

BIOLOGY OF HEMATOPOIETIC STEM CELLS AND PROGENITORS: Implications for Clinical Application

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■ **Abstract** Stem cell biology is scientifically, clinically, and politically a current topic. The hematopoietic stem cell, the common ancestor of all types of blood cells, is one of the best-characterized stem cells in the body and the only stem cell that is clinically applied in the treatment of diseases such as breast cancer, leukemias, and congenital immunodeficiencies. Multicolor cell sorting enables the purification not only of hematopoietic stem cells, but also of their downstream progenitors such as common lymphoid progenitors and common myeloid progenitors. Recent genetic approaches including gene chip technology have been used to elucidate the gene expression profile of hematopoietic stem cells and other progenitors. Although the mechanisms that control self-renewal and lineage commitment of hematopoietic stem cells are still ambiguous, recent rapid advances in understanding the biological nature of hematopoietic stem and progenitor cells have broadened the potential application of these cells in the treatment of diseases.

INTRODUCTION

Stem cell is a term commonly used to describe a cell that can differentiate into multiple cell types and maintain self-renewal activity. Stem cells may be categorized into two different types. Pluripotent stem cells can differentiate into cells of all three germ layers: endoderm, ectoderm, and mesoderm. Examples of such stem cells are embryonic stem and embryonic germ cells. Embryonic stem cells, which are derived from the inner cell mass of the blastocyst, can be cultured in

vitro almost infinitely. Because embryonic stem cells can differentiate into all cell types in the body, they are commonly employed in the generation of gene-targeted mice, using the homologous recombination technique. Unlike embryonic stem cells, multipotent stem cells, which may be isolated from various tissues in fetal and adult animals, are lineage specific and include hematopoietic stem cells (HSCs), neuronal stem cells, hepatic stem cells, etc. In this review we consider HSCs as a prototype of this category.

High-dose total body irradiation leads to death through various mechanisms, one of which is bone marrow failure. In 1949 Jacobson et al. found that fatal marrow aplasia can be rescued by shielding the spleen, where hematopoiesis occurs in mice even into adulthood (1). In 1951 two groups showed that injection of spleen cells or bone marrow cells can rescue lethal-dose irradiated animals (2, 3). Injection of allogeneic bone marrow usually causes graft-versus-host disease (GVHD), which is induced by transferred donor T cells that recognize host cells as nonself (4). However, despite this potentially problematic side effect, allogeneic bone marrow transplantation may be useful for inducing tolerance to secondary transplantation of donor organs or tissues (5). Although it was clear early on that cellular, not humoral, factors play an important role in chimera formation after bone marrow transplantation (6), it was not clear during this era which cell populations within bone marrow are the major contributors to the reconstitution of the hematopoietic system following transplantation.

The presence of hematopoietic stem or progenitor cells in the body was predicted by the evidence of clonogenic mixed colony (composed of granulocyte/macrophage and erythroid cells) formation in the host spleen after injection of bone marrow cells into irradiated mice (7). Occasionally spleen colony-forming cells include cells that are further transplantable and reconstitute the hematopoietic system following secondary transplantation into irradiated mice (8). Although it is known now that day 8 spleen colony-forming units (CFU-S) are formed not by HSCs but by more mature cells such as myeloid progenitors (9), the finding of CFU-S is a landmark in the research of hematopoietic cell development, and it triggered the pursuit of the identity of HSCs. In this review we provide an overview of the characterization of HSCs and downstream hematopoietic progenitors, their biological nature, and their potential therapeutic utility.

BIOLOGY OF HEMATOPOIETIC STEM AND PROGENITOR CELLS IN EXPERIMENTAL SYSTEMS

Mouse Bone Marrow Hematopoietic Stem Cells and Progenitors

Bone marrow HSCs are functionally defined by their unique capacity to self-renew and to differentiate to produce all mature blood cell types. Becker and colleagues first reported the clonal origin of hematopoietic cells in 1963 (10). Later, evidence of the presence of HSCs was obtained by tracking progeny *in vivo* from transplanted

mouse fetal liver cells marked with retroviruses (11). Although some properties of HSCs could be determined by examining (by Southern blotting) the proviral integration pattern in HSC progeny, the surface phenotype or morphology of HSCs was not clear at that time.

Enrichment of HSCs was extensively performed by size fractionation with density gradient centrifugation and elutriation, injection of cell-cycle active drugs such as 5-fluorouracil (5-FU), and fluorescence-activated cell sorting. Currently fluorescence-activated cell sorting is commonly used to purify HSCs. The HSC pool may be separated into distinct subpopulations based on both surface marker expression and self-renewal capacity (12, 13). All HSC activity in adult bone marrow is contained within the lineage marker^{-/lo} (Lin^{-/lo}, c-Kit⁺, Sca-1⁺ subset of marrow cells (14). Single HSCs of the Thy1.1^{lo} Lin⁻Sca-1⁺ c-Kit⁺ subsets can give rise to long-term multilineage reconstitution and self-renewal in irradiated mice (15, 16). Reciprocal expression of the markers Thy-1.1 and Flk2 is seen as HSCs mature from a population with extensive self-renewal potential (long-term (LT)-HSC, Thy1.1^{lo}Flk2⁻) to a multipotent progenitor population with limited self-renewal potential (Thy1.1⁻Flk2⁺) (17). As discussed below, human HSCs are highly enriched in the CD34⁺ bone marrow fraction; however, mouse LT-HSCs express CD34 at the level of negative to low, not high (18). The presence of CD34⁻ HSCs in other animals is suggested (19). In addition to cell surface staining, HSC-enriched populations can be identified within the side population using the supravital stain Hoechst-33342 (20).

The downstream progeny of HSCs have also been characterized, and lineage-restricted oligopotent progenitor cells for lymphoid [common lymphoid progenitor (CLP)] and myeloid [common myeloid progenitor (CMP), granulocyte-monocyte progenitor (GMP), and megakaryocyte-erythrocyte progenitor (MEP)] lineages have been identified (21, 22) (Figure 1).

Early Hematopoiesis

During development hematopoiesis occurs sequentially in distinct anatomical locations. Both blood and endothelial progenitors first emerge in the extra-embryonic yolk sac blood islands at about embryonic day 7.5 (E7.5) (23). The yolk sac predominantly supports the generation of primitive hematopoietic cells, consisting mainly of nucleated erythrocytes. Definitive hematopoietic cell types may be assayed by *in vitro* culture from both the yolk sac and the aorta/gonad/mesonephros (AGM) region of the embryo proper prior to the onset of circulation (at ~E8.5); however, the precise relationship between primitive yolk sac HSCs and definitive HSCs remains controversial (24–26). Whereas some studies suggest independent origins of primitive and definitive HSCs (27–29), others provide evidence for a common precursor population, which arises in the yolk sac to provide primitive hematopoiesis for the early embryo but also seeds the AGM and fetal liver as these sites become competent to support HSC and hematopoietic cell development (30–34). However, regardless of their precise lineal relationship, it is clear that there

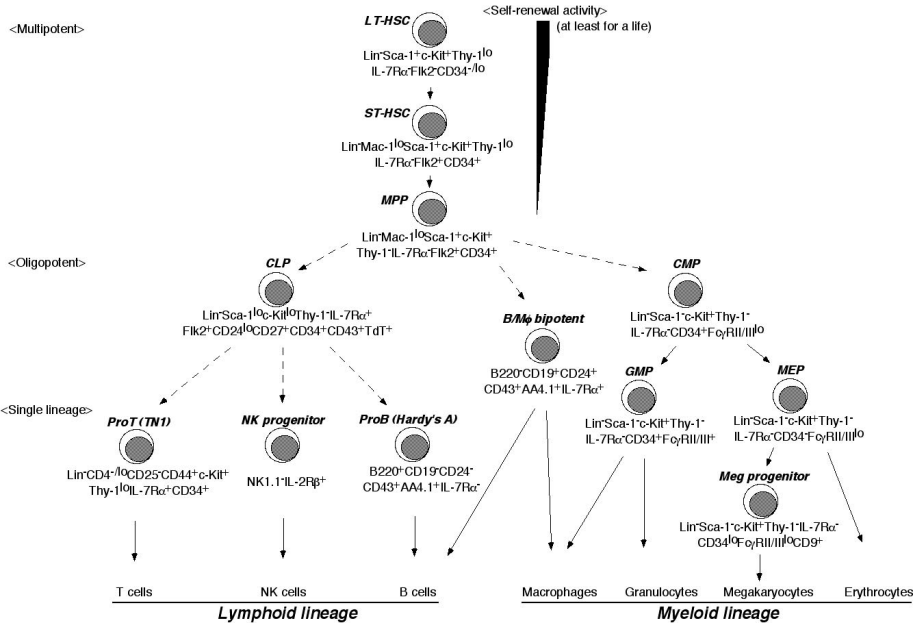


Figure 1 Conceptual hematopoietic trees in adult mice. Indicated cell populations can be purified based on the cell surface phenotype. Not all of the linear relationships in this figure have been proved. Multipotent progenitors (MPPs), at least at the population level, can differentiate into all types of hematopoietic cells, but have no detectable self-renewal potential in vivo. Megakaryocyte progenitors have recently been identified (22a). ProT cells are present in the thymus. For dendritic cell differentiation, please refer to (22b).

are distinct requirements for primitive and definitive hematopoiesis, including differential dependence on particular transcription factors [e.g., AML1 (35), jumonji (36)] and secreted factors [e.g., Epo (37, 38), steel factor (SLF) (39)]. In addition, whereas fetal liver and bone marrow HSCs efficiently engraft lethally irradiated adult animals, HSCs isolated from yolk sac (or AGM prior to E11) cannot (40–43), possibly reflecting a lack of expression of receptors required for bone marrow homing or for subsequent productive interactions with bone marrow stroma or secreted factors (24, 44). Yolk sac HSCs may require transit to or through the fetal liver to “activate” their ability to engraft irradiated adults (45), as E8 or E9 blood island cells injected into the yolk sacs of synchronized fetuses provide HSCs for life (30, 33), and E9 or E10 yolk sac HSCs are capable of engrafting sublethally irradiated newborn mice (46–48), which retain hematopoietic activity in the liver for 1–2 weeks following birth (49–51). Adult-engrafting HSCs may also be derived from yolk sac HSCs by coculture in vitro with the AGM-S3 stromal cell line, derived from the E10.5 AGM region (52).

Kyba et al. (53) recently provided new insight into the molecular differences underlying the differential engraftment capacity of primitive and definitive HSCs.

These authors demonstrated that enforced expression of the homeobox gene *HoxB4* in either yolk sac- or embryonic stem cell-derived HSCs conferred upon these cells the capacity to functionally engraft and contribute to the long-term, multilineage reconstitution of lethally irradiated adult recipients. *HoxB4* previously was implicated in promoting self-renewing divisions of bone marrow HSCs in vitro and in expanding the HSC population in vivo (54–58) (see below). Expression of *HoxB4* in yolk sac HSCs is only transiently required to confer adult engraftment potential and induces the precocious expression of at least two markers of definitive HSCs: the chemokine receptor, CXCR4, and the transcription factors TEL/ETV6, both of which may be involved in the seeding of bone marrow hematopoiesis by fetal liver HSC (53, 59–61). Elucidation of the upstream inducers and downstream targets of *HoxB4* that permit the acquisition of definitive hematopoietic function in yolk sac- or embryonic stem cell-derived HSCs will certainly be a focus of intense future research.

Colonization of fetal liver by yolk sac- or AGM-derived HSCs begins at ~E10 or E11, and by E12 the fetal liver is the major site of hematopoiesis. Fetal liver HSCs eventually migrate to the bone marrow at ~E16–E17 [(62, 63); J.L. Christensen, D.E. Wright, A.J. Wagers, I.L. Weissman, unpublished results], and bone marrow becomes the predominant site of hematopoietic cell development soon after birth and continuing into adult life (64, 65). Fetal liver HSCs express cell surface markers that are distinct from adult HSCs [including Mac-1(63) and AA4.1(66)] and generate several mature blood cell types that cannot be generated by adult HSCs. These include Ly-1⁺ B-1a cells (67), which provide the first wave of B cells during embryonic development and comprise the majority of B cells in the newborn and V γ 3⁺ and V γ 4⁺ T cells (68, 69). Finally, CD4⁺CD3⁻ lymphotoxin- β ⁺ integrin α 4 β 7⁺ cells arise exclusively in the fetus and give rise to dendritic antigen presenting cells (APCs), natural killer (NK) cells, and follicular dendritic cells (FDC), but not B or T cells (70). These cells may be FDC precursors, or may support the development of FDC, and are implicated in the organogenesis of lymph nodes (67).

When compared with bone marrow HSCs, fetal liver HSCs show a more rapid and robust capacity for reconstitution of lethally irradiated adults (66, 71–74), suggesting that the fetal liver HSC population contains a higher proportion of long-term reconstituting (LT-) HSCs than the bone marrow HSC pool, or that these cells possess an intrinsically greater capacity for bone marrow homing, lodgement, or expansion. Interestingly, the LT reconstituting potential of bone marrow HSCs declines further with age, as bone marrow HSC isolated from 24-month-old mice, while increased in absolute number, exhibit about fivefold reduced competitive engraftment ability (75).

The Hemangioblast

The concurrent emergence of hematopoietic and endothelial precursors in the embryonic yolk sac, as well as their overlapping patterns of gene expression (25, 26, 76), provides circumstantial evidence for the derivation of these cell types

through a common progenitor, or hemangioblast. Furthermore, multiple genetic deficiencies [e.g., Flk-1 (77, 78), TGF- β 1 (79)], resulting in selective defects in the generation or organization of both blood and vascular cells, reveal an apparent interdependence of vasculogenesis and hematopoiesis throughout development. Some evidence for hemangioblast activity in the developing embryo derives from studies of cultured mouse embryonic stem cells, in which populations of vascular endothelial growth factor (VEGF)-responsive precursors with bipotent potential for hematopoietic and endothelial cell development may be isolated (80, 81). In addition, *in vitro* culture of TIE2/TEK⁺ cells isolated from murine AGM (82), or Flk-1⁺ populations isolated from differentiating embryonic stem cells or E9.5 yolk sac (83–86) generates both blood and endothelium. However, whereas many studies clearly indicate a close relationship between hematopoiesis and vasculogenesis, a precise, clonal characterization of their proposed common precursor *in vivo* has yet to be accomplished.

Hematopoietic Stem Cell Self-Renewal and Hematopoietic Differentiation

HSCs are an asynchronously dividing cell population (87); however, because in the absence of overt injury the size of the total pool of HSCs remains roughly the same, about half of all HSC divisions must, at the population level, be self-renewing. The cellular signals that influence the choice between self-renewal and differentiation are incompletely defined, but several candidate molecules have been suggested. The identification of genes that promote HSC self-renewal has been a long-standing goal, as these molecules potentially represent a means for maintaining expansion of HSCs *in vitro*, a feat that would have significant impact on the collection of HSCs for transplantation (particularly in cases in which HSC numbers are limiting, as in the use of cord blood for adult transplantation) and on current gene therapy strategies.

Although some studies have described modest, transient expansion of LT-HSC *in vitro* in response to particular cytokines (including SIF, Flt3L, Tpo, and IL-3), either alone or in combination, in most cases the proliferation of HSCs *in vitro* inevitably leads to hematopoietic differentiation or death, with an overall loss of long-term repopulating HSCs (88–96). Recently, however, several developmental regulators of cell fate determination including Wnts [(97); T. Reya, A.W. Duncan, L.A. Ailles, J. Domen, D.C. Scherer, K.W. Willert, L. Hintz, R. Nusse, I.L. Weissman, unpublished results], Notch (98, 99), and Sonic hedgehog (Shh) (100) have been shown to promote expansion of HSCs *ex vivo*. In addition, impressive expansion (~40-fold) of *ex vivo* cultured HSCs, which retain oligoclonal lymphomyeloid differentiation potential upon transplantation to lethally irradiated adult recipients, has been accomplished by retrovirus-mediated expression of HOXB4 (55). HOXB4, as well as HOXA9, also enhances HSC expansion *in vivo* when introduced via retroviral transduction into HSCs prior to transplantation (54, 57, 101). Identification of the upstream inducers and

downstream targets of these molecules in HSCs will certainly be a focus of many future investigations.

Telomere shortening may be one factor that limits HSC self-renewal potential. Telomerase, an RNA/protein complex responsible for extending telomeric DNA, is expressed by mouse fetal liver and bone marrow HSC (102). Telomerase activity appears to correlate with self-renewal capacity and is reduced as HSCs differentiate to multipotent progenitor populations (102). Studies of human "candidate" HSCs (CD34⁺ CD45RA^{lo} CD71^{lo}) have suggested that telomerase activity may be induced in cycling HSCs (103). However, despite constitutive telomerase activity in HSCs, telomeres still shorten with HSC division *in vivo* (104–106), indicating that whatever telomerase activity is present in HSCs is not sufficient to maintain telomere length. In addition, serial transplantation of HSCs in mice is limited to ~5–7 rounds (107, 108), which many indicate that HSCs cannot self-renew indefinitely. However, it is important to note that serial transplantation is not simply a measure of HSC lifespan; it is also a measure of HSC homing to hematopoietic tissues, chemotaxis to hematopoietic microenvironments, and establishment of appropriate cell-cell interactions within these microenvironments. HSC transplantation always involves the transfer of cells into lethally irradiated hosts, and the irradiated environment is far from normal. For example, in normal young mice about 4% of LT-HSCs have >2n DNA, and about 8% per day enter the cell cycle (87). In contrast, HSCs in transplanted mice are more frequently in cycle, and this enhanced proliferation of HSCs lasts for at least 4 months after transplant (105). HSCs with >2n DNA, whether from young normal mice (109), transplanted hosts (110), or aged mice, (75) are less efficient as transplantable entities. Thus, serial transplantation introduces an artifact(s) unrelated to lifespan, so direct inferences regarding HSC lifespan from these studies must take such caveats into account.

Apoptosis

Programmed cell death (apoptosis) also regulates the size of the HSC pool (111). Ectopic expression of the antiapoptotic protein BCL2 in transgenic mice leads to an increase in the steady-state frequency of HSCs and progenitor cells in the bone marrow and an increase in competitive repopulation potential (112). In addition, BCL2-expressing HSCs show enhanced survival *in vitro* and may be maintained in serum-free media containing only SIF, IL-3, or Tpo (113). Importantly, BCL2 transgenic HSCs are not prevented from differentiating under such conditions, and each of the single cytokines capable of evoking HSC proliferation *in vitro* differentially biases the outcome of such divisions. The precise physiologic regulators of apoptosis in HSCs have remained elusive. RT-PCR analysis showed that murine HSCs do express BCL2 family members; however, BCL_{xL}, rather than BCL2 itself, appears to be the primary antiapoptotic protein expressed by HSC (112). CD95 (Fas), which can trigger apoptosis of cells after ligand binding, is not expressed by murine HSCs (114), and bone marrow hematopoiesis does not appear to be affected by Fas deficiency (115). However, Fas expression may be

inducible on HSCs or hematopoietic progenitors following exposure to certain cytokines including IFN- γ or TNF- α (115–117) and in these cases appears to reduce hematopoietic repopulating potential, although rigorous studies of purified HSC populations have not been reported.

Hematopoietic Stem Cell Migration: Physiological Circulation and Enforced Mobilization

As discussed above, HSC migration is an intrinsic aspect of the development of the hematopoietic system and is critically required for the success of bone marrow and peripheral blood progenitor (PBPC) transplantation in the treatment of multiple hematopoietic and nonhematopoietic diseases. While the capacity of HSCs to migrate from blood to bone marrow (homing) and from bone marrow to blood (mobilization) has been conserved through evolution, the biological role of this phenomenon in HSC function remains unknown. Surprisingly, migration of HSCs to and through the circulation appears to occur physiologically in normal animals. Using genetically marked parabiotic mice, which are surgically joined such that they develop a shared circulatory system, we recently demonstrated that HSCs rapidly migrate through the blood and play a functional role in the reengraftment of unconditioned bone marrow (118). Thus, in the steady state HSCs redistribute via the bloodstream among distinct anatomical locations and therefore are likely to be found in all tissues of the body. Importantly, their presence in tissues may confound interpretation of studies designed to detect hematopoietic activity from nonhematopoietic tissues, as unless purified populations are used, hematopoietic activity derived from itinerant HSCs may be attributed to developmental plasticity of tissue-specific progenitor cells (119, 120). The constant flux of HSCs in the circulation further suggests an explanation for the unexpected success of bone marrow transplantation. This clinically important process likely exploits an already existing mechanism of HSC migration that in unmanipulated animals allows the constitutive recirculation of HSCs through bone marrow, blood, and other tissues. Likewise, the induced mobilization of HSCs, stimulated by treatment with cytotoxic agents and/or cytokines, may occur via an amplification of normal HSC migration, either increasing HSC exit from the bone marrow or inhibiting HSC reentry into the tissues from the blood.

Yet the question remains: What is the physiological relevance of constitutive circulation of HSCs in adults? One possibility is that the capacity for migration, which is vital to the seeding of HSCs to appropriate hematopoietic organs in the developing fetus, is retained, somewhat by default, into adulthood. Alternatively, constant flux of adult HSCs may provide an immediate source of rapidly recruitable progenitor cells for initiating extramedullary hematopoiesis in case of catastrophic blood loss. Migration could also be a fundamental step in HSC development that is required to determine HSC cell fate decisions (i.e., differentiation), via the relocation of daughter HSCs to distinct marrow niches. Finally, as HSCs have, in some instances, been shown to contribute to the regeneration of chronically injured

nonhematopoietic tissues, circulating HSCs may represent a source of pluripotent cells in normal animals, which can be recruited for repair of damaged tissue under appropriate circumstances (see below).

Given the clinical significance of HSC migration in transplantation and in mobilization regimens, much research has focused on identifying the molecular mediators of this process. As in paradigms described for the migration of mature inflammatory cells involving the sequential action of tethering receptors, activating chemoattractants and strongly adhesive proteins (121, 122), HSC migration appears to invoke the function of particular cell adhesion and chemokine receptors. HSC homing to bone marrow likely begins by the initial tethering of cells to endothelium, which may involve the function of vascular selectins (E- and/or P-selectin) and/or the integrin very-late-adhesion molecule-4 (VLA-4, $\alpha 4\beta 1$) (123–125). Bone marrow endothelial cells constitutively express the VLA-4 ligand, vascular cell adhesion molecule-1 (VCAM-1), and E-selectin (126, 127), and irradiation enhances adhesion molecule expression by bone marrow endothelium (126).

Murine and human HSCs express the chemokine receptor CXCR4 (128, 129), and in *in vitro* chemotaxis assays, murine HSCs show selective responsiveness to the CXCR4 ligand SDF-1 α (129). Consistent with a role for SDF-1 α /CXCR4 interactions in HSC homing to bone marrow, engraftment of NOD/SCID mice by human HSCs is blocked by inhibitory antibodies to CXCR4 (130). SDF-1 is constitutively expressed by bone marrow endothelium (131, 132), and its expression in both the bone marrow and spleen increases following irradiation (133). Although mice deficient in either CXCR4 or SDF-1 α exhibit perinatal lethality (134–136), CXCR4-deficient HSCs appear to be capable of seeding the fetal liver, and CXCR4^{-/-} fetal liver HSCs can successfully engraft the bone marrow of irradiated recipients and undergo multilineage differentiation (59, 60). Interestingly, CXCR4-deficient hematopoietic progenitors are present at an increased frequency in the circulation of chimeric mice, perhaps indicating that these cells are not properly retained in the bone marrow in the absence of CXCR4/SDF-1 α interactions (60).

In addition to its role as a chemoattractant, SDF-1 α may also directly affect the proliferation and differentiation of primitive hematopoietic cells (137, 138) and may enhance the activity of adhesion receptors, particularly integrins, on both HSCs and progenitor cells (131, 139). In particular, SDF-1 α induces the function of VLA-4 and LFA-1 on HSC-enriched human cord-blood cells (139) and enhances their NOD/SCID repopulating ability following *in vitro* culture (140). SDF-1-mediated integrin activation may play an important role in converting rolling adhesion to firm arrest, thereby allowing extravasation of circulating HSCs into the bone marrow extravascular space (131). During normal development, colonization of fetal liver, bone marrow, and spleen by HSCs requires the expression and function of $\beta 1$ integrin, likely to direct the homing of yolk sac- or AGM-derived HSCs to these tissues (141, 142). In the adult transplantation setting, function-blocking antibodies against VLA-4, VLA-5 ($\alpha 5\beta 1$), or their shared subunit $\beta 1$ integrin prevent engraftment of NOD/SCID mice by human HSC-enriched CD34⁺ cord

blood, and inhibition of VLA-4 blocks bone marrow homing and engraftment by murine HSCs (139, 143, 144). However, the targeted deletion of either $\alpha 4$ or $\alpha 5$ integrin fails to block the localization of stem cells and early hematopoietic progenitor cells to the bone marrow (145), although fetal liver chimeras generated with $\alpha 4^{-/-}$ fetal liver cells show cell-autonomous defects in lympho- and myelopoiesis (146). Thus, combinatorial or compensatory functions of $\alpha\beta$ integrin heterodimers are likely involved in stem and progenitor cell homing from blood to bone marrow.

HSC and progenitor cell mobilization can be stimulated by systemic treatment with certain cytotoxic drugs (including cyclophosphamide (Cy), hydroxyurea (HU), and 5-fluorouracil (5-FU)) and/or cytokines (including G-CSF, GM-CSF, IL-11, IL-3, IL-8, SIF, Flt3L, and others), which substantially increase the frequency of HSCs and progenitor cells in the bloodstream (147). Induced HSC mobilization is often associated with increased HSC proliferation (148–150), and in some cases, HSC division may be required for mobilization (149). Interestingly, the same molecules that play a role in stem and progenitor cell homing to bone marrow have often been implicated in the pharmacological mobilization of these cells from the bone marrow. For example, following cyclophosphamide/G-CSF treatment of mice, expression of both VLA-2 ($\alpha 2\beta 1$) and VLA-4 is significantly reduced on mobilized peripheral blood (MPB) HSCs (144, 151). In addition in vivo administration of blocking antibodies against the integrin VLA-4 induces the mobilization of colony-forming cells (CFC), CFU-S, and long-term repopulating activity in both mice and primates (152–154). Conversely, blocking antibodies to the integrin LFA-1 appear to prevent progenitor cell mobilization induced by IL-8 (155). In addition, induced overexpression of SDF-1 α in the circulation mobilizes stem and progenitor cells (156), and antibodies against CXCR4 or SDF-1 α can block mobilization induced by G-CSF administration (132).

Regulated proteolysis has recently emerged as an important mediator of induced HSC egress from the bone marrow and of hematopoietic recovery following cytoreductive treatment. In vivo treatment of mice with various HSC-mobilizing agents including G-CSF, SIF, and cyclophosphamide correlates with an increase in neutrophil-associated proteolytic activity within the bone marrow (132, 157, 158). Neutrophil function had previously been implicated in induced HSC mobilization, as neutropenic mice are unable to mobilize hematopoietic progenitors in response to multiple agents (159, 160). Neutrophil-expressed proteases degrade bone marrow-expressed VCAM-1 and SDF-1 α in vitro, and reduced expression of these proteins in the bone marrow correlates well with increased progenitor frequency in the circulation (132, 157, 158). Furthermore, in vivo treatment of mice with an inhibitor of neutrophil elastase significantly inhibits G-CSF-induced progenitor cell mobilization and ameliorates the loss of SDF-1 α in the bone marrow (132). Similarly, proteolytic release of soluble SIF from membrane-bound SIF by the matrix metalloproteinase MMP-9 is induced following 5-FU, SDF-1 α , VEGF, or G-CSF administration and appears to be required for efficient mobilization of hematopoietic progenitor cells and for hematopoietic recovery following 5-FU-induced cytoreduction (150). These data indicate that, in addition to direct

effects on HSCs and progenitors, mobilizing stimuli also cause profound changes in the bone marrow microenvironment that ultimately influence HSC motility and function.

Hematopoietic Stem Cell Plasticity

The idea that the developmental potential of HSCs may not be limited to hematopoietic outcomes has emerged from several published reports indicating that cells derived from bone marrow are capable of giving rise to multiple “unexpected” cell types. These include neural cells (161–163), skeletal muscle (164–166), cardiac muscle (166–169), and hepatic cells (170–173), as well as epithelia of the gut, skin, lung, and kidney (174). Some investigators have suggested that “transdifferentiation” of bone marrow HSCs underlies these events; however, direct evidence supporting such claims is, for the most part, lacking. Most studies have assayed unpurified or partially purified cell populations, and almost none have performed analysis on single cells, a requirement for rigorous proof of multipotency. Bone marrow harbors both hematopoietic and mesenchymal stem cells, which give rise to multiple differentiated cell fates, and it is conceivable that additional tissue-specific stem cells also reside there. Furthermore, recent evidence suggests that apparently multipotent progenitor cells, which may contribute to nearly every tissue in the body, can be isolated from adult bone marrow (175), providing further impetus for future studies to employ only highly purified, well-characterized cell populations.

To try to clarify the true potential of HSCs, we have analyzed the progeny of single, rigorously purified and transplanted HSCs (16) and found little evidence to support the idea that these cells contribute significantly to the production of nonhematopoietic cells, at least in the steady state (i.e., in the absence of any acute or chronic tissue injury aside from the initial irradiation required for HSC engraftment). Our data argue against the hypothesis that bone marrow HSCs possess a robust, intrinsic capacity for the production of nonhematopoietic cell outcomes; however, we cannot rule out the potential of HSCs to be recruited into atypical functions in the face of selective pressure induced by tissue injury. Strong selective pressure may facilitate HSC-derived nonhematopoietic cell outcomes, whether as a result of transdifferentiation or cell fusion with endogenous progenitors, by rescuing host cells with donor-derived gene products. Spontaneous fusion *in vitro* of embryonic stem cells with bone marrow- or brain-derived cells, with subsequent acquisition of “stem cell” function in the hybrid cells, has recently been demonstrated (176, 177), but the possibility that such a mechanism may underlie transdifferentiation *in vivo* has yet to be addressed.

Gene Expression Profiling at the Population and Single Cell Levels

The hallmark of stem cells is their ability to balance self-renewal and differentiation. Whether the differentiation of HSCs through lineage-restricted progenitors

to mature effector cells occurs as the result of exogenous or intrinsic signals remains unclear, but in either case the molecular mechanisms of this process are reflected in the gene expression profiles of the stem and progenitor cells along the differentiation hierarchy. At each decision point, genes associated with the adopted pathway are upregulated, while the genes necessary for the lineage(s) not chosen are silenced. Insight into these mechanisms has been provided by studies examining the relative expression levels of known lineage-associated genes.

Early studies used multipotential hematopoietic cell lines to model HSC behavior. Factor-dependent cell Patterson mix cells (178) have been widely used for this purpose, as they are karyotypically FDCP normal, nonleukemogenic cells that respond to cytokines with appropriate lineage readout, i.e., granulocytes and monocytes, in response to G-CSF and GM-CSF; and erythrocytes in response to erythropoietin (EPO) (179). Further studies revealed that the EPO receptor (EPOR) and GATA-1 erythroid transcription factor were expressed at low levels (180, 181) and that hypersensitive sites in the EPOR promoter and β -globin control locus were present, indicating that the chromatin structure of these genes is in an active, open configuration in these cells prior to differentiation (182).

Single-cell RT-PCR analysis of individual FDCP mix cells and human CD34⁺ Lin⁻ progenitors revealed coexpression of erythroid and granulocyte/macrophage-specific genes (183). These results suggested that stem and progenitor cells are "primed" for multilineage differentiation by expressing low levels of lineage-affiliated genes. It has also been suggested that priming may likewise account for the expression of multiple lineage markers on some leukemic cells (184).

More precise analysis has been done with freshly isolated HSCs and lineage-committed progenitors, showing coexpression of lineage-affiliated genes in a single developing cell (185). Sixteen percent of single HSCs coexpressed erythroid-specific (β -globin and EPOR) and granulocyte/macrophage-specific [myeloperoxidase (MPO), granulocyte colony-stimulating factor receptor (G-CSFR)] genes, whereas 39% of single CMPs, the next lineal descendant of HSCs committed to the myeloid lineage (22), show a promiscuous gene expression. Importantly, the downstream progenitors of CMPs, GMP, and MEP only expressed lineage-appropriate transcripts, MPO/G-CSFR, and β -globin /EPOR, respectively. Similarly, 23% of single CLPs coexpressed both B cell-specific (λ 5 and/or Pax5) and T cell-restricted (CD3 or GATA-3) genes, whereas proB and proT cells only expressed T or B lineage-affiliated genes, respectively (22). Taken together these results reveal that low-level, promiscuous expression of lineage-specific genes precedes commitment to a particular lineage and may be requisite of multipotent progenitors. However, it is interesting to note that promiscuous lymphoid and myeloid gene expression has not been reported except in Pax5^{-/-} proB cells as discussed below (186). This may indicate that myeloid differentiation is the default developmental pathway and that the gene expression programs required for lymphoid development must be actively induced.

The reports described above used RT-PCR on few or, most informatively, on prospectively isolated single cells to examine the expression of known

lineage-specific genes. However, precise knowledge of the complete gene expression programs of the stem and progenitor populations is necessary to elucidate fully the molecular mechanisms associated with development and lineage decisions. Recent advances in microarray and genomic approaches have already begun to facilitate these studies and will likely provide global gene expression data. Lemischka and colleagues have used non-PCR-based subtracted libraries of fetal liver HSCs to identify thousands of genes selectively expressed in AA4.1⁺ fetal liver HSCs (187). Many of the transcripts identified correspond to expressed sequence tags (ESTs) that had not been previously characterized. Additionally, known genes not previously detected in HSCs were identified. For example, CD27, previously described in T cells, was identified as selectively expressed in both fetal liver and bone marrow HSCs and has subsequently been shown to be preferentially expressed on short-term repopulating HSCs (188). These studies as well as an additional microarray analysis of enriched short-term and LT-HSCs (189) revealed an overlapping set of genes, including studies of CD34, CD27, and *evi-1* that were preferentially expressed in HSCs in each of the studies. The comparison of gene expression of HSCs to the downstream lymphoid and myeloid progenitors (CLP, CMP, GMP, and MEP) reveals clusters of genes preferentially expressed in each compartment along the developmental hierarchy (A. V. Terskikh, T. Miyamoto, I.L. Weissman, unpublished data). These results nicely document the shifts in the gene expression programs that correlate with the different potentials associated with each of the progenitor populations. Terskikh et al. also showed an overlap in the genes expressed in hematopoietic and neural progenitors, supporting the notion that some genes responsible for stem cell properties, such as those enabling self-renewal, would be shared among stem cells from different somatic tissues (190).

The coordination of the silencing of some genes with the activation of others is the mechanism by which cells choose a differentiation pathway to the exclusion of others. The strength and weakness of these genomic approaches are the large number of genes identified by these methods. What remains to be established are the roles these gene candidates play in shifting the intricate balance that exists between self-renewal, pluripotency, and differentiation and discerning those genes that make the cell competent to receive/respond to differentiating signals and those genes that are deterministic for differentiation to a given lineage.

Many of these studies confirm the currently known model of the hierarchy of blood development by validating the expression of expected genes. In addition to having implications for the roles of the promiscuously expressed genes in the differentiation/commitment process, these studies raise questions as to the mechanisms of the priming itself in terms of the chromatin status of the loci. Additional information gained from stem- and progenitor-specific libraries and microarray analyses are likely to be more powerful in identifying the genes, novel or otherwise, that comprise the self-renewal and differentiation programs. These approaches will not readily reveal the role of genes that are not differentially transcribed as part of the developmental program but that are modified posttranslationally as part of the orchestrated changes.

Lineage Commitment and Plasticity

In general, the process of development from pluripotent progenitors to mature cells with specific functions involves the progressive loss of developmental potential to other lineages. Over the past several decades, researchers have exploited a variety of technological advances in biomedical research to elucidate the precise developmental steps in blood cell formation. From this data, a hierarchy is emerging in which each successive developmental stage loses the potential to become a specific cell type or class of cells (191). This stepwise developmental process has been considered linear in the sense that once a cell has made a developmental choice it cannot revert. We are still a long way from a complete understanding of the molecular basis for lineage commitment in hematopoiesis, but testable models for this complex process have emerged and are currently under investigation. In this section we detail a few specific experimental systems and significant results that provide insight into the mechanisms that control lineage commitment, thereby shedding light on the issue of lineage infidelity.

The earliest known lymphoid-restricted cell in adult mouse bone marrow is the common lymphocyte progenitor (CLP) (21), and the earliest known myeloid-restricted cell is the common myeloid progenitor (CMP) (22). Importantly, these cell populations possess an extremely high level of lineage fidelity in *in vitro* and *in vivo* developmental assays. The only "unexpected" developmental outcome observed to date from either of these progenitor populations is the infrequent generation of low numbers of B cells from CMPs *in vivo*. It is not known whether this result is due to lineage infidelity of CMPs or impurities (i.e., B cell progenitors) in the sorted CMP population. Regardless, the prospective isolation and developmental characterization of these progenitors support the linear model of hematopoiesis in which a cell that loses the potential to develop into a specific lineage never regains that potential. As it now stands, the earliest lineage-potential decision that a developing HSC/multipotent progenitor population must make is whether to become a lymphoid or myeloid cell type, and once it does, that decision is permanent.

Developmental hierarchies have been elucidated to a remarkable extent for both the T cell and B cell lineages with the number of developmental stages growing still. Unfortunately, this level of understanding has not yet been achieved in regard to NK cell development. To begin to explore the signals that promote NK cell development, CLPs genetically engineered to express the human IL-2 receptor β chain (hIL-2R β) were studied (192). This receptor was chosen because it had previously been demonstrated that IL-15 was indispensable for NK cell development, the receptor for which is composed of three chains: the IL-15R α chain, the common γ_c chain (γ_c), and the IL-2R β chain (193). The hypothesis was that CLPs stimulated through an ectopically expressed hIL-2R β chain (which couples with an endogenous γ_c) would preferentially attain a NK cell fate because this signal would mimic an IL-15R signal. Surprisingly, when hIL-2R β -expressing CLPs were stimulated with hIL-2 *in vitro* they developed into B cells, NK cells,

granulocytes, and macrophages. As mentioned above, myeloid cell development (i.e., granulocytes and macrophages) had never been observed in CLPs in previous experiments. What signal was IL-2 providing that led to this dramatic cell fate conversion? Further investigation determined that hIL-2R β signaling activated the expression of the granulocyte/macrophage colony-stimulating factor receptor (GM-CSFR), which could drive CLP development toward myeloid cell fates when activated. In follow-up experiments, ectopic expression and activation of the GM-CSFR could also induce lineage conversion in CLP, bypassing the need for hIL-2R β . Importantly, this IL-2R-induced lineage conversion effect could be recapitulated in proT cells from the thymus but not in proB cells (192).

What does this data suggest about the process of lineage commitment, at least to the lymphoid lineage? When considering this, it is important to note that in RT-PCR assays the GM-CSFR was detected in HSCs, the only known precursor to CLP (185). CLPs, however, never express the GM-CSFR under normal developmental conditions, whereas it is frequently expressed in CMPs (9, 192). Therefore the simplest model is one in which the first step in commitment to a specific lineage is downregulation of cell-surface receptors that drive development to alternate lineages. At the CLP/CMP lineage checkpoint, HSCs commit to the lymphoid lineage by shutting down expression of the GM-CSFR (and likely other genes), thereby preventing myeloid cell outcomes. Expression and stimulation through the GM-CSFR disturbs this program and can redirect CLPs to the myeloid lineage. Therefore, CLPs have not irreversibly committed to the lymphoid lineage but rather have taken only the first step in this process.

The question then becomes, at what point does a cell become fully committed to a specific lineage? One clue comes from the data mentioned above in which proB cells cannot be diverted from their lymphoid cell fate in response to hIL-2R β signaling, whereas proT cells can (192, 194). Therefore, proB cells must have developed beyond the next developmental hurdle and become stabilized in their lineage choice. What is this stabilizing factor(s)?

Fortunately, an ideal lineage-stabilizing factor candidate has been studied extensively: the transcription factor Pax-5. In a series of elegant studies, Pax-5 was not only shown to play a central role in the early development of the B cell lineage, but it also turned out to be required for stabilizing the commitment process itself (195). In Pax-5^{-/-} mice B cell development is arrested at the transition between the pro-BI and pro-BII stage (prior to V to DJ rearrangement at the IgH gene locus). Unlike their wild-type counterparts, Pax-5^{-/-} pro-BI cells also have the ability to grow extensively in vitro and develop into a number of distinct hematopoietic lineages including T cells, NK cells, and macrophages in various in vitro and in vivo assays (186). The incredible developmental plasticity of Pax-5^{-/-} pro-B cells can be attributed to the role of Pax-5 in silencing and activating specific genes during development. Relevant to this discussion, Pax-5 downregulates genes associated with the myeloid lineage, including c-fms and PD-1, and activates genes that promote B cell development including CD19 and Ig α (186). Therefore, without Pax-5 to orchestrate lineage-appropriate gene expression, pro-B cells from

Pax-5^{-/-} mice are not stabilized (or committed) in their lineage choice. Another important result regarding Pax-5 is that it can block hIL-2R β -induced myeloid differentiation when overexpressed in CLP but not in proT cells (A.G. King, M. Kondo & I.L. Weissman, unpublished observations). This suggests that there also exists a T lineage stabilization factor(s) yet to be identified (although there are candidates). It is not known what signal(s) is responsible for controlling subsequent lineage fate decisions downstream of CLP (i.e., between B, T, and NK cells), but research into this question is ongoing.

There has been some speculation as to whether the lineage infidelity occasionally observed in acute myeloid leukemia cells occurs during the normal course of hematopoiesis *in vivo*. Evidence for lineage conversions has come from experiments using genetically modified cells or transformed cell lines [reviewed in (196)], and thus has not answered the question of whether this process is common and significant *in vivo*. A more recent approach has utilized technology that allows one to permanently mark cells as soon as they express a specific developmental marker and then determine whether that cell later transdifferentiates into an alternative lineage. This technology relies on the lineage-specific expression of a recombinase (Cre or FLP) that when expressed removes an inhibitory element in a target gene that is then permanently turned on in that cell; GFP and β -galactosidase are common reporters that are used. If such a marked cell decides to switch lineages, it can easily be detected. These studies are no doubt informative, but it is important to keep in mind that multilineage gene expression (or priming) is common in progenitor cells, as mentioned above. If a progenitor has the capacity to develop into both lymphoid and myeloid lineages, it may express myeloid-specific genes prior to committing to the lymphoid lineage. For example, HSCs express the GM-CSFR prior to deciding whether to proceed down the lymphoid or myeloid lineage (185). If the GM-CSFR promoter was used to express the recombinase, it would be expressed in and mark as myeloid-committed an HSC that ultimately became a lymphoid cell. Therefore, one would have the false impression that a committed myeloid cell transdifferentiated to the lymphoid lineage. Although this caveat may not apply to all of these *in vivo* cell-marking strategies, it is an issue that must be factored into the interpretation of such data.

HUMAN HEMATOPOIETIC STEM CELLS AND PROGENITORS

Dilemmas of Experimental Systems

Identification and characterization of human hematopoietic stem and progenitor cells have been impaired by the lack of optimal assay systems. As in the mouse, short-term *in vitro* assays are sufficient to demonstrate clonal myelo-erythroid, B-, NK-, and dendritic cell but not T cell, read out. However, competitive *in vivo* repopulating assays used in mice to demonstrate sustained self-renewing and multipotent differentiation capacity of HSCs, as well as T cell development from

HSCs and committed progenitors, cannot be performed in humans for ethical reasons. Therefore, surrogate assays have been developed.

In various forms of the long-term culture-initiating cell assay (LTC-IC), candidate cells are primarily cultured for 5–10 weeks on adherent, bone marrow–derived stromal cells that presumably resemble a bone marrow–like microenvironment (197, 198). In a second step cells are transferred into semisolid medium containing cytokines. Cells of the primary culture that retain their proliferative capacity, the LTC-IC, will generate myelo-erythroid or B cell colonies (199–201). This assay is very useful to define primitive or primarily quiescent progenitors and provides proof of self-renewal capacity or multilineage differentiation potential.

To study human hematopoiesis in *in vivo* models, two essential prerequisites need to be met: The host should not eliminate the xenograft via an immune reaction and should provide a permissive microenvironment for engraftment and multilineage differentiation of donor cells. Spontaneously occurring immunodeficient mouse strains partially meet these criteria and have been modified to improve their model function. Early studies were done in SCID mice that display a T and B cell defect (202, 203) and beige/nude/xid (bnx) mice that display an NK, T, and B cell defect (204). However, both mice strains can mediate rejection of xenografts owing to macrophages and residual NK cells. Therefore, SCID mice were backcrossed to nonobese diabetic (NOD) mice that display partially deficient NK cell, antigen-presenting cell, and macrophage functions (205). To improve the microenvironment for human cells and consecutively, proliferation and multilineage read out, SCID mice were transplanted with human fetal bone, thymus, liver, or lymph nodes (SCID-hu model) (203, 206), and recipient mice were injected with recombinant human cytokines (204, 207) or were genetically modified to produce human cytokines (208). Owing to good engraftment capacity (10–20 times better than SCID), easy handling, ready availability, and economic aspects, the NOD-SCID mouse is currently used by most groups studying human hematopoiesis in *in vivo* models, and engrafting cells are termed SCID repopulating cells (SRCs) (92, 209–211). However, SCID mice have a high radiation sensitivity, human hematopoiesis in the NOD-SCID model shows a bias towards B-cell development, T cell development is rare, and engraftment can only be monitored for about 6 months owing to the limited life-span of these mice. To address some of these deficiencies additional mouse models such as NOD-SCID $\beta 2$ microglobulin knockout mice (212, 213), NOD-RAG1 knockout mice (214), NOD/SCID/ γc triple-mutant mice (214a), and RAG2/common cytokine receptor γ chain (γc) double-mutant mice (215, 216) have been developed. The specific utilities of these mouse strains still need to be determined.

As an alternative to the murine xenotransplantation models, Zanjani et al. established a large animal transplantation model in which human hematopoietic stem cells are transplanted intraperitoneally into unconditioned, early gestational sheep fetuses (217–219). In this model low numbers of selected progenitors can engraft, and myelo-erythroid as well as T and B lymphoid read out can be monitored over several years.

Isolation of Candidate Human Hematopoietic Stem Cells

Since the generation of monoclonal antibodies against the sialomucin CD34 almost two decades ago (220), the CD34 antigen has become the major positive marker for human hematopoietic stem and progenitor cells. Among nonhematopoietic tissues, CD34 is expressed on endothelial cells of small vessels and is a ligand for L-selectin (CD62L) (200, 221). The biological function of CD34 on hematopoietic cells is poorly understood: Expression of human CD34 in mouse hematopoietic cells suggests a role of CD34 in adhesion to the stromal microenvironment (222), and CD34 mutant mice show reduced colony-forming activity in bone marrow; however, mutant mice keep up normal peripheral blood counts and respond to hematopoietic stress as well as wild-type mice, showing that CD34 is not essential for hematopoiesis (223). Of hematopoietic cells in human fetal liver, cord blood, and bone marrow, 0.5–5% express CD34 (220, 224). CD34⁺ cells harbor virtually all *in vitro* clonogenic potential (220, 224, 225) (see also Table 1); however, the CD34⁺ population is heterogeneous. Only a small fraction (1–10%) of CD34⁺ cells that do not express mature lineage markers (Lin⁻, as CD3, CD4, CD8, CD19, CD20, CD56, CD11b, CD14, and CD15) and CD38 (226) contains single cells with *in vitro* bilineage, lymphoid (B/NK) and myeloid differentiation potential (227–230) (Table 1). The majority of CD34⁺ cells (90–99%) coexpress the CD38 antigen, and this subset contains most of the lineage-restricted progenitors (discussed below). CD34⁺CD38⁻ cells and not CD34⁺CD38⁺ cells are highly enriched for LTC-IC (201, 231) and contain SCID-hu-repopulating (227) and NOD-SCID-repopulating cells (209, 210), with some of them able to read out even in secondary NOD-SCID transplants (211, 232). However, the Lin⁻CD34⁺CD38⁻ cell fraction is still very heterogeneous with regard to surface marker expression and biological functions.

Single Lin⁻CD34⁺Thy-1⁺ cells and not Lin⁻CD34⁺Thy-1⁻ cells generate B/myeloid progeny in culture and produce B/myeloid progeny in SCID-hu mice transplanted with 10⁴ sorted cells (227). Also, Lin⁻CD34⁺Thy-1⁺ cells and only few if any Lin⁻CD34⁺Thy-1⁻ cells generate T cells in SCID-hu thymi (227). Although not evaluated in this study, virtually all Lin⁻CD34⁺Thy-1⁺ cells reside in the CD38⁻ fraction. The highest LTC-IC activity (63%), NOD-SCID (SRC 1/5) and fetal sheep repopulating ability, resides in a CD34⁺KDR (VEGFR2)⁺ fraction (0.1–0.5% of CD34⁺ cells) in cord blood, bone marrow, and G-CSF-mobilized peripheral blood (211). CD34⁺KDR⁺ cells but not CD34⁺KDR⁻ cells generated myeloid, T, B, and NK cells in mice and myeloid/T cells in primary and secondary fetal sheep transplants. These results show a >100 × enrichment of SRC in CD34⁺KDR⁺ cells compared with CD34⁺CD38⁻ cells. Only ~30% of CD34⁺KDR⁺ cells are CD38⁻, suggesting that depletion of CD38⁺ cells would further increase SRC purity. With this high purification of potential human HSCs, it will now be essential to do critical clonal *in vivo* experiments, as has been done for mouse HSC (18).

An alternative approach to assess clonal read-out is to mark putative HSCs genetically with retroviral vectors that randomly and permanently integrate into

TABLE 1 Surface marker expression on candidate human hematopoietic stem and progenitor cells

Tissue	Surface marker	% frequency	Assay system	Read-out	Citation
Candidate HSC					
FBM	lin ⁻ CD34 ⁺ Thy-1 ⁺	0.05-0.1 ^a	SCID-hu	T/B/myeloid	(227)
FBM	CD34 ⁺ CD38 ⁻ HLA-DR ⁺		Long-term culture	Clonal B/myeloid	(228)
CB	CD34 ⁺ CD38 ^{lo} CD10 ⁻ CD19 ⁻		Seq. colony formation	Clonal B/myeloid	(255)
CB	CD34 ⁺ CD38 ⁻ CD33 ⁻ CD10 ⁻	0.01 ^a	Liquid culture	Clonal B/myeloid	(229)
BM	lin ⁻ CD34 ⁺ CD38 ⁻		Seq. Colony formation	Clonal B/NK/myeloid	(230)
CB/BM	lin ⁻ CD34 ⁺ CD38 ⁻		NOD-SCID	B/myeloid, SRC (1/10 ⁶)	(209, 210)
CB	lin ⁻ CD34 ⁻ CD38 ⁻		NOD-SCID	B/T/myeloid, SRC (1/10 ⁸)	(245)
BM	lin ⁻ CD34 ⁻		Fetal sheep	T/myeloid, primary+ secondary host	(244)
CB/BM/MPB	lin ⁻ CD34 ⁺ KDR ⁺	0.1-0.5 ^b	LTC-IC	Frequency 63%	(211)
NOD-SCID	B/NK/T/myeloid,	SRC(1/5)			
CB	lin ⁻ CD34 ⁺ CD38 ⁻ CD133 ⁺	88 ^c	Fetal sheep	T/myeloid, primary+ secondary host	(254)
CB	lin ⁻ CD34 ⁻ CD38 ⁻ CD133 ⁺	0.2 ^d	NOD-SCID	B/myeloid	(254)
CB	lin ⁻ CD34 ⁺ , retrovirally marked		Pre-culture, NOD-SCID	Clonal B/myeloid SRC Long/short-term SRC	(232)
Candidate lymphoid progenitors					
FBM/BM	lin ⁻ CD34 ⁺ CD38 ⁺ CD10 ⁺	0.09 ^a	Stroma cell culture	Clonal B/NK/DC, no myeloid T cells	(263)
CB	CD45RA ⁺ Thy-1 ⁻ HLA-DR ⁺		SCID-hu		
CB	CD34 ⁺ CD38 ⁻ CD7 ⁺ IL-7R α ⁻	8 ^c	Stroma cell culture	Clonal B/NK/DC, no myeloid	(264)
	CD45RA ⁺ Thy-1 ⁻ HLA-DR ⁺				

(Continued)

TABLE 1 (Continued)

Tissue	Surface marker	% frequency	Assay system	Read-out	Citation
Candidate myeloid progenitors BM	CD34 ⁺ CD45RO ⁻		Methylcellulose, LTC-IC	CFU-GM enriched*	(273)
	CD34 ⁺ CD45RO ⁺		Methylcellulose, LTC-IC	LTC-IC/BFU-E enriched*	
	CD34 ⁺ CD45RA ⁺		Methylcellulose	CFU-GM enriched*	(274)
CB/BM/PB	CD34 ⁺ CD45RA ⁻		Methylcellulose	CFU-GEMM/BFU-E enriched*	
Fetal BM/BM	CD34 ⁺ CD64 ⁻ M-CSFR ^{hi}		Liq. cult./methylcell.	CFU-G/M/GM no E/Mix*	(275, 276)
	CD34 ⁺ CD64 ⁺ M-CSFR ^{hi}		Liq. cult./methylcell.	CFU-M enriched no E/Mix*	
	CD34 ⁺ CD64 ⁻ M-CSFR ^{lo}		Liq. cult./methylcell.	CFU-G enriched no E/Mix*	
FL/CB/MPB	CD34 ⁺ IL-3R ^{lo}	60–80 ^b	Methylcellulose	All myeloid colonies*	(277)
	CD34 ⁺ IL-3R ⁺	11–17 ^b	Methylcellulose	CFU-GM enriched*	
	CD34 ⁺ IL-3R ⁻	7–20 ^b	Methylcellulose	BFU-E enriched*	
CB/BM	CD34 ⁺ Fli3 ⁺	60–90 ^b	Methylcellulose	CFU-GEMM/GM enriched*	(278)
	CD34 ⁺ Fli3 ⁻	10–40 ^b	Methylcellulose	BFU-E enriched*	
CB	CD34 ⁺ CCR1 ⁺	80 ^b	Methylcellulose	CFU-GM enriched*	(279)
	CD34 ⁺ CCR1 ⁻	20 ^b	Methylcellulose	BFU-E enriched*	
BM/CB	lin ⁻ CD34 ⁺ CD38 ⁺ CD45RA ⁻ IL-3R ^{lo}	0.28 ^a	Methylcellulose	All myeloid colonies	(271)
	lin ⁻ CD34 ⁺ CD38 ⁺ CD45RA ⁺ IL-3R ^{lo}	0.35 ^a	NOD-SCID	All myeloid progeny	
	lin ⁻ CD34 ⁺ CD38 ⁺ CD45RA ⁻ IL-3R ⁻	0.13 ^a	Methylcellulose	CFU-G/M/GM restricted	
			NOD-SCID	BFU-E/CFU-Meg restricted	
			No B/NK progeny detected	Erythroid progeny	

Percentage of frequency of progenitors: in ^aMNCs, ^bCD34⁺ cells, ^cCD34⁺CD38⁻ cells, ^dlin⁻CD34⁺CD38⁻ cells.

*No lymphoid read-out tested.

FL, fetal liver; FBM, fetal bone marrow; CB, cord blood; BM, bone marrow; PB, peripheral blood; MPB, mobilized peripheral blood; SRC, SCID repopulating cells.

the host genome, allowing the follow-up of single-cell progeny (233, 234). A disadvantage of this method is that viral integration requires cycling of target cells, which could induce commitment and loss of HSC capacities. Using this approach, Guenecha et al. showed for the first time that single-marked Lin⁻CD34⁺ clones transplanted into NOD-SCID animals together with other Lin⁻CD34⁺ cells generated multilineage B and myeloid progeny (232). This study also suggests that short-term (<3 months) and LT- (≥3 months) HSCs contribute to SRCs, and it shows that in retroviral-marked SRC exhaustion occurs in secondary transplant recipients. The use of lentiviral vectors might avoid these limitations imposed by retroviral marking.

In autologous and allogeneic hematopoietic cell transplantation enrichment/purification of HSCs and depletion of immunologically reactive cells or tumor cells in the graft could benefit the patient as discussed below. Selected hematopoietic transplants are being evaluated in humans because it was shown that human CD34⁺ hematopoietic cells contain all colony formation and LTC-IC activity and reconstitute xenotransplantation models as mouse and sheep; it was also shown that baboons successfully transplanted with human CD34⁺ cells (235), CD34⁺, and CD34⁺Thy-1⁺. Indeed, multiple trials in the autologous and allogeneic setting show that CD34⁺ and CD34⁺Thy-1⁺ cells can successfully reconstitute and maintain hematopoiesis (236–242).

Although it was known that at least some murine HSCs are CD34^{-/lo} (18, 243), the finding that human Lin⁻CD34⁻CD38⁻ cells contain SRC and fetal-sheep repopulating cells at very low levels (1 CD34⁻ SCR in 10⁸ cord blood mononuclear cells (MNCs) compared with 1 CD34⁺ SRC in 10⁶ cord blood MNCs) (19, 244, 245) came as a surprise to most basic and clinical researchers. Whereas Lin⁻CD34⁻CD38⁻ cells contain no or very rare CFC, they give rise to CD34⁺ cells in vivo and in vitro and subsequently are able to generate CFU (244–247). This suggests that Lin⁻CD34⁻CD38⁻ cells, which were formerly missed owing to their lacking direct in vitro activity, might be upstream of CD34⁺ cells in the hematopoietic hierarchy, leading to concern that CD34⁺ transplanted patients might not receive essential CD34⁻ HSCs and could experience late graft failures. By showing that CD34 expression on mouse HSCs is reversible, Ogawa's group recently added valuable data to this issue: Normal HSCs of young mice are mostly CD34⁺, whereas adult mouse HSCs (>10 weeks) are mostly CD34⁻ but acquire CD34 expression upon "activation" through G-CSF mobilization or 5-FU treatment. Finally, transplanted CD34⁺ HSCs can revert to a CD34⁻ phenotype that upon secondary transfer displays full HSC potential [(248–250); reviewed in (251)]. If the murine CD34⁺ expression on HSCs model CD34 expression on human HSCs, cord blood up to teenage HSCs and G-CSF as well as chemotherapy-mobilized HSCs should be mostly CD34⁺. Only untreated adult bone marrow HSCs would be mostly CD34⁻.

However, Okuno et al. suggested that human and mouse HSCs differentially regulate the CD34 gene (252): Artificial expression of the entire human CD34 genomic locus in mice revealed that HSCs of 10-week-old mice were murine CD34⁻ but expressed human CD34.

If CD34⁺ and CD34⁻ human HSCs exist, is there any other marker that would positively identify both? CD133, a recently described glycoprotein, might meet this criterion. CD133 is expressed in all CD34⁺CD38⁻ and on some CD38⁺ progenitors (253). Also, 0.2% of Lin⁻CD34⁻CD38⁻ cells express CD133, and these are the only cells within the Lin⁻CD34⁻CD38⁻ fraction with SRC potential (254). Therefore, it was suggested that this CD34⁺ and CD34⁻ “unifying” antigen might be a better target for HSC enrichment than CD34 (254). To clarify this issue, further preclinical studies are needed. All or most clinical trials that have tested CD34⁺ enrichment methods in an autologous or allogeneic transplantation setting used chemotherapy and/or cytokine-mobilized CD34⁺ cells and according to both mouse models should harbor most of the HSCs. Indeed, to date, no higher incidence of late graft failure has been reported.

Identification of Human Early Lineage Committed Progenitors

The existence of clonal lymphoid- and myeloid-committed progenitors that harbor all lymphoid and myeloid potential, respectively, has long been proposed and was finally shown in the mouse model (21, 22). This also suggests that HSCs or multipotent progenitors do not commit directly to monospecific lymphoid or myeloid progenitor cells. Although good assays for myeloid development are available, combined T, NK, and B cell read-out from single or low numbers of cells remains a major unresolved challenge. Because early lymphoid and myeloid commitment is discussed in the mouse section of this review, we focus here on studies that addressed clonal read-out of proposed progenitors in human. All described bipotent lympho-myeloid progenitors reside in the CD34⁺CD38⁻ fraction (see above) (227–230, 255). To segregate those from the earliest lymphoid or myeloid progenitors, additional candidate antigens need to be identified. Based on combined phenotypic and functional analysis of fetal liver and fetal and adult bone marrow, terminal deoxynucleotidyl transferase (TdT), CD7, CD10, and IL-7R α are such candidate antigens (256–262). So far, the closest definition of a potential human CLP population showed that fetal and adult bone marrow Lin⁻CD34⁺CD38⁺CD10⁺ cells generate no myeloid progeny and contain clonal progenitors of B, NK, and dendritic cells (263). Also, these cells as a population give rise to T cells in the SCID-hu thymus assay (263) (see Table 1). In another similar rigorous study it was shown that cord blood CD34⁺CD38⁻CD7⁺ cells contain ~40% clonal B, NK, and dendritic cell precursors (264). However, T cell read-out was not evaluated. Because the described CD7⁺ progenitors reside in the CD38⁻ fraction and ~30% of them are CD10⁻, it was suggested, but not formally proven, that these cells are developmentally upstream of the CD34⁺CD38⁺CD10⁺ cells (264).

In mice genetic disruptions of IL-7 or the IL-7R α chain demonstrate nonredundant, essential functions of this cytokine/cytokine receptor pair for T and B cell development (265), IL-7R α expression distinguishes the earliest lymphoid committed cells (21), and in vivo IL-7 administration might improve T, B, and NK

cell reconstitution after murine allogeneic bone marrow transplantation (266). In humans, however, IL-7 signaling is not essential for normal B cell development because *in vitro* B cell development is possible without IL-7 (267), and IL-7R disruption *in vivo* causes T cell but not consistently B cell deficiencies [268, 269; reviewed in (270)]. It would be of interest to know whether human early lymphoid progenitors express IL-7R α and depend on IL-7 signaling. Lin⁻CD34⁺IL-7R α ⁺CD19⁻Pax-5⁺ bone marrow cells are reported to contain cells with high B-lymphoid developmental capacity, but clonal and multilymphoid potential was not tested (260). In contrast, CD34⁺CD38⁻CD7⁺ lymphoid progenitors are reported to be cell surface IL-7R α ⁻, IL-7R α transcripts were only occasionally detected, and Pax-5 was not detectable by PCR (264). Although no data about IL-7R α and Pax-5 expression was reported in the original publication of the lymphoid-committed Lin⁻CD34⁺CD38⁺CD10⁺ cells, we now know that this population contains cells that express IL-7R α and Pax-5 as assessed by RT-PCR (271). Furthermore, we can subdivide the Lin⁻CD34⁺CD38⁺CD10⁺ cells into a CD10⁺IL-7R α ⁻ and a CD10^{lo}IL-7R α ⁺ fraction, with the latter being highly enriched in clonal B-cell progenitors (M.G. Manz & I.L. Weissman, unpublished observations). It shall be important to determine whether CD10^{lo}IL-7R α ⁺ cells, but possibly not CD10⁺IL-7R α ⁻ cells, contain both T and B cell progenitors and whether thymus seeding cells share some of these phenotypes [for review of human early thymocyte development see (272)].

As in the case of lymphoid progenitors, multiple studies report on surface marker-associated enrichment of myeloid-colony forming cells (273–279) (see Table 1). Collectively this data shows that CD34⁺ cells that are either CD45RO⁻, CD45RA⁺, CD64⁺, IL-3R α ⁺, flt3⁺, or CCR1⁺ are enriched for CFU-GM-forming cells, whereas CD34⁺ cells that are either CD45RO⁺, IL-3R α ⁻, flt3⁻, or CCR1⁻ are enriched for erythroid-colony forming cells (see Table 1). When reported, cloning efficacy ranged between 10–35% (274) and 26–50% (276). No data on alternative lymphoid potential are available from these studies.

In an attempt to identify human early myeloid commitment as precisely as in murine bone marrow (22), we identified three cell populations that are likely counterparts of the murine CMPs, GMPs, and MEPs (271). These cells are CD34⁺CD38⁺, they are negative for multiple mature lineage markers including early lymphoid markers such as CD7, CD10, and IL-7R α , and they are further distinguished by the markers CD45RA, an isoform of CD45 that can negatively regulate at least some classes of cytokine receptor signaling (280), and IL-3R α , a cytokine receptor that when activated supports proliferation and differentiation of primitive progenitors (96, 281, 282). CD45RA⁻IL-3R α ^{lo} (CMPs), CD45RA⁺IL-3R α ^{lo} (GMPs), and CD45RA⁻IL-3R α ⁻ (MEPs), display cloning efficacies of 84%, 75%, and 87%, respectively, and show no *in vitro* B or NK cell read-out. Importantly, CD45RA⁻IL-3R α ^{lo} cells give rise to GMPs and MEPs and at least one third generate both GM and MegE colonies on a single-cell level. By focusing on a limited number of hematopoiesis-associated genes, we found expression profiles generally consistent with the distinct *in vitro* read-out capacities of the different progenitors. This

data, documenting for the first time myeloid progenitor purification and placement in a developmental hierarchy, is largely in line with previous reports on enrichment of myeloid progenitor fractions (Table 1).

Clinical Implications of Hematopoietic Stem Cells

The current common clinical application of hematopoietic cell transplantation (HCT) is for patients with malignancies, bone marrow failure states, and immunodeficiencies. Two types of transplantations are performed—autologous and allogeneic. Patients who receive autologous grafts have an underlying malignancy that is either at high risk of relapse or has already failed following standard chemotherapy. Thus, the therapeutic principle behind an autologous transplantation is that significantly increased doses of radiation and/or chemotherapy can be delivered to the patients to achieve maximal tumor kill, with the dose-limiting toxicity being death of the hematopoietic organ, and the patients are rescued by the HCT. Dose escalation studies have been performed to determine the maximum tolerated doses that result in ablation of malignancies and host hematopoiesis without conferring untoward toxicities to the other organ systems (283). Standard clinical practice is to use mobilized peripheral blood (MPB), which is collected from patients following cytoreductive cycles of chemotherapy to minimize the tumor burden.

Mobilization schemes generally involve the administration of cyclophosphamide in conjunction with G-CSF or with G-CSF alone. The MPB product is collected by apheresis and stored frozen. Most transplant centers do not manipulate the autologous product; thus the potential exists for the MPB product to be contaminated with tumor cells. Whether or not these tumor cells contribute to relapse in patients that fail autologous transplantation remains controversial. Often individuals who fail autologous transplantation relapse in the sites of their original disease, suggesting that the lack of efficacy for those individuals was owing to insufficient ablation of the tumor by the preparative regimen rather than reinfusion of tumor cells. With the advent of devices that allow selection of hematopoietic stem and progenitor cells using the CD34⁺ marker, it is becoming more prevalent to perform CD34⁺ cell selection attempts to obtain grafts that are reduced in tumor cell burden. A more stringent method for purifying human HSCs that can result in significantly greater purging of tumor cells was described above and utilizes the same approach taken to isolate HSCs from mouse bone marrow. Human HSCs are enriched by magnetic bead selection for CD34⁺ cells and are isolated by fluorescence-activated cell sorting for CD34⁺ Thy-1⁺ cells (227).

Three clinical trials have been performed using HSCs from MPB that were isolated by CD34⁺Thy-1⁺ selection: The first cohorts were patients with widely metastatic (stage IV) breast cancer (241), the second, patients with multiple myeloma (240), and the third, patients with subsets of non-Hodgkin's lymphoma (284). The goals of these studies were to determine if adequate numbers of CD34⁺Thy-1⁺ HSCs could be collected and purified from patients with these different malignancies to yield rapid and sustained hematopoietic cell engraftment, to assess the tumor contamination in the MPB product, and to test for

treatment-related toxicities. The amount of CD34⁺Thy-1⁺ cells collected was based upon mouse studies (285) that indicated $>2-4 \times 10^5$ HSCs (Thy-1^{lo}Lin^{-lo}Sca-1⁺c-Kit⁺) per kg recipient body cells would be sufficient for rapid engraftment. In all three studies it was possible to collect and purify adequate numbers of CD34⁺Thy-1⁺ cells. The median time to obtain absolute neutrophil counts $>500/\text{mm}^3$ was between 10 and 12 days, and the best results were seen if doses $>8 \times 10^5$ CD34⁺Thy-1⁺ cells/kg were infused. Significant reduction in tumor contamination was observed in MPB products that showed evidence of tumor cells prior to the HSC isolation. In the breast cancer studies 37% of MPB samples had contaminating cytokeratin⁺ cells, whereas none of the purified products had evidence of contaminating breast cancer cells as determined by an assay sensitive to one in one million cells (241). In the lymphoma studies tumor contamination was reduced between 3 and 6 logs (284), and in the myeloma studies the reduction was between 2 and 4 logs (240).

The clinical outcome was most impressive in the stage IV breast cancer patients, who with a median follow-up time of 1.4 years still had a survival rate of $\sim 60\%$ (241). A recent reanalysis of data from these stage IV breast cancer patients from one institution (Stanford University) demonstrated that with a median follow-up time of >4 years the results continue to look promising. These data are particularly impressive given a prior stage IV breast cancer study (286) that demonstrated a median event free survival time of 9.6 months for patients treated with high-dose chemotherapy plus autologous MPB rescue as compared with 9.0 months for patients who received conventional dose chemotherapy. In this study $>90\%$ of the patients died by 2 years posttransplantation. Taken together, these three clinical studies demonstrated that it is possible to obtain sufficient numbers of CD34⁺Thy-1⁺ cells with significant reduction in tumor burden and achieve rapid and sustained hematopoietic cell engraftment.

Allogeneic Hematopoietic Stem Cell Transplants

The therapeutic concept for allogeneic HCT differs from that of autologous HCT because hematopoietic cells obtained from an appropriate HLA-matched donor not only can rescue patients who undergo myeloablative radiation combined or not with chemotherapy, but the allogeneic graft also can confer an effect that has been termed graft-versus-tumor (287–289). Unmanipulated allogeneic bone marrow contains $\sim 10^7$ CD3⁺ cells per kilogram of recipient weight, whereas MPB contains one log greater (10^8) T cells per kilogram. The significance of the T cell content contained within a graft is multifaceted. One of the major complications of an allogeneic transplantation using an unmodified hematopoietic graft is graft-versus-host disease (GVHD) (290–292). GVHD is caused by mature T cells that recognize host antigens as foreign and mount an immune attack against the host organs. For this reason all allogeneic transplantations are placed on systemic post-transplant immunosuppressive therapy. Attempts to reduce the morbidity and mortality of GVHD by depletion of T cells from the hematopoietic grafts resulted in reduced GVHD, but other untoward complications were noted. In clinical trials

T cell depletion of bone marrow resulted in significantly increased incidences of graft failure (293, 294). Furthermore, it has been observed that immune reconstitution as well as loss or reduction of graft-versus-tumor activity is increased in recipients of T cell-depleted grafts. Thus, the current clinical standard is to transplant either allogeneic bone marrow or MPB into recipients with the knowledge that the mortality owing to GVHD and related complications is ~15–20% in HLA-matched sibling transplants and even greater in other more genetically disparate transplants.

Studies were performed in mice to resolve the problem of resistance to engraftment of purified allogeneic HSCs (295). These studies showed that there are at least three ways to achieve successful allogeneic HSC engraftment: (a) Escalation of the numbers of HSCs transplanted allows engraftment across most genetic disparities. Doses in excess of 30–60-fold the amount required to only rescue lethally irradiated mice across congenic barriers are often needed to rescue mice transplanted across major and minor histocompatibility complex barriers, although the doses of fully allogeneic HSCs required for rapid (<12 days) engraftment are only two-fold higher than the doses of syngeneic HSCs required for rapid engraftment (285). It is important to note that at even the highest dose of allogeneic HSCs no GVHD resulted, as there were no contaminating T cells. (b) A second approach is to treat recipients with antibodies directed against immune cell subsets in addition to the radiation conditioning. When major histocompatibility complex (MHC) differences exist between donor and recipient mice, the addition of antibodies directed against NK cells and others against residual T cells markedly reduces the barrier (295–298). It will be important to compare these mouse studies with human studies that use antithymocyte or anti-T/NK cell antibodies or lymphoablative drugs such as fludarabine. (c) Studies in mice comparing transplantation of purified HSC transplantation versus unmodified bone marrow demonstrated that bone marrow contains a non-HSC population that can facilitate the engraftment of allogeneic HSCs (299–301). It was shown that resistance to engraftment of purified HSCs could be overcome by cotransplantation of candidate-facilitating populations with HSCs (299–301). An extensive phenotype analysis was performed on mouse bone marrow and demonstrated that one of the salient features of the HSC facilitating population was expression of the CD8⁺ molecule (299). Furthermore, within the CD8⁺ population heterogeneity of morphology as well as expression of the $\alpha\beta$ T cell receptor was noted. These data suggest that conventional CD8⁺ T cells and another CD8⁺ cell type confer facilitating activity. At the doses of CD8⁺ facilitating-cell cotransplanted, there was no evidence of GVHD. It will be important to determine if a homologous human population exists that might be used to augment engraftment of purified HSCs.

Hematopoietic Stem Cell Transplantation for Induction of Tolerance to Autoantigens and Alloantigens

In addition to the applications of HCT for the treatment of malignancies there is extensive data in the experimental literature showing that HCT can be used to

induce tolerance to solid organ grafts and to treat severe autoimmune diseases. The classic studies of Billingham et al. (302) first demonstrated that infusion of allogeneic hematopoietic cells into newborn mice allowed permanent acceptance of donor-matched solid organs in these recipients when they reached adulthood. More pertinent to these studies, Main & Prehn (5) found that mice surviving bone marrow transplants after lethal irradiation were specifically, permanently tolerant of donor-strain skin grafts without further immunosuppression. However, the clinical use of simultaneous solid organ and HCT has been prevented by the complications associated with the HCT procedure, primarily GVHD. Because HSC grafts are devoid of mature immune cells and are themselves immunologically naive, such grafts will not cause GVHD. Studies were performed to formally test if purified allogeneic HSCs such as bone marrow grafts could induce tolerance to donor-matched heart grafts (303, 304). HSC engraftment into MHC-mismatched recipients permitted long-term survival of donor-matched neonatal heart grafts, whereas third-party grafts were rejected. One proposed mechanism by which the HSC grafts induced tolerance was by altering negative T cell selection. These studies further suggested that, contrary to the conventional view that positive T cell selection is mediated by radio-resistant host elements, donor hematopoietic elements dictate positive T cell selection in HSC-chimeric mice when MHC restriction is tested by an *in vivo* assay (304). The clinical implication of these studies was that purified HSCs can induce organ transplantation tolerance without the possibility of causing GVHD.

The other relatively unexplored clinical application for HCT is in the treatment of severe autoimmune diseases such as type 1 diabetes, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus. Case reports in the clinical literature demonstrate that patients with malignancies and a preexisting autoimmune disease achieved cure of both maladies following allogeneic HCT (305–311). Although extensive research has been done in animal models, most of these studies have examined the efficacy of MHC-mismatched bone marrow transplants in blocking autoimmune pathogenesis, a donor/recipient combination that is not easily translatable to clinical practice. However, in a series of studies in rodents that had either an induced form of multiple sclerosis (experimental allergic encephalomyelitis) or experimentally induced arthritis it was observed that affected rodents appeared to benefit from high dose therapy and rescue with syngeneic hematopoietic cells (312–315). Given the higher risks associated with the allogeneic versus autologous HCT procedure, and the possibility that autologous HCT may provide benefit, the current focus of all of the HCT-related human trials for autoimmune disease use the autologous HCT approach.

In theory, autologous HCT can alter autoimmune pathogenesis by the combined effect of the conditioning regimen that may eradicate the repertoire of autoreactive cells and the replacement of the immune system with grafts that contain no or limited numbers of mature immune cells. Indeed, studies in patients with severe systemic lupus erythematosus who received high-dose cyclophosphamide and rescue with CD34-selected autologous cells have shown promising results (316).

However, whether or not long-lasting remissions will be achieved requires further follow-up. It should also be noted that CD34 selection results in an ~ 3 -log depletion of T cells from MBP grafts; thus, such grafts contain $\sim 10^5$ CD3⁺ cells/kg. CD34⁺Thy-1⁺ cells contain far fewer CD3⁺ cells, on the order of 10^1 – 10^2 CD3⁺ cells/kg, and thus may be considered a superior graft source for autoimmune patients.

Congenic Versus Allogeneic Hematopoietic Stem Cell Transplantation for Treating Autoimmune Diabetes

To address the question of whether or not transplantation of purified congenic versus allogeneic HSCs could block the pathogenesis of autoimmune diabetes in nonobese diabetic (NOD) mice we performed comparative transplantation studies (316a). NOD mice develop spontaneous autoimmune diabetes, and the pathology is characterized by a T cell-mediated lymphocytic infiltration of their pancreatic islets beginning at ~ 4 weeks of age. The cell infiltration progresses over the course of several months, resulting in overt hyperglycemia at ~ 6 months of age. For the congenic HSC transplantation studies, the HSC source was NOD.Thy-1.1 mice. These mice have the Thy-1.1 allele bred onto the NOD background (317) and like conventional NOD mice still develop hyperglycemia at a high frequency. This congenic difference allowed both the isolation of HSCs based on the Thy-1.1 molecule and assessment of hematopoietic cell engraftment. Successfully engrafted mice demonstrate Thy-1.1 cells in the peripheral blood, and the Thy-1.2 allele is expressed by conventional NOD strain mice. Adult NOD recipients underwent transplantation with lethal radiation and infusion with congenic NOD-Thy1.1 HSCs at an age at which their islets were already inflamed. Despite this lympho- and myeloablative radiation conditioning and rescue with purified congenic HSCs, most of the transplanted NOD (78%) mice succumbed to diabetes with a short delay when compared with unmanipulated control NOD (316a) (Figure 2). These mice had evidence of persistent circulating Thy-1.2 recipient T cells. Thus, we tested to see if T cells that survived lethal irradiation could cause diabetes after transplantation by utilizing HSCs isolated from NOD.SCID mice. NOD.SCID mice do not produce mature T or B lymphocytes; thus, HSC grafts from such donors could not contribute pathogenic cells. Hence, if NOD.SCID HSC-transplanted mice developed diabetes, the disease process could only be mediated by the remaining host T cells. NOD.SCID HSC recipients developed diabetes even though their peripheral T cell levels were still fourfold reduced compared with unmanipulated mice at the time of diabetes onset (316a) (Figure 2).

The effect of allogeneic HSC transplantation was studied in the same NOD mouse model. Adult NOD mice were conditioned with lethal radiation plus antibodies against NK and CD4 cells. These antibodies were added to the preparative regimen because it was previously demonstrated that radiation plus antibody targeting of these cell subsets, but not radiation alone, permitted engraftment of MHC-mismatched HSCs in NOD mice (295). Transplantation of purified MHC-mismatched HSCs resulted in 100% disease protection (Figure 2). This

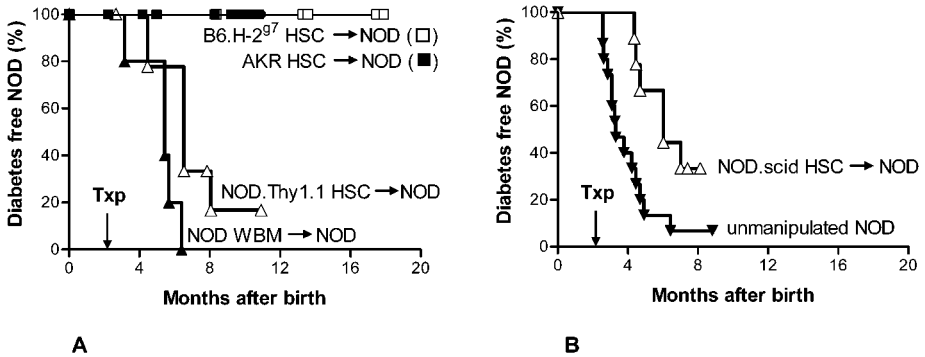


Figure 2 Diabetes-free survival after congenic or allogeneic hematopoietic stem cell (HSC) transplantation. (A) Prediabetic NOD mice (8 weeks) received a pretreatment of α -ASGM1 and α -GK1.5 antibodies plus lethal irradiation and either allogeneic AKR ($n = 13$) or B6.H-2^{g7} ($n = 6$) HSCs or received congenic NOD.Thy1.1 ($n = 9$) HSCs or syngeneic NOD whole bone marrow (WBM) ($n = 5$). Allogeneic HSC transplantation was protective, whereas NOD mice receiving NOD WBM or NOD.Thy1.1 HSCs succumbed to autoimmune diabetes. (B) To test whether the remaining host T cells could cause diabetes onset, NOD.scid HSCs (which do not give rise to B and T cells) were transplanted into lethally irradiated prediabetic NOD mice ($n = 9$). Six of nine recipients developed disease. This emphasizes that the remaining host T cells can cause diabetes even when present in low numbers at time of disease onset. Incidence of diabetes in unmanipulated female NOD mice ($n = 15$) is 93%. Copyright © 2003 American Diabetes Association From Diabetes, Vol. 52, 2003 Reprinted with permission from *The American Diabetes Association*.

disruption of the autoimmune process was not due to the additional antibody pretreatment because control NOD mice that received syngeneic bone marrow all succumbed to disease. Because the one gene associated with diabetes susceptibility in NOD mice is a class II MHC molecule, it was possible that diabetes pathogenesis was abrogated, as the transplanted HSCs gave rise to cell populations expressing the different donor MHC class II molecule. MHC class II molecules mediate T cell selection and antigen presentation, and thus these elements could be altered by donor cells, thereby interrupting autoreactivity. A more clinically relevant study in which donors were matched at the MHC but different in other background genes was then performed. This model resembles human-matched unrelated donor transplantation. Again, none of the MHC-matched HSC-engrafted NOD mice developed diabetes (Figure 2) despite the fact that recipients still had evidence of significant levels of persistent NOD T cells ($\sim 16\%$) in their peripheral blood following transplantation (316a). These studies show that the donor hematopoietic graft does not necessarily need to express a different class II MHC molecule in order to successfully block autoreactivity.

In summary, these studies of HSC transplantation for the treatment of spontaneously arising autoimmune disease demonstrated that even highly purified

congenic HSCs (analogous to autologous transplantation) could not effectively block disease pathogenesis. Thus, one should be aware that the genetic predilections for autoimmune diseases including autoimmune T cell-mediated diseases may not have been eliminated by autologous transplants. In contrast, allogeneic HSCs—either MHC-mismatched or more importantly, MHC-matched—prevented progression of the autoimmune process. Thus, we favor an approach of allogeneic HCT for the treatment of severe autoimmune diseases. The morbidity of clinical allogeneic HCT has been dramatically reduced by the emergence of nonmyeloablative transplantation regimens (318–320). The future will surely include the use of nonmyeloablative allogeneic HSC transplantations for the induction of immune tolerance to autoantigens and solid organs.

CONCLUDING REMARKS

Regenerative medicine is a new field in the life sciences, which can be applicable to many diseases that have no effective treatment right now. Knowledge of stem cell biology forms the fundamental basis of regenerative medicine and allows us to develop new therapeutic methods. Although remarkable advances have been made in HSC biology in the past 10 years, the number of unsolved fundamental questions is considerable. Regulation of self-renewal activity of HSCs, for example, is a major issue that must be clarified, not only because of scientific interest but also to increase the number of patients who may be effectively treated. Further accumulation of basic knowledge regarding the biology of HSCs and their downstream progenitors will continue to provide a solid base for understanding stem cell biology.

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ERRATA

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