following treatment with a variety of natural or molecularly engineered cytokines, chemokines, and adhesion molecule antagonists (Table 1) (see References 103–123). Humans display wide variations in mobilization efficiency following G-CSF treatment, the most commonly used modality in the clinic, suggesting that this process is influenced by genetic factors that are not currently known. Based on QTL analysis, loci on murine chromosomes 2 and 11 have been implicated as regulators/modifiers of G-CSF-induced mobilization (124), the former acting extrinsically on cells of the BM microenvironment and the latter acting intrinsically on hematopoietic cells (125). The molecular mechanism(s) governing the enforced egress of stem/progenitor cells from the BM into the blood are complex, but for at least two cytokines, namely, G-CSF and IL-8, the accumulation of activated neutrophils in the BM appears necessary for the mobilization phenotype (126,127). Following G-CSF treatment, granulocytes release proteolytic enzymes such as neutrophil elastase and cathepsin,

which cleave VCAM-1 (and other adhesion molecules) that normally serve to anchor clonogenic cells in the BM, resulting in their release into the periphery (126). Distinct mechanisms almost certainly operate for other mobilizing agents and await detailed characterization.

Numerous studies in rodents, non-human primates, and humans have established the utility of mobilized peripheral blood (MPB) cells for reconstituting hematopoiesis after myelosuppressive or ablative conditioning and precipitated its preferred use for auto- and allotransplantation (127). Hematological recovery generally occurs more rapidly in patients transplanted with MPB than BM cells (duration of severe neutropenia and thrombocytopenia shortened from ~25 to ~9 days), this difference being largely attributable to the higher average number of CD34<sup>+</sup> cells or granulocyte/macrophage colony-forming units (CFU-GM) transplanted per kilogram of recipient body weight with optimized MPB grafts (127). However, when normalized for compositional differences, murine G-CSF/cyclophosphamide MPB cells actually engraft slower than BM cells (128) and exhibit a 10-fold reduced capacity for long-term competitive repopulation (129). Compared to human BM CD34<sup>+</sup>Lin<sup>-</sup> cells, the *in vitro* proliferation and differentiation capacity of CD34<sup>+</sup>Lin<sup>-</sup> cells from normal and mobilized PB is also inferior (130,131), and higher numbers of MPB cells are required to establish levels of engraftment comparable to BM cells in xenogeneic transplant models (132,133). Mobilized stem/progenitor cells are predominantly in G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle (134) and quiescent cells typically mediate superior long-term engraftment (57,59). However, cycling and quiescent cells do not differ in the rate at which they regenerate circulating blood cells during the initial weeks after transplantation (135). Murine MPB progenitors home to the BM much less efficiently than do BM-derived cells within the first several hours after IV-transplantation (128,136), although they may accumulate in the marrow in greater numbers at later times. Reduced homing of mobilized stem cells is associated with their significantly lower expression of multiple integrins that are implicated in the homing process (128,136). Similar changes in adhesion molecule phenotype have been noted in GM-CSF MPB cells (137) and are consistent with the proteolytic cleavage of adhesion molecules that is suggested as the predominant mechanism of mobilization following treatment with cytokines that induce neutrophilia (126).

During fetal development and early postnatal life, the HSC population expands dramatically and begins to undergo subtle changes that continue into adult life. Due to their higher rate of proliferation compared to BM stem cells (37), HSCs collected from human fetal liver (FL) at between 6 and 22 weeks of gestation have a significantly higher expansion potential *in vitro*. For example, single FL long-term culture-initiating cells (LTC-IC) give rise to ~fivefold more CFCs at 5–6 weeks than do BM-derived LTC-IC (138). The average

**Table 1.** Molecules that mobilize hematopoietic stem/progenitor cells into the blood

Agent	References
Natural cytokines	
IL-1	103
IL-6	104
IL-7	105
IL-8	106
IL-11	107
IL-12	108
IL-17	109
G-CSF	110, 111
GM-CSF	112
SCF	110
Flt3/flk2 ligand	113, 114
Engineered cytokines	
Daniplestim (IL-3 receptor agonist)	115
Leridistim (dual IL-3 and G-CSF receptor agonist)	116
Myelopoietin (dual IL-3 and GM-CSF receptor agonist)	117
Promegapoietin-1 $\alpha$ (dual IL-3 and mpl receptor agonist)	118
Progenipoietin-1 (dual G-CSF and flt3 receptor agonist)	119
Chemokines	
MIP-1 $\alpha$	120
SDF-1	121
Adhesion agonists	
VLA-4 antibody	122
AMD3100 (CXCR4 agonist)	123

proliferative output of circulating blood cells, primitive progenitors and secondary CRU by murine and human FL CRU *in vivo* is also significantly higher than that of ontogenically older CRU (38–40). Interestingly, although the marrow homing/seeded efficiency of murine FL CRU is comparable to that of BM CRU (40), alterations in homing become evident as stem cells differentiate into the progenitor compartment. Nearly 10-fold fewer FL CFCs can be recovered from the BM within the first day after IV-transplantation compared to progenitors procured from adult marrow (128,139). This decline in homing efficiency with differentiation of fetal stem cells is associated with a diminished ability of FL CFCs to adhere to BM stromal cells *in vitro*, possibly due to the presence on fetal cells of low affinity forms of the integrins  $\alpha 4\beta 1$  and  $\alpha 5\beta 1$  (140). Defective homing may account in part for the extremely slow and transient engraftment of FL and fetal BM cells observed clinically (141). Although, in contrast to adult HSCs, such defects appear unrelated to the rapid cycling of fetal SRC, which does not appear to deleteriously affect their ability to engraft NOD/SCID mice (142).

Umbilical cord blood represents an attractive source of transplantable HSCs because of its primitive origin in ontogeny, ready availability, ease of procurement, absence of risk to donors, and the possibility of cryopreserving cells at birth for use as an autograft in persons requiring hematopoietic rescue later in life. Due to the immaturity of the newborn's immune system, lymphocytes present in CB have proven to be more tolerant of human leukocyte antigen (HLA) mismatches and consequently provoke less acute and chronic graft vs. host disease (GVHD) in an allotransplant setting (143). This allows more patients to find suitable donors, especially among ethnic and minority groups that are under-represented in the National Marrow Donor Program's Transplant Registry. The major drawback associated with the use of CB cells for hematopoietic rescue is significantly delayed engraftment, with a median 32 and 81 days to neutrophil and platelet recovery, respectively, compared with 18 and 29 days, respectively, for unrelated BM transplants (143). This is due to the small size (~80 mL) of most CB units which, despite containing a ~twofold higher frequency of LTC-IC (144) and CRU (39) than BM cells, provide a suboptimal dose of HSCs for transplantation of adults of average body weight. Fortunately, compared to adult cells, CB stem/progenitor cells produce larger colonies and expand to a much greater extent *in vitro* (37) and on a per-cell basis engraft NOD/SCID mice at 10- to 50-fold higher levels even in the absence of administered human growth factors (39). These properties should theoretically compensate for the relatively low numbers of HSCs contained in a single CB donation. Moreover, if proliferation is initiated *ex vivo* prior to infusion in order to circumvent the requirement of immature CB stem cells to undergo more cell divisions before differentiation to myeloid progenitors, it may in the future

be possible to use cultured CB cells to facilitate rapid engraftment in myeloablated patients (145).

### Phenotypic Characterization of Hematopoietic Stem Cells

Although LTR HSCs are present at a very low frequency in hematopoietic tissues, they can now be isolated in nearly pure form by selection on the basis of a variety of physical and immunophenotypic characteristics. Many stem cell markers have been described over the past 20 years and are reviewed in detail elsewhere (146–149). Briefly, murine HSCs are characterized by their high expression of stem cell antigen (Sca)-1, class I major histocompatibility (MHC) antigen H-2K, and in the case of FL HSCs, AA4.1. HSCs express low to intermediate levels of Thy-1 and c-kit and are characterized by the absence of lineage (Lin) antigens expressed predominantly on terminally differentiated lymphocytes (CD45R/B220, CD3, CD4, CD8), myeloid (CD11b/Mac-1, Ly-6G/Gr-1), and erythroid (TER-119) cells. Notably, however, low levels of CD4, B220, Gr-1, Mac-1, and TER-119 can be induced on some stem cells, particularly following activation by 5-FU treatment (45,150,151). Human HSCs are characterized by high expression of CD34, intermediate expression of c-kit and Thy-1, and low or no expression of CD38, HLA-DR, CD45RA, and CD71. Murine and human HSCs also express high levels of P-glycoprotein (P-gp), a membrane efflux pump responsible for multidrug resistance in tumor cells and which mediates export of several fluorescent dyes including Rhodamine-123 (Rh-123) (152). Thus, LTR HSCs exposed to Rh-123 are characterized by low fluorescence, while their more mature progeny are Rh-123-bright (76–78).

In addition to these "classical" phenotypes, several novel markers of primitive HSCs have been discovered over the past several years (Table 2). Following the initially provocative demonstration that a significant proportion of murine LTR HSCs are CD34 negative (17,153,154), it was shown that CD34 antigen is expressed on c-kit<sup>+</sup>Lin<sup>-</sup> stem cells from fetuses and neonates, but is reversibly down-modulated in association with their age and activation state after birth (155–157). Reversibility of CD34 expression was subsequently documented on human stem cells capable of regenerating hematopoiesis upon transplantation into immunodeficient beige/nude/xid (*bnx*) mice (158). Differences in the expression patterns of CD38 antigen between mice and humans and its modulation with HSC activation state have also confounded characterization of stem cells between the two species. Current evidence indicates that all human HSCs are CD38<sup>-</sup>, whereas more mature progenitor cells are CD38<sup>+</sup>. In contrast, when murine Sca-1<sup>+</sup>c-kit<sup>+</sup>Lin<sup>-</sup> BM or FL cells were fractionated according to CD38 expression, most day-12 CFU-S were contained within the CD38<sup>-low</sup> fraction, whereas the population that expressed higher levels of CD38

**Table 2.** Phenotypes of human and murine hematopoietic stem and progenitor cells

Stem cells	Progenitor cells
Adult mouse BM	
Sca-1 <sup>+</sup>	Sca-1 <sup>-/+</sup>
H-2K <sup>+++</sup>	H-2K <sup>+</sup>
Thy-1 <sup>lo a</sup>	Thy-1 <sup>-</sup>
c-kit <sup>lo b</sup>	c-kit <sup>+</sup>
CD34 <sup>- b</sup>	CD34 <sup>+</sup>
CD38 <sup>+ b</sup>	CD38 <sup>-/lo</sup>
Rh-123 <sup>lo (P-gp<sup>+</sup>) b</sup>	Rh-123 <sup>hi (P-gp<sup>-/lo</sup>)</sup>
HO <sup>lo (Bcrp1/Abcg2<sup>+</sup>); SP</sup>	HO <sup>hi</sup>
ALDH <sup>+</sup>	ALDH <sup>-</sup>
Flk-2 <sup>-</sup>	Flk-2 <sup>+</sup>
Lin <sup>- c</sup>	Lin <sup>-</sup>
Endoglobin <sup>+</sup>	Endoglobin <sup>-/lo</sup>
Mouse fetal liver	
AA4.1 <sup>+</sup>	AA4.1 <sup>-</sup>
Mac-1 <sup>+</sup>	Mac-1 <sup>-</sup>
Rh-123 <sup>hi</sup>	Rh-123 <sup>hi</sup>
Human	
CD34 <sup>- and + b</sup>	CD34 <sup>+</sup>
CD38 <sup>-</sup>	CD38 <sup>+</sup>
Thy-1 <sup>lo</sup>	Thy-1 <sup>-</sup>
c-kit <sup>lo</sup>	c-kit <sup>+</sup>
HLA-DR <sup>-/lo</sup>	HLA-DR <sup>+</sup>
CD45RA <sup>-/lo</sup>	
CD71 <sup>-/lo</sup>	
AC133 <sup>+</sup>	
Rh-123 <sup>lo</sup>	Rh-123 <sup>hi</sup>
HO <sup>lo</sup>	HO <sup>hi</sup>
KDR <sup>+</sup> (Flk-1 <sup>+</sup> )	KDR <sup>-/lo</sup>
ALDH <sup>+</sup>	ALDH <sup>-</sup>

<sup>a</sup>Only in Thy-1.1 strains of mice. HSCs in Thy-1.2 mouse strains are distributed roughly equally between Thy-1.2<sup>-</sup> and Thy-1.2<sup>lo</sup> fractions; <sup>b</sup>Antigen expression is reversibly modulated by HSC activation state so that reciprocal phenotypes can be observed following chemotherapy or mobilization; <sup>c</sup>Upregulation of lineage antigens such as CD4, B220, Gr-1, Mac-1, and TER-119 has been observed on some stem cells following 5-FU treatment.

contained few CFU-S and virtually all LTR HSCs (159). When stem cells are activated by injection of 5-FU, mobilized by G-CSF or exposed to cytokines *in vitro*, CD38 expression is turned off in conjunction with the expression of CD34 (160). Thus, the majority of HSCs in normal mice are CD34<sup>-</sup>CD38<sup>+</sup> (161), but reciprocity in their expression patterns raises the possibility that similar changes in stem cell phenotype may apply to human HSCs and must be considered in future strategies for stem cell purification.

AC133 (CD133) is a transmembrane glycoprotein antigen of unknown function that is selectively expressed on the majority of CD34<sup>+</sup> cells from human FL, BM, and normal and mobilized PB (162). Although largely overlapping in their expression patterns, discordant expression of AC133 and CD34 has been demonstrated. For example, AC133<sup>+</sup>CD34<sup>-</sup>Lin<sup>-</sup> cells have been identified in CB and fractionated according to their expression of CD7, a marker typically associated with lymphoid-restricted stem cells. AC133<sup>+</sup>

CD7<sup>-</sup> cells are highly enriched for progenitor activity and distinguished from CD7<sup>+</sup> cells by their ability to generate CD34<sup>+</sup> cells in liquid cultures. Cultured AC133<sup>+</sup>CD7<sup>-</sup> cells also produce human progeny in the BM of NOD/SCID mice 8 weeks after transplantation, whereas 400-fold greater numbers of the AC133<sup>-</sup>CD7<sup>-</sup> subset had no engraftment ability (163).

Although its expression is implied by the established responsiveness of primitive HSCs to flk-2/flt3 ligand, recent studies have clarified expression of the flk-2/flt3 receptor tyrosine kinase on murine Sca-1<sup>+</sup>c-kit<sup>+</sup>Lin<sup>-</sup> BM cells. Three phenotypically and functionally distinct populations have been identified: Thy-1<sup>lo</sup>Flk-2<sup>-</sup> cells contain stem cells with LTR potential, Thy-1<sup>lo</sup>flk-2<sup>-</sup> cells are short-term repopulating stem cells, and Thy-1<sup>-</sup>flk-2<sup>+</sup> cells are multipotent progenitors that lack self-renewal potential (74). Interestingly, HSCs from murine FL are contained within both the flk-2<sup>-</sup> and flk-2<sup>+</sup> populations (74). CRU from murine FL can also be distinguished from BM CRU by virtue of their expression of AA4.1, Mac-1, and capacity to retain high levels of Rh-123 dye (164). These changes in HSC phenotype during ontogeny parallel observations of functional differences between these two populations of CRU and provide new opportunities for the isolation and characterization of pluripotent cells at distinct stages of development.

Between 0.1 and 0.5% of CD34<sup>+</sup> cells from human BM, CB, and normal or mobilized PB express vascular endothelial growth factor receptor 2 (VEGFR2), also known as KDR or Flk1 in mice. Unipotent or bipotent progenitor cells are restricted to the KDR<sup>-</sup> fraction of CD34<sup>+</sup> cells, whereas HPP-CFCs are detectable in both CD34<sup>+</sup>KDR<sup>+</sup> and CD34<sup>+</sup>KDR<sup>-</sup> fractions (73). In contrast, more primitive 12-week LTC-ICs are enriched 300-fold in CD34<sup>+</sup>KDR<sup>+</sup> cells and are absent in the KDR<sup>-</sup> subset. CD34<sup>+</sup>KDR<sup>+</sup> LTC-ICs can be further amplified by supplementing cultures with VEGF, suggesting that KDR plays a functional role in HSC self-renewal. Limiting-dilution repopulation assays in NOD/SCID mice indicate a frequency of one HSC per five CD34<sup>+</sup>KDR<sup>+</sup> cells, and as few as 3,000 cells can generate significant numbers of human cells of multiple lineages after primary and secondary transplantation into fetal sheep (73). Murine studies suggest that embryonic Flk1<sup>+</sup> cells have hemoangiogenic potential but lack long-term HSC activity (165). Despite this difference between mice and humans, it has been suggested that the CD34<sup>-</sup>KDR<sup>+</sup> and CD34<sup>+</sup>KDR<sup>+</sup> phenotypes might define the postnatal and prenatal “hemoangioblast”, the progenitor of the blood and endothelial lineages (73).

A further link between angiogenesis and hematopoiesis is suggested by the differential expression of endoglobin, an ancillary transforming growth factor  $\beta$  receptor, within a fraction of Lin<sup>-</sup> cells referred to as “side population” (SP) cells. Due to their ability to efficiently efflux the dye Hoechst 33342 (HO), this small population exhibits a characteristic

pattern of fluorescence in the far red and blue emission channels when analyzed by flow cytometry (81). Endoglobin<sup>+</sup> cells account for 20% of total BM SP cells, and this population contains all HSCs with competitive LTR and self-renewal potential (166), thereby enabling a fivefold enrichment of HSCs with this marker alone. Endoglobin null embryos die at day 10 to 10.5 of gestation, the time at which repopulating stem cells first appear, due to defects in blood vessel and cardiac development, and in yolk-sac hematopoiesis (167). Future studies of the developmental potential of endoglobin null ES cells in chimeric mice will be required to clarify its role in HSC differentiation.

As noted previously, HSCs express high levels of P-gp, a plasma membrane transporter encoded by the multidrug resistance 1 (*MDR1*) gene (152). Enforced expression of *MDR1* in murine BM cells causes stem cell expansion *in vitro* and a myeloproliferative disease in mice transplanted with expanded grafts (168). HSCs from mice with targeted disruptions in *Mdr1a* and *Mdr1b*, the murine homologs of the human *MDR1* gene, lack the ability to expel Rh-123 but exhibit normal numbers of SP cells following staining with HO dye. This finding led to the identification of a distinct class of ABC transporters that determine the SP phenotype. One such transporter, *Bcrp1* (or *Abcg2*), is expressed at high levels in SP cells from murine BM, skeletal muscle, and cultured ES cells, and in BM from rhesus monkeys (169). Retrovirus-mediated over-expression of *ABCG2* (the human gene) in murine BM cells causes a significant expansion of SP cells *in vitro* (to 60% of infected cells vs. 0.05% in normal or vector-only transduced cells), and up to 50% of BM cells exhibit the SP phenotype 15 weeks after transplantation of *ABCG2*-expressing cells into lethally irradiated mice (169). Interestingly, unlike cells over-expressing *MDR1*, BM cells that over-expressed *ABCG2* exhibited an 80% decline in cloning efficiency *in vitro* and were unable to generate significant numbers of spleen colonies or contribute to long-term engraftment *in vivo*. These findings suggest that *Bcrp1* is a molecular determinant of the SP phenotype but when expressed at high levels inhibits hematopoiesis at an early developmental stage.

In addition to immunophenotypic analyses of antigens that are expressed on the surface of hematopoietic cells, an alternative strategy for HSC purification is to develop a fluorescent substrate for an intracellular enzyme that is differentially expressed in stem cells and their more mature progeny. This approach has been utilized successfully with aldehyde dehydrogenase (ALDH), a cytosolic enzyme that confers resistance of HSCs to alkylating agents such as cyclophosphamide and 4-hydroxyperoxycyclophosphamide (4-HC). Jones et al. enriched HSCs from murine BM on the basis of their small size (using counterflow centrifugal elutriation [CCE]) and AA4.1<sup>-</sup>Lin<sup>-</sup> phenotype and then separated this population into ALDH<sup>+</sup> and ALDH<sup>-</sup> fractions by sorting cells that were stained with dansyl aminoacetaldehyde (DAAA), a fluorescent substrate for ALDH (93).

ALDH<sup>+</sup> cells contained very few CFCs or CFU-S and failed to protect mice from the lethal effects of radiation-induced marrow aplasia. However, when combined with more mature cells with short-term repopulating potential, as few as 10 ALDH<sup>+</sup> cells were able to regenerate and maintain multilineage hematopoiesis for up to 23 months in primary and secondary hosts (93). These findings provide some of the strongest evidence that LTR HSCs are not able to provide short-term engraftment when isolated in sufficiently pure form, despite claims to the contrary (170–172). Lin<sup>-</sup>ALDH<sup>+</sup> cells isolated from CB are also enriched 50- to 100-fold for primitive hematopoietic cells detected in short- and long-term BM cultures, thus validating this approach for human HSC selection (173).

### Pathways Regulating HSC Self-Renewal

As discussed previously, the ability to self-renew is a defining property of HSCs. But the molecular mechanisms that determine how these rare cells sustain hematopoiesis over a lifetime without being depleted have remained elusive, transforming the search for self-renewal factors into a virtual “holy grail” of experimental hematology. Recently, several secreted proteins and transcription factors have been shown to induce self-renewal of adult HSCs and our understanding of this complex pathway in both normal and leukemic stem cells has expanded significantly (174). Members of the *Hox* homeobox gene family were among the first to be implicated as critical regulators of lineage specification and stem cell development in a number of tissues, including the hematopoietic system. Notable among these, the transcription factor *HOXB4* was first observed to be expressed at high levels among human CD34<sup>+</sup>CD38<sup>-lo</sup>CD45RA<sup>-</sup>CD71<sup>-</sup> BM cells that are highly enriched in LTC-ICs, but was absent in more mature progenitor populations (175). Retrovirus-mediated over-expression of *HOXB4* in cultured mouse BM cells results in a dramatic increase in the number of day-12 CFU-S and CFCs able to generate large colonies in primary and secondary cultures (176). Furthermore, unlike in normal BM transplants in which HSC numbers typically recover to only 5–10% of pretransplant levels after many months, the CRU compartment is regenerated to normal levels when *HOXB4*-transduced BM cells are injected into lethally irradiated mice, resulting in 50-fold greater numbers of totipotent HSCs detectable in serially transplanted primary and secondary hosts (176). Importantly, *HOXB4*-transduced HSCs do not expand above the levels observed in unmanipulated mice, indicating that its overexpression does not override the regulatory mechanisms that maintain the HSC pool size within normal limits (177).

*Bmi-1* is a transcriptional repressor of the Polycomb group that is highly expressed in phenotypically primitive BM cells of mice and humans. Although the number of HSCs in FL of *Bmi-1* null embryos is normal, such mice die within 2

months of birth due to a profound and progressive depletion of all blood cells, including primitive progenitors. The fact that FL and neonatal BM cells obtained from *Bmi-1*<sup>-/-</sup> mice contribute only transiently to hematopoiesis in lethally irradiated wild-type recipients suggests that failure of HSCs to self-renew is the primary manifestation of *Bmi-1* deletion. BM cells harvested from these primary animals 6 weeks after *Bmi-1*-deficient stem cell transplantation are unable to regenerate *Bmi-1*<sup>-/-</sup> cells in secondary mice, thus failing a classic test of HSC self-renewal (178,179). Expression of several stem cell associated genes, cell survival genes, transcription factors, and genes that modulate cell proliferation are altered in BM cells from *Bmi-1*<sup>-/-</sup> mice (178). In particular, Park et al. have suggested that the effects of *Bmi-1* are mediated through its repression of the genes encoding p16<sup>Ink4a</sup> and p19<sup>Arf</sup>, which respectively inhibit cell proliferation and enhance cell death, so that in *Bmi-1* null mice one would predict premature exhaustion of the stem/progenitor cell pool (178).

The Hedgehog (Hh) pathway is another cascade that plays a pivotal role in HSC proliferation distinct from the differentiation-inducing cytokines. Sonic hedgehog (Shh) is one of three transmembrane proteins that comprise the Hh family in humans and that mediates signaling either through cell-to-cell contact between adjacent cells expressing the Patched (Ptc) receptor, or by diffusion of the soluble ligand through the microenvironment and interacting with distal cells. Shh, Ptc, and Smo (Smoothed, another Hh receptor) are expressed in primitive human CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> cells, mature myeloid cells, B and T lymphocytes, BM stromal cells, and umbilical vein endothelial (HUVEC) cells (180). CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> cells, stromal and HUVEC cells, but not end cells, also express three Gli transcription factors (Gli-1, -2, and -3) that act downstream of Hh signaling. This suggests that Hh signaling is not essential to hematopoietic cell maturation. Addition of neutralizing anti-Hh antibodies to cytokine-supplemented cultures of CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> cells inhibits the proliferation of total cells and cells with primitive phenotypes and maintains their clonogenic capacity (180). Paradoxically, CFCs are also expanded when soluble Shh ligand is added to such cultures, although differences in cell proliferation and the amount of progenitor expansion elicited via anti-Hh and Shh suggests an alternative mechanism of action of the two molecules. This was further illustrated when anti-Hh or Shh was added to cultures initiated with 10<sup>3</sup> CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> cells, containing 1 SRC. When the contents of such cultures were transplanted at limiting-dilution into NOD/SCID mice 7 days later, cells treated with anti-Hh were still found to contain only one SRC, indicative of HSC maintenance. Cells treated with soluble Shh were able to engraft several mice and generated greater numbers of human hematopoietic cells *in vivo*, suggesting that HSCs had undergone self-renewal divisions in response to Hh signaling (180). In *Drosophila*, Shh activity is associated with control of bone morphogenetic protein (BMP) signaling. Noggin, a

natural inhibitor of BMP-4, was capable of inhibiting the Shh-induced proliferation of phenotypically primitive hematopoietic cells in a manner similar to anti-Hh, although anti-Hh had no effect on BMP-4-induced proliferation. Thus, Shh appears to function as a regulator of HSCs, albeit in combination with other growth factors, via mechanisms dependent on downstream BMP signals.

The evolutionarily conserved Notch signaling pathway also plays a critical role in cell fate decisions in a variety of organisms including *Caenorhabditis elegans*, *Drosophila*, and mice. Two Notch receptors, Notch1 and -2, are expressed throughout hematopoietic maturation. In general, Notch activation leads to transcriptional suppression of lineage-specific genes, inhibiting differentiation in response to inductive signals and leaving some progenitors uncommitted but competent to adopt alternative fates (181). Interestingly, whereas either Notch1 or 2 is capable of inhibiting myeloid differentiation in 32D cells, they do so in a cytokine-specific manner: Notch1 in response to G-CSF and Notch2 in response to GM-CSF (182). Notch receptors bind to transmembrane proteins that serve as ligands containing a highly conserved Delta-Serrate-Lag-2 (DSL) domain in the extracellular region. One such ligand, Jagged1, is expressed by BM stromal cells and its role in promoting the maintenance or expansion of stem/progenitor cells has been established by several studies. When CD34<sup>+</sup>c-kit<sup>+</sup> cells isolated from the aorto-gonadal mesocephros (AGM) region of mouse embryos were cultured on S17 stromal cells that were engineered to express Jagged1, the number of CFCs increased fourfold compared to control stroma (183). Human CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> CB cells cultured with Jagged1 in the absence of any other cytokines were able to engraft NOD/SCID mice at higher levels than freshly isolated cells, suggesting an expansion of SRC (184). Varnum-Finney et al. transduced murine Sca-1<sup>+</sup>c-kit<sup>+</sup>Lin<sup>-</sup> BM cells with a retrovirus expressing a constitutively activated form of Notch1 and were able to generate immortalized “stem cell” lines that retained a primitive morphology, phenotype, and the capacity to differentiate into lymphoid or myeloid progeny *in vitro* and in competitively repopulated recipients (185). Taken together, these data establish the Notch signaling pathway as an important regulator of HSC self-renewal.

Wnt proteins represent another important class of intercellular stem cell regulators about which much has been learned over the past few years. Previously, characterization was hampered by the fact that unlike Hh proteins and BMPs, Wnt proteins are inefficiently secreted and relatively insoluble due to their hydrophobicity. These technical issues were recently overcome, leading to the isolation of purified mouse Wnt3a (186). Addition of the protein to single c-kit<sup>+</sup>Thy-1<sup>lo</sup>Sca-1<sup>+</sup>Lin<sup>-</sup> mouse BM cells increased the frequency of cells responding to limiting doses of SCF by ~sixfold and 30% of these cells maintained a HSC phenotype. Expanded cells were also injected into lethally irradiated mice, which were then analyzed for multilineage engraftment 6 weeks later

(186). Limiting-dilution assays predicted that if HSCs were maintained in such cultures, only 10% of the recipients would be reconstituted successfully. In contrast, 100% of the animals injected with cultured cells contained donor-derived progeny, suggesting that HSCs had undergone self-renewal in response to purified Wnt3a. Retrovirus-mediated overexpression of constitutively active  $\beta$ -catenin (a downstream activator of the Wnt signaling pathway) in long-term cultures of enriched HSCs also expanded the pool of transplantable stem cells determined both by phenotype and *in vivo* repopulating ability (187). Conversely, ectopic expression of axin or a frizzled ligand-binding domain, inhibitors of Wnt signaling, led to inhibition of HSC proliferation, increased death of HSCs *in vitro*, and reduced engraftment in transplanted mice (187). Interestingly,  $\beta$ -catenin-transduced HSCs expressed ~three- to fourfold higher levels of HoxB4 and Notch, suggesting that genes previously identified as regulators of HSC self-renewal may be related and act in a molecular hierarchy.

### Summary and Future Directions

The concepts discussed in this review can be summarized as a set of propositions that comprise the framework for future studies directed at exploiting HSCs for cell therapies, tissue engineering, and elucidation of the molecular mechanisms leading to hematological disease. First, the most primitive HSCs possess a unique capacity for long-term repopulation and self-renewal that can only definitively be measured by rigorous quantitative transplantation assays. Second, LTR HSCs exhibit considerable functional heterogeneity as a result of differences in tissue origin, cell cycling, or activation state. Third, strategies for stem cell enrichment, which have been refined by many investigators over the past 40 years, have culminated in the definition of a complex but relatively precise phenotype that now facilitates the isolation of essentially pure HSCs. Fourth, with “pure” HSCs in hand, it has been possible to begin to identify the genes that control self-renewal and lineage specification, providing opportunities for intervention of dysregulated signaling pathways that lead to aberrant hematopoiesis and cancer. Advances in stem cell biology over the past 5 years have been staggering, and it is difficult to imagine a more exciting time to be in this field. In particular, the tantalizing notion that HSCs may find applications in the treatment of diseases or the repair of damaged or aged tissues that are not conventionally hematological in nature, such as myocardial infarction, ischemia, or cirrhosis, should stimulate intense investigations and rapid progress in tissue engineering that, in the wake of the sequencing of the human genome, arguably represent the next major advance in modern medicine. But experimental findings must be analyzed critically and basic researchers should exercise caution in reporting findings that because of their provocative nature often spur

premature translation to the clinic. The constantly evolving interpretation of evidence for stem cell “transdifferentiation” highlights the need for rigorous experimentation before rewriting the dogma. Regardless, it is clear that there are important connections among normal hematopoiesis, tissue repair, and cancer. Continued studies of HSCs will provide important insights into the biology of many regenerating cellular systems and will ultimately yield new approaches to treat a variety of human diseases.

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