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Limiting Factors in Murine Hematopoietic Stem Cell Assays

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Hematopoiesis arguably provides the most well-defined role of stem cells in tissue development, maintenance, and repair, largely because of the experimental methods developed over decades of investigation. Assays of hematopoietic stem and progenitor cell potential were developed in the late 1950s–1960s with the first reports of in vivo transplantation into lethally irradiated recipients (Ford et al., 1956; McCulloch and Till, 1960) and clonal growth of hematopoietic bone marrow cells in vitro (Bradley and Metcalf, 1966). These two major assays have undergone substantial refinement but remain the foundation for defining hematopoietic stem cell biology. Here, we provide a brief overview of methods commonly used to analyze hematopoietic stem and progenitor cell content in mice, discuss the limitations of these assays, and provide an in-depth review of the limiting dilution assay (Szilvassy et al., 1990), the best single assay for quantitating HSC content.

Introduction

All blood cells are derived from hematopoietic stem cells (HSCs), which are multipotent cells that under permissive conditions are capable of either self-renewal (hence forming another HSC) or differentiation into all blood cell lineages. Hematopoiesis is now among the best-defined differentiation cascades in mammalian tissues due to the ease of access and morphologic distinctiveness of many of its members. The advent of antibody technology, culture capability, transplantation, and finally genetic engineering of mice has enabled a detailed understanding of processes involved in the commitment and differentiation of HSCs into progenitor cells and ultimately mature blood cells. Furthermore, combining antibody-based subselection of cells with transplantation has made possible the identification of progenitors and HSCs. A range of different methods for characterizing stem and progenitor cell populations are now available for use in hematopoietic cell research. A review and commentary of these methods seems fitting for a new journal in the stem cell field. This review will focus on techniques applied to murine hematopoiesis, with a particular emphasis on analyzing HSC content in mutant mice or in non-steady-state conditions.

Surrogate Short-Term In Vivo and In Vitro Assays for Detecting HSCs and Their Progeny

Often the first steps many investigators take toward analyzing hematopoietic cell content in mice involve short-term assays. These studies provide much quicker results than the long-term repopulating HSC assay, providing data on either the day of analysis or within a couple of weeks of initiating experiments. Such studies can be useful screens to determine whether it is worthwhile performing long-term repopulating HSC assays, which not only require patience due to the length of time required to monitor the mice posttransplant but require significant numbers of experimental animals and are consequently costly. The caveat to using the majority of these short-term studies is that, while informative, these assays do not measure HSCs and may not provide information that corresponds to the quality or quantity of HSCs in the sample studied. Examples of routinely used surrogate methods for screening HSCs and progenitor cells follow.

Immunophenotypical Analysis of HSC/Progenitors

In the last 20 years, a number of different methods whereby HSCs and progenitor cells can be identified have emerged (Goldschneider et al., 1978; Spangrude et al., 1988; Visser and Bol, 1982; Visser et al., 1981). All rely on fluorescence-activated cell sorting (FACS)-based methods. The purity of the populations of HSCs achieved using these methods has increased within recent years, such that approximately 50%–96% of single cells in certain purified populations can give rise to long-term reconstitution after transplantation (Matsuzaki et al., 2004; Yilmaz et al., 2006). The most commonly used FACS-purified populations of HSC/progenitor cells include the following:

Thy1.1[lo], Lin− Sca-1+ Cells

The Weissman laboratory has been a driving force in defining methods for the purification of HSCs by FACS, providing the first evidence in 1988 that short-term repopulating HSCs (as opposed to FACS methods isolating CFU-S [Goldschneider et al., 1978; Visser and Bol, 1982]) could be isolated based on their expression of stem cell antigen-1 (Sca-1), low expression of Thy1.1, and lack of expression of lineage markers (Spangrude et al., 1988). The same population was shown to contain long-term repopulating HSCs in a subsequent study (Uchida and Weissman, 1992). These Thy1.1[lo], lineage-negative (lin−)

Sca-1+ cells are still used in studies by different research groups internationally. The population was further purified by the expression of the stem cell factor receptor, c-*Kit*, in 1992 (Ikuta and Weissman, 1992). However, there are limitations of using this population in research laboratories: Thy1.1 is expressed by the C57BL/Ka-Thy1.1 mice, but not C57BL/6 mice, which are the congenic strain of mice most commonly used for HSC transplantation studies worldwide (Spangrude and Brooks, 1992). Furthermore, the Thy1.1 counterpart in C57BL/6 mice (Thy1.2) does not have the same pattern of expression on HSCs as does Thy1.1 (Spangrude and Brooks, 1992).

Lin− c-Kit+ Sca-1+ Cells
The expression of c-*Kit* on hematopoietic progenitor cells was first described by Ogawa et al. in 1991 (Ogawa et al., 1991). In 1992, Suda's laboratory reported the isolation of HSCs from C57BL/6 mice in the lin− c-*Kit*+, Sca-1+ (LKS+) population, verifying that a similar protocol to that described by Weissman's lab (Ikuta and Weissman, 1992) did enrich for HSCs in this congenic mouse strain (Okada et al., 1992). However, this population is very heterogeneous and consists predominantly of progenitor cells with less than 10% of it representing HSCs. More recently, the expression of CD34 (Osawa et al., 1996) and Flt3 (CD135) (Adolfsson et al., 2001; Yang et al., 2005) has been used to further purify long-term repopulating HSCs (LKS+ CD34− Flt3−) from short-term repopulating HSCs (LKS+ CD34+ Flt3−) and multipotent progenitors (LKS+ CD34+ Flt3+) (Yang et al., 2005).

Common problems associated with these methods include the restriction of the use of Sca-1 to certain strains of mice (Spangrude and Brooks, 1993) and discrepancies between the expression of these antigens and their functional potential in older mice, in HSCc in different stages of the cell cycle, post-5-FU or G-CSF treatment and post-transplantation (Morrison et al., 1996; Spangrude et al., 1995; Walkley et al., 2005b). Moreover, the expression of CD34 can depend upon the developmental stage of the mouse, as CD34 is expressed on HSCs of mice younger than 8 weeks (Matsuoka et al., 2001). Reliability of these markers is also questionable in mutant mice, as the phenotype of HSCs in such mice has been shown to not always correlate with the numbers or quality of HSCs when they are measured using more stringent functional studies (Purton et al., 2006; Walkley et al., 2005a, 2007b). Caution should therefore be used when relying on these markers to assay HSC content in non-steady-state or mutant mouse models.

Fluorescent Dyes Capitalizing on High Drug Efflux Properties of HSCs: Rhodamine 123, Hoescht 33342, and the Side Population
Two different vital dyes, the mitochondrial-binding dye rhodamine 123 (Rh123) and DNA-binding dye Hoescht 33342 (Ho 33342), have also been used either alone or in combination to isolate HSCs (Goodell et al., 1996; Li and Johnson, 1992; McAlister et al., 1990; Phillips et al., 1992; Wolf et al., 1993). Both of these dyes are retained at very low intensity in HSCs due to high efflux of the dyes from HSCs, as shown by studies utilizing the drug

verapamil. This drug blocks the activity of the ATP-binding cassette (ABC) transporter superfamily (membrane pumps that efflux drugs from cells and are highly expressed in HSCs) and prevents both of these dyes from being effluxed, resulting in the disappearance of the low-retaining Rh123 and Ho 33342 populations (Goodell et al., 1996; Phillips et al., 1992).

A more common method used in laboratories today to isolate HSCs is the Ho 33342 side population (SP) (Goodell et al., 1996). The SP cells are visualized by emitting Ho 33342 at two wavelengths simultaneously, resulting in a distinct "tail" profile, which disappears with verapamil treatment (Goodell et al., 1996). The major problem with this method is that it is highly sensitive to slight modifications in staining techniques, hence leading to discrepancies in the quality of the HSC populations obtained using this method in different laboratories (Lin and Goodell, 2006). Furthermore, different batches of Ho 33342 have been reported to vary significantly in their ability to accurately delineate the SP population; hence they need to be screened to select a suitable batch for experiments (Matsuzaki et al., 2004). The SP population is also not as pure as HSCs enriched by other methods such as LKS+ CD34− Flt3− cells, although it can be used in combination with other markers such as Sca-1, c-*Kit*, and CD34 to further purify HSCs with extreme efficiency (Lin and Goodell, 2006; Matsuzaki et al., 2004). Notably, cells with an SP profile have also been detected in many other organs but with inconsistent functional correlation with stem cell-like functions (Challen and Little, 2006). This method should not be assumed to yield stem cells in other tissue types or even in species other than the mouse.

SLAM Family Members
SLAM proteins are a family of cell surface glycoproteins in the immunoglobulin superfamily with specific SLAM antigens (CD150+ CD244− CD48− cells) identified as useful to purify a population of which approximately 50% of single cells reconstituted lethally irradiated animals (Kiel et al., 2005). Unlike the limitations presented by those isolated using Thy1.1 or Sca-1 expression, the SLAM receptors appear to be expressed by many mouse strains (Kiel et al., 2005), and more faithfully detect HSCs in older, mobilized, or transplanted mice (Yilmaz et al., 2006). The SLAM method for isolating HSCs is yet to be tested in mutant mice to prove this population accurately detects HSCs in such mice. Regardless, the functional potential of HSCs and progenitors cannot be measured merely based on surface expression markers; hence, where possible, investigators should not rely solely on immunophenotypical analysis to measure HSC content in mice.

In Vitro and Short-Term In Vivo Assays for Detecting Functional Potential of HSCs and Progenitors
In vitro and short-term in vivo assays are also often used for measuring HSC and progenitor cell content but are generally a reflection of the more mature progenitor cells as opposed to the immature HSCs.

Colony-Forming Cell Assays

The colony-forming cell (CFC) assays measure progenitor cells in a given population using semisolid agar- or, more commonly, well-defined methylcellulose-based culture media, which are commercially available. The majority of CFCs consist of lineage-restricted colonies: erythroid-restricted burst-forming units-erythroid (BFU-E), which are more immature than the colony-forming units-erythroid (CFU-E); megakaryocyte-restricted CFU-Mk; colony-forming units-granulocytes (CFU-G), colony-forming units-monocytes/macrophages (CFU-M); and colony-forming units-granulocytes/macrophages (CFU-GM). The most immature (multipotent) CFC measurable contains granulocytes, erythrocytes, macrophages, and often megakaryocytes (CFU-GEMM) and is usually measured at day 12 after culture initiation. This CFC is also often called CFU-mixed, as it may not always contain megakaryocytes but does contain erythroid and granulocyte/macrophage cells. B and T lymphocyte in vitro CFC potential are more difficult to assess, usually requiring specialized coculture systems (Schmitt and Zuniga-Pflucker, 2002; Whitlock and Witte, 1982), and hence are not routinely used, although there are now commercially available methylcellulose-based colony assays to measure pre-B cells. While informative about the progenitor cell content of a population of interest, the CFCs do not measure HSCs.

Cobblestone Area-Forming Cells/Long-Term Culture-Initiating Cells

The cobblestone area-forming cells (CAFC) and long-term culture-initiating cell (LTC-IC) assays are coculture systems that some investigators use to predict HSC frequencies. They are described in more detail in a recent review by van Os et al. (2004). The reliability of the use of these assays to measure HSCs remains controversial in the field, though it can fairly be said that they reflect a more immature or primitive population than the CFC. Another issue with these assays is the interlaboratory variability often observed due to varying feeder layers and specific culture conditions. They may be useful, however, in limiting dilution format for quantitating primitive cells when other features such as homing capacity or other functions required for in vivo engraftment may compromise the reliability of transplant assays.

Short-Term In Vivo Assays

Colony-forming unit-spleen (CFU-S) cells are cells that, once injected into an irradiated recipient, home to the spleen and form macroscopic colonies that provide very short-term (usually 1–3 weeks) in vivo repopulation of the mouse (Till and McCulloch, 1961). The CFU-S are therefore early engrafting cells, providing radioprotection to the mouse and allowing it to survive more readily in the first 2–3 weeks posttransplantation when pancytopenia usually occurs. These progenitors are more immature than CFCs but are more mature than HSCs.

Investigators should therefore be cautious about their conclusions regarding HSC content from studies that rely on the surrogate assays outlined above. The gold standard for measuring HSCs is the long-term repopulating assay.

In Vivo Assays to Measure HSC Numbers and Their Functional Potential

There are various types of long-term repopulating assays, the most common of which is the competitive repopulation assay (Harrison, 1980). This assay measures the functional potential of the unknown source of HSCs against a set known number of HSCs (usually whole bone marrow cells from congenic wild-type mice). In brief, the number of repopulating units (RU) in the donor cell population (source of unknown HSC content being measured) can be determined by the following formula: donor RU = % donor cells × C/(100 − % donor cells), where C = the number of competing RU and 1×10^5 whole bone marrow cells = 1 competing RU (Harrison et al., 1993; Yuan et al., 2005). While providing information about the function of HSCs in their capacity to repopulate compared to the competing bone marrow, this study provides qualitative or at best semiquantitative information about the HSCs within a given population—it cannot distinguish between the number of HSCs or their quality (progeny produced per HSC). Furthermore, caution should be used when designing competitive repopulation assays, as it has been shown that the reliability of this assay is critically dependent on the numbers of HSCs present in the populations being assessed: when too few or too many HSCs (recipients of $<1 \times 10^5$ or $>2 \times 10^7$ bone marrow cells each from donor and competing sources) are present, the data may not be meaningful (Harrison et al., 1993).

The frequency of HSCs (from which the number of HSCs can be calculated) is commonly measured using the limiting dilution assay, which is a variation of the competitive repopulation assay. In this assay, a series of dilutions of the unknown source (donor "test" cells) are competed against a set number of competing bone marrow cells. The number of mice negative for reconstitution in each cell dose is then measured, and the frequency of HSCs (competitive repopulating units, CRU) is estimated using Poisson statistics (Szilvassy et al., 1989, 1990; Taswell, 1981). The success of this assay therefore relies on the survival of the transplanted mice. To achieve statistical significance, it is best to have at least eight recipient mice per cell dose, with a minimum of three (but preferably four or more) cell doses (Szilvassy et al., 1990). Note that CRU, which measures the quantity of HSCs, is distinct from RU, which measures the functional quality of HSCs. To aid in data analysis of limiting dilution assays, StemCell Technologies Inc. have developed a program, L-Calc, the software for which is free to download from their website. This program calculates the frequency of HSCs within the test cell population, provides the 95% confidence intervals for these calculations, and can also be used to perform statistical comparison between two or more test samples.

As will be discussed further below, there are two major types of limiting dilution assay-based CRU assays routinely used today to measure HSCs in studies performed by numerous investigators. The major difference between these assays is the number of HSCs present in the competing cell source. The first, termed the CRU

assay (Szilvassy et al., 1990), uses a minimal number of HSCs as the competing source of cells. This allows for the optimal detection of HSCs at the single level but does not allow for the quality of the HSCs being measured to be compared to a known standard of wild-type HSCs. The second assay, also generally termed a CRU assay, which can create confusion, incorporates a standard, sufficient number of HSCs in the competing cell population. The test cells are therefore measured relative to their ability to effectively compete against this standard number of HSCs: if the test cells have fewer or more HSCs than wild-type control donors, this will generally be reflected in the results of the assay. The major limitation of this CRU assay is that it may not accurately detect HSCs at the single cell level.

The most stringent test of HSC potential is the serial transplant assay. The HSC compartment has been shown to be heterogeneous, comprising a hierarchy of HSCs that can be identified by their functional capacity. The most immature HSC in this hierarchy is capable of sustaining hematopoiesis throughout serial transplantation (Lemischka et al., 1986b; Purton et al., 2006; Rosendaal et al., 1979). Hence, in this assay, the source of HSCs is transplanted into sequential serial transplant recipients, and the ability of this population to sustain hematopoiesis by presumptive self-renewing divisions is determined. Limitations to this assay are its dependence upon homing and engraftment processes that may be perturbed without altering stem cell function per se, in particular mutant mouse strains. Therefore, serial dilution is most useful where homing and engraftment are not affected. Duration of engraftment, a process that over prolonged intervals requires stem cell self-renewal, may be considered an alternative measure of stem cell function in contexts where homing or engraftment may be altered.

The limiting dilution assay utilizing bone marrow cells containing a sufficient, standard number of HSCs (such as 2×10^5 bone marrow cells) can be used to incorporate all three different types of long-term repopulating HSC assays. If the competing cells are whole bone marrow cells, the numbers of RUs can be measured for each of the test cell doses injected. Furthermore, secondary transplants can be performed after the primary transplant has been monitored long-term. There are, however, some important factors critical to the analysis of the data in limiting dilution assays, which, if not standard across laboratories, can lead to discrepancies in the results obtained. These include the source of competing cells, the source of donor cells, the percent cutoff used to determine the frequencies of negative mice, and the time posttransplant at which the data are analyzed. We discuss these below.

Important Factors to Consider in Limiting Dilution Assays
Competing Cells
The source of competing cells is a variability that can lead to different results between laboratories. Initially, Szilvassy et al. used compromised bone marrow as the competing source for the CRU transplants (Szilvassy et al., 1990). This population was obtained by serially transplanting bone marrow into lethally irradiated recipients for two successive transplants (Szilvassy et al., 1990). Problems associated with this method were the cost and time required to obtain the compromised bone marrow, and the possibility of variability between different populations of compromised bone marrow. In addition, this population of competing cells was depleted of HSCs and hence did not provide a good source of known HSC content for competitive reconstitution.

The use of compromised bone marrow for competing cells was subsequently replaced by the use of W^{41}/W^{41} mice as recipient mice (Miller and Eaves, 1997; Trevisan et al., 1996). The hematopoietic cells (including HSCs) of W^{41}/W^{41} mice have a mutated c-Kit gene, resulting in partial impairment of their c-Kit receptor kinase activity (Nocka et al., 1990). The hematopoietic cells of these mice therefore do not have a normal response to the c-Kit ligand, stem cell factor, which is produced by the bone marrow microenvironment and so exhibit reduced numbers of HSCs (Geissler and Russell, 1983; Reith et al., 1990). CRU studies performed by Trevisan et al. (1996) demonstrated that sublethally irradiated W^{41}/W^{41} mice provide a more sensitive host environment whereby small numbers of HSCs could be reliably detected without the need for additional support marrow to be provided to the recipients.

More recently, investigators have used whole bone marrow cells from mice that have not been previously transplanted for their competing cells, which is a method that is more reproducible between laboratories and allows for a direct comparison of the quality and quantity of HSCs, such as when comparing HSC number and function in mutant versus wild-type mice. In our laboratories, we have found that 2×10^5 competing bone marrow cells have provided a good standard of competing marrow for limiting dilution assay studies (Janzen et al., 2006; Purton et al., 2006; Walkley et al., 2005a). This finding has also been independently confirmed in studies from at least three other laboratories (Ema et al., 2005; Lawrence et al., 2005; Liang et al., 2007). In each of these studies, the data obtained were a good fit to the Poisson distribution and independently determined that the number of long-term multilineage repopulating HSCs per 1×10^5 wild-type bone marrow cells was approximately three to four, a finding also consistent with that reported by others using various means (Abkowitz et al., 2000).

Test Cells, Unknown HSC Potential
As for the competing cells, the source of test cells is also an important factor to consider when designing a limiting dilution assay. An example is single LKS+ CD34− cells, which were previously transplanted together with LKS+ CD34+ cells, a population enriched for short-term repopulating HSCs but devoid of long-term repopulating HSCs (Osawa et al., 1996). More recently Ema et al. transplanted single LKS+ CD34− cells together with 2×10^5 competitor bone marrow cells and assessed their multilineage competitive reconstituting ability (Ema et al., 2005, 2006). In each of two different experiments, they reported that 24

of 96 or 48 of 135 transplanted mice showed multilineage reconstitution at 16 or 12 weeks posttransplant, respectively. These values equate to 75% or 64% of mice in each group being negative for engraftment, which is outside the range of the 37% negative mice value required to calculate one HSC using Poisson statistics (Szilvassy et al., 1990). It is therefore possible that the sufficient numbers of HSCs within 2×10^5 competing whole bone marrow cells could mask the repopulating potential of the single test HSCs coinjected. In this instance, the use of the W^{41}/W^{41} recipient-based CRU may provide a more sensitive means of HSC detection.

Furthermore, while single cell transplants are important when testing the ability of new strategies to purify HSCs, the use of immunophenotypical methods to isolate HSCs for comparative studies may not provide accurate results when assessing HSC content in mutant mouse models (or HSCs that might not otherwise be in a steady state condition), as discussed earlier in this review. Single cell studies also require very large numbers of recipient mice for accurate results (>100 for highly purified HSC sources, even more for HSC populations that are not as pure)—such expensive studies are often therefore not feasible in smaller laboratories that have restricted funding. A recent study of extensive single cell transplants performed in combination with serial transplantation has revealed a previously unappreciated, stable heterogeneity within the HSC-containing population (Dykstra et. al., 2007). An excellent overview of single HSC transplants, including detailed methods, has been previously reported by Ema et al. (2006).

In our experience, the best source of donor cells in HSC studies of adult mutant or non-steady-state mice is whole bone marrow cells. This avoids any problems associated with potential discrepancy between HSC phenotype and function and allows an easy way to enumerate the numbers of CRU within a set amount of bone marrow, e.g., CRU per femur. In our experiments, each recipient mouse receives cell doses ranging from 8×10^3 to 2×10^6 whole bone marrow cells from our "unknown" source of HSCs together with 2×10^5 competing congenic bone marrow (Janzen et al., 2006; Purton et al., 2006; Walkley et al., 2005a). We have found these cell doses sufficient to detect both reductions (Purton et al., 2006) and increases (Janzen et al., 2006; Walkley et al., 2005a) in HSC numbers in different mutant mice.

Donor Cell Percentage Used to Determine the Number of Negative Mice

The limiting dilution assay is based on Poisson statistics: instead of relying on the functional quality of the test source, the assay relies on the frequency of mice that are considered to have negative reconstitution by the test source. The original report by Szilvassy et al. (1990) transplanted male test cells together with female competing cells into female recipients, relying on detection of the Y chromosome by Southern blot analysis. Using this procedure, they could reliably detect 5% donor (test) reconstitution; hence, negative reconstitution was defined as being <5% test-derived cells (Szilvassy et al., 1990).

This method is less sensitive than that now obtained by FACS using congenic transplants, which are now more routinely used for these assays. It is now more typical to analyze limiting dilution assays with negative reconstitution being <1% test cell reconstitution. Some researchers have even assessed limiting dilution assays with <0.1% test cell reconstitution being considered to be negative; however, the accuracy of reliably detecting such low donor cell reconstitution remains questionable. In all situations, the test cells should repopulate all lineages, best assessed by determining the % test-derived cells contributing to B lymphopoiesis, T lymphopoiesis, and myeloid (granulocytes and macrophages) cell reconstitution in the peripheral blood. Peripheral blood erythroid cell reconstitution cannot be measured using the congenic system, as mature erythrocytes do not express CD45; however, it is generally accepted that if the other lineages are repopulated by the test source, the erythroid lineage will be as well.

Time Posttransplant at which the Results Are Analyzed

Another critical contributor to the correct analysis of HSCs via the limiting dilution assay (which also applies to any in vivo HSC repopulating assay) is the time point at which the assays are analyzed. Yang et al. (2005) recently demonstrated that as few as 50 LKS+ CD34+ Flt3− short-term repopulating HSCs competed against 2×10^5 congenic bone marrow cells could reconstitute multiple lineages (>0.01% myeloid, >1% B and T lymphoid) for at least 16 weeks posttransplant. Furthermore, 50 multipotent progenitor cells (LKS+ CD34+ Flt3+) could competitively reconstitute T (>0.01%) and B (>0.1%) lymphopoiesis for 16 weeks, with some myeloid reconstitution also detectable at 16 weeks (Yang et al., 2005). These are important observations, as currently many investigators tend to report results of long-term HSC assays that are analyzed at or less than 16 weeks posttransplant. It also raises the question of whether or not these cells are also detected in the W^{41}/W^{41} recipient-based CRU assay, as these studies have not yet been done.

In addition, an elegant study by Jordan and Lemischka (1990) analyzed the fate of individual oncoretroviral-marked HSCs and assessed their contribution to multilineage hematopoiesis posttransplantation. The results demonstrated that there were frequent fluctuations in clonal contribution to hematopoiesis for the first 4–6 months posttransplant, after which a few multipotent clones gave rise to stable hematopoiesis (6–12 months posttransplant). These studies suggest that 6 months posttransplant should be the optimal time point at which long-term hematopoiesis should be assessed in these assays. Recent studies by Dykstra and colleagues demonstrate that single cell transplants of putative stem cells can show gradual decline in reconstituting capacity up until 16 weeks, after which time the contribution was stable, while other subsets exhibit the opposite reconstitution kinetics (Dykstra et al., 2007). Therefore, a minimum of 16 weeks is required and an optimal 6 months is suggested for monitoring long-term reconstitution posttransplant.

Other Factors to Consider in Limiting Dilution Assays

Cultured Cells

The limiting dilution assay (and other primary competitive repopulation assays) may not reliably detect HSC content in cultured cell populations. It is now well-recognized that there is a hierarchy of HSCs based on their functional repopulating capacity (Dykstra et al., 2007; Lemischka et al., 1986a; Purton et al., 2006; Rosendaal et al., 1979; Yang et al., 2005). Cultured HSCs may represent a range of HSC potential, depending on whether the culture conditions have increased the numbers of immature HSCs or expanded their progeny, which may have better competitive repopulating potential in recipient mice. Hence, in instances in which cultured cells are used, serial transplant studies should be performed to confirm the conclusions of the primary transplant data, such as shown by Zhang et al. (2006).

Homing to the Bone Marrow Niche

Limiting dilution assays also do not account for the homing capacity of the HSCs. Hence, data assessing HSC content where HSCs have impaired homing capacity, such as calcium sensing receptor null HSCs (Adams et al., 2006) and HSCs deficient for the Rho GTPase, Rac1 (Cancelas et al., 2005), may be misleading. In such instances, conditional mutant mice would be valuable to study, as deletion of the gene can occur after the HSCs have been transplanted, hence minimizing any effect of impaired homing capacity (Cancelas et al., 2005).

Impaired Differentiation of the HSC into a Hematopoietic Lineage

Another assumption made when utilizing the limiting dilution assay is that the HSCs have normal differentiation potential into all lineages. Arrested or impaired differentiation into one or more of the lineages (B lymphocytes, T lymphocytes, granulocytes/macrophages) could markedly impact the results of the limiting dilution. For example, the differentiation of megakaryocytes and lymphocytes is severely impaired in conditional AML-1 mice (Ichikawa et al., 2004). Competitive transplants of bone marrow from these mice revealed normal contribution to myelopoiesis at 12 weeks posttransplant but no reconstitution of T or B lymphopoiesis (Ichikawa et al., 2004). Additional studies of the transplanted mice revealed that the AML-1-deficient bone marrow showed significant competitive repopulation of the double negative 2 (DN2) fraction of immature thymocytes in these mice, but there was a significant impairment in the contribution of AML-1-deficient cells to the more mature DN3 thymocytes in these mice (Ichikawa et al., 2004). In other situations in which this may occur, investigators can determine lineage contribution of the HSCs to all of the other lineages aside from the affected lineage(s), in addition to assessing repopulation of immature progenitor cell content such as in the studies of Ichikawa et al. described above (Ichikawa et al., 2004). This may help to determine the correct frequency of HSCs within the population. Potential difficulties do arise if there is a block in myeloid cell production, as both B and T lymphocytes have long life spans; hence, the limiting dilution assay may not accurately measure HSCs in primary recipients in such a scenario. In such instances, secondary transplants, together with other in vivo and in vitro studies investigating progenitor cell content as discussed above may help to delineate whether there is a defect at the HSC level.

Altered Proliferative Kinetics of Progenitors

The productive capacity of any given stem cell may be affected by the proliferative kinetics of its descendent progenitor cells. If a progenitor population is capable of increased proliferation, it may exponentially increase the production of mature cells that are scored in CRU assays. It would follow, therefore, that under conditions of limiting dilution, some animals would be scored positively, not because of increased HSC numbers, but rather due to the productivity of their offspring progenitors.

Defects in Hematopoietic Microenvironments and Their Potential Impact on HSCs

Other defects that may not be accurately assessed using standard limiting dilution assays include those that occur when HSCs have an impaired interaction with the bone marrow microenvironment such as observed for Rac2-deficient HSCs (Jansen et al., 2005). In such instances, the increased likelihood of mobilization of the HSCs away from the HSC niche will impact the results obtained if using whole bone marrow cells as the test source of HSCs. Furthermore, microenvironment-specific defects may contribute to similar phenomena as shown recently for Rb mutants. Conditional mutant studies have shown that there are no intrinsic HSC defects in Rb null HSCs (Walkley and Orkin, 2006). In contrast, loss of Rb in both the bone marrow microenvironment and myeloid cells resulted in an aberrant microenvironment, which induced a profound mobilization of HSCs out of the bone marrow (Walkley et al., 2007b). In the latter instance, the effects of the microenvironment on HSCs should be assessed such as we recently reported for RARγ null mice, which also have an impaired microenvironment that markedly impacts on hematopoiesis (Walkley et al., 2007a).

Method of Detecting Donor versus Host Cells

While the congenic CD45.1/CD45.2 transplant system is the preferred one for use in assaying murine HSCs, in some situations investigators are unable to use this system due to the donor mouse strains being incompatible to this system. Backcrossing mice onto the C57BL/6 congenic CD45.1/CD45.2 strains is recommended but is also very time consuming and can delay experiments for more than 1 year. Hence, in these instances, investigators more commonly rely on the use of PCR-based techniques (such as use of the Y chromosome to detect male donor cells in female hosts) to determine the extent of donor versus host reconstitution. This can pose issues, especially if the peripheral blood cells are not fractionated into myeloid and lymphoid lineages prior to analysis, as lymphoid cells have a long life span, as discussed above. In these situations, a more accurate assessment of donor cell-derived contribution to hematopoiesis may be achieved by determining peripheral blood myeloid cell reconstitution in addition to bone marrow-derived immature B

lymphocyte reconstitution in recipient mice at 6 months posttransplant.

Concluding Remarks

In conclusion, while remaining one of the better assays to assess HSC content, investigators should also be aware of the potential problems associated with analyzing limiting dilution assays. Caution should also be taken when forming conclusions based on data obtained using shorter-term approaches commonly used to analyze HSC content. It is recommended that investigators assessing HSC content in mutant and nonsteady state populations use long-term in vivo-repopulating assays together with a range of the other short-term in vitro and in vivo assays to accurately determine the number and functional potential of HSCs and their progenitors in mice. Furthermore, the recommended 16–26 weeks posttransplant required for analysis have cost implications that are obvious and extremely problematic. Finally, the long interval required for analysis of these assays impedes progress in the field. Alternative means of assessing and quantitating HSCs are clearly needed but may be more of a dream than a reality.

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