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Mouse Hematopoietic Stem Cell Identification And Analysis

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Abstract

Hematopoietic stem cells (HSCs) remain the most well-characterized adult stem cell population both in terms of markers for purification and assays to assess functional potential. However, despite over 40 years of research, working with HSCs in the mouse remains challenging because of the relative abundance (or lack thereof) of these cells in the bone marrow. The frequency of HSCs in bone marrow is about 0.01% of total nucleated cells and approximately 5000 can be isolated from an individual mouse depending on the age, sex and strain of mice as well as purification scheme utilized. Adding to the challenge is the continual reporting of new markers for HSC purification, which makes it difficult for the uninitiated in the field to know which purification strategies yield the highest proportion of long-term, multi-lineage HSCs. Here, we will report will review different strategies for hematopoietic stem and progenitor purification identification. We will also discuss methods for rapid flow cytometric analysis of peripheral blood cell types, and novel strategies for working with rare cell populations such as HSCs in the analysis of cell cycle status by BrdU, Ki-67 and Pyronin Y staining. The purpose of this review is to provide insight into some of the recent experimental and technical advances in mouse hematopoietic stem cell biology.

INTRODUCTION

Hematopoietic stem cells have tremendous therapeutic potential and have been harnessed in the clinic for more than 40 years in the context of bone marrow transplantation. Multipotent long-term HSCs (LT-HSCs) reside in the bone marrow and can self-renew to sustain the stem cell pool or differentiate into short-term HSCs (ST-HSCs) and lineage-restricted progenitors that undergo extensive proliferation and differentiation to produce terminally differentiated, functional hematopoietic cells. ST-HSCs or multipotent progenitors (MPPs) are only able to sustain hematopoiesis in the short term, while the LT-HSCs must persist for the lifespan of the organism to perpetually replenish the hematopoietic system. HSCs can be isolated from bone marrow or peripheral blood using enrichment (magnetic cell separation – MACS) and / or single-cell sorting (fluorescence-activated cell sorting – FACS) based on cell surface markers and / or vital dye staining. The HSC has served as the paradigm for adult stem cell populations by virtue of a well-defined differentiation cascade with distinct intermediaries connecting the differentiation of LT-HSCs into mature, functional hematopoietic cells. Many of the stages of HSC differentiation can be purified from the bone marrow or peripheral blood using characteristic cell surface markers which has greatly facilitated the study of hematopoietic biology and revealed important signaling molecules and molecular pathways crucial to HSC function. In this review, we will discuss a range of methods for characterizing HSCs, progenitors, and mature hematopoietic cells which can then be applied to the analysis of mutant mice or non-steady state conditions.

Hematopoietic Stem Cell Purification Schemes

Purification of HSCs has been remarkably improved in the past decades owing to the technical advances in flow cytometry and the development of monoclonal antibodies. While there is no single marker to distinguish HSCs from the other cells in the bone marrow, highly purified HSCs can be obtained with combinations of cell surface markers, and/or with vital dye staining. The canonical cell strategy used to enrich HSCs includes first removing differentiated cells with markers identifying differentiated blood cells, the so-called lineage cocktail with antibodies against about 8 differentiation markers, termed Lin⁻ selection, combined with positive selection for marker known to be expressed on HSCs, such as c-Kit⁺ (K) and Sca-1⁺ (S). This strategy selects a population of cells, the LKS (also KSL, or KLS) that includes HSC, but is still heterogeneous and also contains lineage-primed multi-potent progenitors in addition to short term and long term HSCs. Only ~10% KSL cells contain long-term hematopoietic reconstitution activity, so this population is better termed “hematopoietic stem and progenitors” than HSCs. To obtain HSCs of higher purity, several additional selection strategies have been developed by different laboratories. Here, we will review and compare major strategies for identifications of HSC as KLS-CD34⁻Flk-2⁻[1], KLS-CD150⁺CD48⁻ cells[2], the Hoechst-effluxing side population (SP) [3] and the associated variations on that theme (e.g. CD45^{mid}Lin⁻Hoechst^{low}Rhodamine^{low} [4]). In addition, the corresponding methods to purify the various short-term HSC and committed progenitor populations will be discussed. A summary of cell surface phenotypes and the hematopoietic cell types they enrich for is presented in Table 1.

Our lab typically uses the fluorescent vital dye Hoechst 33342 staining to purify mouse HSCs. This dye binds to DNA in live cells, so it has been used to identify replicating or quiescent cell populations. The Hoechst dye is retained at low levels in HSCs due to their ability to efflux the dye via membrane transport pumps, which are highly active in these cells compared to other bone marrow cell types. When Hoechst dye fluorescence is displayed at two emission wavelengths, Hoechst blue (450nm) and Hoechst red (675nm), the HSCs are distinctly present in the side of the fluorescent profile, hence the term “side population” (SP) (Supplemental figure 1). The high dye efflux activity of SP cells is attributed to their high expression of multidrug-resistance ABC transporters, such as transporter *p*-glycoprotein (Mdr1) and Abcg2. This has been validated in part by the demonstration that the *Abcg2* knockout mouse has a severe reduction in the SP population [5]. Several studies have shown that this side population is highly enriched for HSC activity, and most of the SP cells bear other makers, such as KLS (Figure 1). In addition, transplantation assays show that almost all the hematopoietic repopulating activity is confined to the SP fraction [3, 6]. However, SP staining efficiency is sensitive to variations in the preparation process. To avoid under-staining, KLS antibody staining is often used to complement SP staining to ensure a highly pure HSC population. Thus purified cells, with the phenotype of SP⁺c-Kit⁺Sca-1⁺Lineage⁻, are termed as SP^{KLS} (or “SParKLS”). We also commonly include SLAM markers such as CD150 along with the SP to facilitate comparison of these HSC with those identified via other means. We have performed SP^{KLS} purification on multiple instrument setups for cell sorting and analysis, including MoFlo (Dako), Aria (BD) and LSR II (BD). A comparison of staining profiles on different machines is presented in supplemental Figure 1. While it is possible to observe an SP with a violet laser, we obtain the best results using UV lasers. When setting up this template on any new machine, the Hoechst profile should be examined carefully (the different instrument parameters are presented in supplementary Table 1).

SP cells were shown to be sensitive to Verapamil, an ABC transporter protein inhibitor. When this drug is included in the Hoechst staining and washing buffers, the SP fraction disappears. Accordingly, when establishing the SP method, inclusion of a Verapamil (50 μ M) control can be used to ensure that the correct population is being identified [3].

Regarding where to draw the SP gate, particularly how far towards the top of the SP, we prefer a conservative SP gate, while attempting to maximize cell number yield and minimize contamination from non-HSCs. Mouse SP cells typically comprise about 0.01% of whole bone marrow within a gate excluding dead cells and red blood cells. However, this value varies depending on the mouse age, sex and strain. For example, the percentage of SP cells steadily increases with age [7]. If the Hoechst staining is performed correctly, more than 80% of SP cells should be positive for c-Kit and Sca-1 and negative for lineage markers. In addition to the mouse bone marrow, SP cells have also been found in hematopoietic tissues of other species including rhesus monkeys and humans[8], in some cell lines, and in various primary tumor cells [9-11].

By global gene expression profiling studies on HSC, a continually growing arsenal of markers is becoming available to stem cell biologists to purify their populations of interest. Those include Thy1.1 [12], CD34 [13], Flk-2 [14], Tie-2 [15], endoglin [16], Epcr [17], and CD150 and CD48 [18]. As each new HSC marker is published, each laboratory should validate that it works in their hands before proceeding with large scale experiments or abandoning more traditional isolation strategies. We find that SP^{KLS} cells are homogeneously CD34^{-low}, Flk-2⁻, and CD48⁻ [19, 20] (Figure 1). However, when we use the signaling lymphocytic activation molecule (SLAM) family-based purification scheme in combination with SP^{KLS} staining, we found that the SP was surprisingly heterogeneous for CD150 staining [21]. CD150⁺ cells are more prevalent in cells towards the lower tip of the SP, whereas CD150⁻ cells were more prevalent towards the top of the SP; this appears to be the first marker that subdivides the SP and may identify different functional sub-sets of HSCs [21]. We also showed that both the CD150⁺ and CD150⁻ subsets of SP^{KLS} have substantial functional HSC activity, as defined by long-term multilineage engraftment, although the CD150⁻ cells have somewhat diminished potential, relative to the CD150⁺ subset [22]. We have previously shown that cells from the bottom tip of the SP show better long-term engraftment than cells from the top of the SP [8], and so this distribution of HSC activity relative to CD150 staining is consistent with the observation that there appears to be a gradient of HSC activity along the length of the SP, perhaps representing a continuum of HSCs with slightly different functional properties. Based on these findings, SP^{KLS} in conjunction with the CD150^{high} marker are used to obtain HSC with higher activity. Three different purification strategies (SP^{KLS}CD150⁺, KLS-CD34⁻Flk-2⁻ and KLS-CD150⁺CD48⁻) are presented in Figure 2.

We also examined the overlap between the three different schemes. By gating KLS-CD34⁻Flk-2⁻ and KLS-CD150⁺CD48⁻ cells to a Hoechst plot, we found that more than 90% of those cells are SP cells (Figure 2). These results suggest that different purification strategies result in very similar purified HSC populations, which should thus be largely comparable functionally and molecularly. Each method has its advantages and drawbacks, however, with regard to staining requirements, or cytometer set up in terms of available lasers and fluorochromes. Furthermore, all of the methods are subject to potential problems incurred due to poor staining conditions (e.g. a dim marker paired with a dim fluorochrome on a non-optimally set-up cytometer), resulting in populations with low purity and activity. Thus, we encourage investigators to perfect the HSC purification strategy they wish to follow, and to benchmark it against other published reports using both surface marker staining and functional tests.

HSC changes throughout development

Between 3 and 4 weeks after the birth of the animal, mouse HSCs undergo an intrinsically timed change [23]. In the fetal liver and fetal bone marrow, as well as in newborn mice up to 3 weeks of age, the HSCs are actively cycling, but after the switch, most become quiescent

[24]. To purify HSCs from fetal liver, fetal bone marrow, or bone marrow from newborn mice, a different approach must be taken than those described above. These active HSCs do not efflux dye like quiescent adult HSCs do, so SP cannot be used. Like active adult HSCs, these fetal and newborn HSCs express Mac1, which can therefore not be used as one of the lineage markers for depletion or negative gating.

As in adult bone marrow, SLAM markers can be used to enhance purification of fetal liver HSCs: 37% of lethally irradiated mice injected with one Sca-1+Lin⁻ Mac+CD150+CD48⁻ fetal liver derived cell exhibit long-term multilineage reconstitution. Using just CD150+CD48⁻CD41⁻, possible with a two-color stain, 18% of purified cells will give long-term multilineage reconstitution [25]. Other markers can enhance purification of fetal HSCs as well. Within the KLS fraction of fetal liver cells, the EPCR⁺ cells, but not the EPCR⁻ cells, are capable of long-term reconstitution and multilineage engraftment in irradiated mice [26]. Our lab has used a combination of SLAM markers and EPCR (KLS CD150+CD48⁻EPCR⁺) to isolate a population of fetal liver cells that is highly enriched for HSCs (Figure 3). In adult bone marrow, approximately 90% of SP^{KLS} are also EPCR⁺, but only 6-8% of EPCR+Lin⁻ cells fall in the SP gate upon backgating analysis, indicating that EPCR is insufficient alone [17].

Differences Between Mouse and Human HSC Purification

The mouse is the most widely used animal model for studying human diseases and many molecules show a high degree of conservation between mouse and human. While many aspects of HSC biology are shared between the species, the purification strategies used differ slightly for experimental isolation of mouse HSCs and purification of human HSCs for therapeutic applications. Although the mouse bone marrow SP represents remarkable enrichment for HSC activity, a previous study showed that human cord blood or adult bone marrow SP cells have very low hematopoietic activity in vitro [8]. In addition, typically human bone marrow CD34⁺CD38⁻ cells are isolated for therapeutic applications [27], and mouse SP cells have been conclusively shown to express low levels of CD34. It was reported that Lin⁻/CD34⁻ cells isolated from human bone marrow and cord blood are capable of long-term multilineage repopulation in SCID mice [28], but their clinical relevance has never been established.

Despite these differences in HSC markers, clinical hematology has been greatly facilitated by advances in flow cytometry. The different cell surface markers on stem cells, erythrocytes, leukocytes, and platelets make them readily amenable to flow cytometric analysis [29, 30] Moreover, many leukemias can be identified by abnormal CD45 expression and side scatter properties with co-expression of other molecules used to discriminate the phenotype, for example CD34⁺CD13⁺ for acute myeloid leukemias [31].

Hematopoietic Progenitor Cell Analysis

As mentioned previously, the markers CD34 and Flk-2 are commonly used to separate the KSL compartment into long-term HSCs (KLS-CD34⁻Flk-2⁻), short-term HSCs (KLS-CD34⁺Flk-2⁻) and multipotent progenitor (MPP) cells (KLS-CD34⁺Flk-2⁺). The distribution of these antigens on hematopoietic cell populations can also be demonstrated by labeling of Hoechst-stained bone marrow with these antibodies. Gating to a CD34 / Flk-2 plot shows that the SP^{KLS} cells are negative for both these markers, but the KLS non-SP[±] population contains both short-term HSCs (CD34⁺Flk-2⁻) and multipotent progenitors (CD34⁺Flk-2⁺). Thus, the Hoechst-stained population can be used to simultaneously isolate long-term and short-term HSCs as well as MPPs.

HSCs differentiate into their terminal lineages through many steps of progenitor stages. While they are ultimately replaced from the HSC, differentiated blood cells are more directly generated from committed progenitors. Homeostasis affects the number of these progenitors, as well as the balance of HSCs that are quiescent vs. differentiating. When investigating the effects of a mutation or a treatment on the hematopoietic system, it is useful to examine the impact on the progenitors, as well as the HSC and differentiated progeny, which may reveal fine differences between the long-term self-renewing HSC and their immediate (non-self-renewing) progeny. The initial differentiation step of HSCs that gives rise to committed progenitors is of particular interest, as the regulatory events that are involved in the early stages of commitment are key to homeostasis. Cell fate decisions in the intermediate stages occur through the regulation of gene expression and epigenetic changes.

The molecular mechanism of developmental fate decision has been studied intensively and expression signatures were discovered for identifying, isolating and characterizing progenitor and mature lineage cells. In 1997, Weissman and colleagues pioneered the analysis of short-term HSCs and hematopoietic progenitor cells. Detailed phenotypes exist to identify common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs), as well as the next successive progenitors of each population comprised of pro-T cells and pro-B cells, and megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs) respectively [32, 33]. The various hematopoietic progenitor cell compartments of bone marrow can be identified by flow cytometry. In 2008, Scadden and colleagues developed a 7-color FACS all-in-one analysis of progenitor population in BM (Figure 4). Common lymphoid progenitors can be identified in whole bone marrow using the combination of KLS with $Il7\alpha^+$; these typically occur at a frequency of approximately 0.02% in mouse bone marrow. The myeloid progenitor population can be identified in the Sca-negative portion of the KSL stain, and are $Il7\alpha^-$. This fraction can be further subdivided into CMP ($CD34^+CD16/32^-$), MEP ($CD34^+CD16/32^-$) and GMP ($CD34^+CD16/32^+$). In 2005, Jacobson and colleagues succeeded in identifying lymphocyte-primed multipotent progenitors (LMPP) as $Flt3^{high}$ cells that have lymphoid as well as granulocyte and macrophage progenitor (GMP) potential but little or no megakaryocyte and erythrocyte progenitor (MEP) potential (Figure 4) [34]. Although these detailed analysis schemes are well documented, careful attention must be applied in instrument setup when using a large number of parameters, particularly compensating for spectral overlap when fluorochromes with close emission wavelengths are used.

Rapid Methods for Peripheral Blood Lineage Analysis

HSC function is generally monitored in terms of functional contribution to blood cell generation. This typically involves transplanting test cells into recipient animals in which their hematopoietic system has been ablated by irradiation. The activity of the transplanted test cells is observed by taking peripheral blood samples from the recipients at various timepoints after the transplant. The level of chimerism and test cell contribution is typically discriminated by having the test and recipient cells carrying distinct markers; frequently different alleles of the CD45 antigen (test cells are typically isolated from CD45.2 mice, as this is the standard C57Bl/6 allele, while recipient mice are normally CD45.1, the congenic strain) which can be readily distinguished using commercially available monoclonal antibodies. The function of test HSCs is described in terms of overall level of contribution to the recipient's peripheral blood (engraftment) and the types of hematopoietic cells generated from the test HSCs (lineage analysis). Typical timepoints for analysis are 4-weeks and 16-weeks after transplantation. After 4-weeks, the peripheral blood cells formed from input test cells can be the progeny of short-term HSCs or long-lived progenitors. However, the only test cells that can self-renew for 16-weeks post-transplant are long-term HSCs; thus, the

peripheral blood components generated from test cells after this time period are the progeny of HSCs. Therefore, long-term HSCs are typically defined as those that can give rise to all the major hematopoietic lineages at least 4 months after transplantation. In some mutants, there may be classically-defined stem cell activity, but a deficiency in the ability to generate a particular hematopoietic lineage, and thus it is also important to track lineage contribution of the donor cells. We typically use antibodies to track three major peripheral blood cell types in transplanted mice – myeloid cells (Gr-1⁺, Mac-1⁺), B cells (B220⁺) and T cells (CD4⁺, CD8⁺). By comparing the distribution of cell types formed from transplanted test cells to wild-type controls, we can determine if the test cells have functional bias for making particular lineages.

With the development of more fluorochromes for flow cytometry, it is now possible to analyze engraftment of the test cell population and multiple peripheral blood lineages with a single tube. We use a dual labeling strategy to simplify the staining scheme and show the distribution of major peripheral blood lineages (myeloid, B cells, T cells) on a single dot-plot. An example of an experiment in which recipient mice (CD45.1) were transplanted with donor HSCs (CD45.2) that had been transduced with a GFP-tagged retrovirus is presented in Figure 5. At 16-weeks after the transplant, peripheral blood was collected, red blood cells were lysed and remaining nucleated cells were stained for flow cytometric analysis with the following antibodies – CD45.2-APC, Gr-1-PeCy7, Mac-1-PeCy7, B220-PeCy7, B220-Pacific Blue, CD4-Pacific Blue and CD8-Pacific Blue. In the analysis, white blood cells were gated for viability based on propidium iodide (PI) staining and displayed on a FITC (GFP) versus APC (CD45.2) dot-plot. Untransduced donor cells appear in the CD45.2⁺GFP⁻ quadrant, and transduced donor cells appear in the CD45.2⁺GFP⁺ quadrant. Some cells appear in the CD45.2⁻GFP⁻ quadrant which represents residual recipient cells that survived irradiation or un-lysed red blood cells (which do not express CD45 at all and should be gated out on FSC/SSC). The CD45.2⁺GFP⁺ population can then be gated to a PeCy7 versus Pacific Blue dot-plot to analyze lineage distribution of transduced donor cells. With our dual labeling strategy, B cells are stained with both B220-PeCy7 and B220-Pacific Blue, which then allows us to observe the three major lineages on a two-color dot-plot. Myeloid cells (Gr-1⁺, Mac-1⁺) appear as PeCy7⁺Pacific Blue⁻, T cells (CD4⁺, CD8⁺) appear as PeCy7⁻Pacific Blue⁺ while the B cells (B220⁺) are the double positive population (Figure 5). This strategy saves time by reducing the number of tubes required to be processed for each sample and the amount of flow cytometric analysis required. The lineage distribution of the transduced (GFP⁺) donor cells can then be compared to control virus or wild-type donor cells to ascertain if the gene harbored by the retrovirus has any effect on lineage differentiation of HSCs. We anticipate this dual labeling strategy could be readily adapted to analyze different tissue samples where multiple cell types are required to be analyzed simultaneously and in which a single antigen identifying a specific cell type can be stained with the same antibody carrying different fluorochromes.

Cell Cycle Status and Proliferation Assays for Purified HSCs

HSCs are a primarily quiescent population, with around 1-3% in cycle when isolated HSC are examined using PI staining [3]. It has become clear that this property is important for maintenance of stem cell activity, as stem cells that are unable to return to quiescence, isolated from mutant mice, exhibit defective repopulating capacity [35]. Thus, when studying HSCs, it is helpful to examine their proliferation status. Standard methods are applicable, such as BrdU labeling, Ki-67 staining and Pyronin Y, and each is useful for addressing slightly different questions. Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2/M), but is absent from resting cells (G0). Ki-67 is an excellent marker to determine the growth fraction of a given cell population at any given point in time. Pyronin Y is an RNA stain which has been used to differentiate the different cell cycle

states of various populations, including HSCs [36], and is most useful for distinguishing cells in G1 and G0, i.e. those that are in real quiescence. These two measures can show the cell cycle status of a given cell population at any given point in time, like a proliferation snap-shot. The recent proliferative history of a cell population can be assayed using the synthetic nucleoside BrdU (bromodeoxyuridine). BrdU can be incorporated into the newly synthesized DNA of dividing cells (during the S phase of the cell cycle), by substituting for thymidine residues during DNA replication, so BrdU can reveal what proportion of stem cells have entered or completed cell-cycle over the entire labeling period, which can range from minutes / hours (generally *in vitro* labeling) to several days (usually *in vivo*); the amount of BrdU incorporation reflects the proliferation history of a cell population over that period.

The analysis of cell cycle kinetics in HSCs is challenging because of the limiting numbers of cells available per mouse. In addition, because SP sorting using the dye efflux phenotype requires cells to be viable, any analysis of intracellular properties cannot be performed simultaneously. As BrdU and Ki-67 staining requires fixation and permeabilization, SP^{KLS} cells must be purified first and then subsequently reanalyzed for these cell cycle assays. Due to small cell numbers associated with HSC sorting, and the cells lost in the subsequent reanalysis processing, we have developed a carrier cell method to alleviate cell loss. With this technique, a given number of carrier cells are added to the purified SP^{KLS} cells before the fix and permeabilization procedure to minimize cell loss when dealing with low cell numbers. Notably, one must choose an appropriate fluorochrome to label carrier cells distinctly (typically B cells from spleen). To distinguish HSCs from the carrier cells, one can choose a fluorochrome that HSCs are lacking (for example the fluorochrome designated for the lineage markers when sorting SP^{KLS} since this is sorted against) or one could use a color that has been spared during the HSC sorting for later intracellular analysis. In the reanalysis of intracellular staining, the cells are then displayed under a two dimensional dot-plot with the carrier fluorochrome (positive for carriers, negative for HSCs) and the Sca-1 or c-Kit fluorochrome (negative for carriers, positive for HSCs) subsequently allowing one to distinguish HSCs clearly from the carrier population (Figure 6). A typical strategy for this would be to pre-sort B220-PeCy5⁺ cells from spleen into a collection tube, then sort SP⁺ / Sca-1-APC⁺ / c-Kit-PE⁺ / Lineage-PeCy5⁻ HSCs into the same tube. The cells are then fixed overnight (or this can be done the same day, but due to the long preparation time for HSC it is convenient to leave these overnight) and the next day permeabilized and stained for anti-BrdU-FITC and reanalyzed. This carrier cell technique has been adapted to also analyze intracellular staining of Ki-67, and Pyronin Y to reveal the proliferation properties of HSCs (Figure 7). It is also worth mentioning that the carrier cells, if chosen wisely, can serve as an internal control for the intracellular staining experiment.

CONCLUSIONS

The hematopoietic system has served as the paradigm for understanding much of adult stem cell biology. In this article we have reviewed some of the common methods for HSC purification by flow cytometry, discussed analysis of hematopoietic progenitors and peripheral blood samples of transplanted mice, and provided novel methods for working with limiting cell numbers when dealing with HSCs. While we have suggested methods for HSC identification and analysis that work well in our laboratory, ultimately each investigator must validate the techniques in their own hands before confidently setting forth on large-scale experimental programs. One caveat to the assays discussed here is that while phenotype can be informative, HSCs are ultimately defined by their functional capacity to repopulate the bone marrow and generate all the major blood lineages in a stem cell-ablated host. The phenotype of stem cells has been well documented to change developmentally as

well as when regeneration is stimulated by agents such as 5-fluorouracil. Thus, phenotype alone cannot be relied upon to definitively identify stem cells.

METHODS AND MATERIALS

Hematopoietic Stem Cell Identification and Isolation

All animal procedures were conducted in accordance with the Baylor College of Medicine (Houston, Texas, USA) institutional guidelines. Whole bone marrow was isolated from femurs and tibias of mice and SP cell staining was performed with the vital dye Hoechst 33342 (Sigma-Aldrich, St Louis, MO) as previously described [3]. Briefly, whole bone marrow was resuspended in staining media at 10^6 cells/mL and incubated with 5 μ g/mL Hoechst 33342 for 90 minutes at 37°C. For Sca-1 enrichment of whole bone marrow prior to sorting, cells were resuspended at 10^8 cells/mL, stained on ice with anti-mouse Sca-1-biotin (eBioscience, San Diego, CA) for 15 minutes, resuspended at 1.25×10^8 cells/mL, incubated with 200 μ L/mL of magnetic antibiotin microbeads (Miltenyi Biotech, Auburn, CA) for 10 minutes at 4°C, rinsed with staining buffer, resuspended at 2×10^8 cells/mL, and magnetically enriched on an AutoMACS instrument (Miltenyi). For antibody staining, cells were suspended at a concentration of 10^8 cells/mL and incubated on ice for 20 minutes with various combinations of the following antibodies (all 1:100 dilution); PeCy5-conjugated Mac-1, Gr-1, CD4, CD8, B220 and Ter119 (eBioscience); FITC-conjugated Mac-1, Gr-1, CD4, CD8, B220 and Ter119 (BD Pharmingen, Franklin Lakes, NJ); Sca-1-APC (eBioscience) –FITC (BD Pharmingen) – PE (BD Pharmingen) –PeCy7 (eBioscience); c-Kit-APC (eBioscience) –FITC (BD Pharmingen) –PE (BD Pharmingen) –AF750 (eBioscience); CD150-PE (BioLegend, San Diego, CA); EPCR-FITC (StemCell Technologies, Vancouver, BC, Canada). Cell sorting and analysis were performed on a MoFlo cell sorter (Dako North America, Carpinteria, CA) and a FACS Aria Cell-Sorting System (BD Biosciences) and additional analysis was accomplished with an LSRII (BD Biosciences).

Fetal liver cells were isolated as previously described [37] from E13.5 embryos. After filtering through a 40 μ m nylon mesh filter, the cells were incubated with anti-lineage (B220, Ter119, CD4, CD8, Gr1)-biotin (eBioscience, San Diego, CA) for 15 minutes, resuspended and incubated with 200 μ L/mL of magnetic antibiotin microbeads (Miltenyi Biotech, Auburn, CA) for 15 minutes at 4°C, rinsed, and magnetically enriched on an AutoMACS instrument (Miltenyi) using a depletion program. The lineage negative fraction was stained and sorted as described for the adult bone marrow samples.

Hematopoietic Progenitor Staining

Whole bone marrow was isolated and stained on ice with various antibody cocktails to identify each progenitor compartment (all antibodies were obtained from BD Pharmingen and used at a concentration of 1:100 unless otherwise indicated). LT-HSC, ST-HSC and MPP were first stained with the vital dye Hoechst 33342 (Sigma-Aldrich). They were then stained with FITC-conjugated lineage markers (Mac-1, Gr-1, CD4, CD8, B220 and Ter119), Sca-1-PeCy7 (eBioscience), c-Kit-APC-AlexaFluor-750 (eBioscience), Flk-2-PE (at 1:50), and CD34-AlexaFluor-647 (at 1:50; eBioscience) for 20 minutes. CLPs were stained with biotinylated lineage markers (Mac-1, Gr-1, CD4, CD8, B220, CD3 and Ter119), IL7 α -PeCy7 (eBioscience), Sca-1-FITC, and c-Kit-PE for 20 minutes. Cells were then spun down, resuspended, and stained with streptavidin-Pacific Blue (1:50) for 20 minutes. CMP, GMP, and MEP were stained with biotinylated lineage markers (Gr-1, Ter119, CD4, CD8, CD3, B220, CD19), IL7 α -PeCy7 (eBioscience), Sca-1-FITC, c-Kit-PE, CD34-AlexaFluor-647 (at 1:50; eBioscience), and CD16/32-AlexaFluor-700 (at 1:50; eBioscience) for 20 minutes. Cells were then spun down, resuspended, and stained with streptavidin-

Pacific Blue (1:50) for 20 minutes. Finally, cells were spun down, resuspended in a propidium iodide solution, and analysis was accomplished on live cells with an LSRII (Becton Dickinson).

Peripheral Blood Analysis

Blood was collected from mice by retro-orbital bleeding and samples were subject to red blood cell lysis. Samples were incubated with the following antibodies (all 1:100 dilution; eBioscience) on ice for 20 minutes – CD4-Pacific Blue, CD8-Pacific Blue, B220-Pacific Blue, B220-PeCy7, Mac1-PeCy7, and Gr-1-PeCy7 for lineage and CD45.1-FITC plus CD45.2-APC (for competitive transplants) or CD45.2-APC alone (for MSCV-GFP over-expression transplants with the FITC channel spared to monitor GFP expression). Cells were then spun down, resuspended in a propidium iodide solution, and analysis was accomplished on live cells with an LSRII (Becton Dickinson).

BrdU Staining

Mice received an initial intraperitoneal injection of BrdU (Sigma-Aldrich; 1 mg/6 g mouse weight) 12 hours prior to sacrifice. Mice were then killed, and SP^{KLS} cells (sparing the FITC channel) were sorted into a previously sorted carrier cell population of 400,000 B220-PeCy5⁺ splenocytes. Samples were prepared for analysis of BrdU incorporation using the FITC BrdU Flow Kit (BD Pharmingen), and samples were reanalyzed by flow cytometry. On reanalysis, HSCs (c-Kit-PE⁺ / B220-PeCy5⁻) were readily distinguishable from carrier cells (c-Kit-PE⁻ / B220-PeCy5⁺) and were gated for analysis of BrdU incorporation.

Pyronin Y Staining

200,000 B220-FITC⁺ splenocytes were pre-sorted into collection tubes. HSCs (more than 1000) were then sorted into the same tube using the gating scheme SP⁺ / Sca-1-APC⁺ / Lineage-FITC⁻. The sorted cells were then incubated for 45 minutes with 20 µg/mL Hoechst 33342 and 50 µg/mL Verapamil (Sigma-Aldrich) in phosphate buffered saline supplemented with 3% fetal bovine serum. Pyronin Y (Sigma-Aldrich) was then added at 1 µg/ml, and the cells were incubated for another 15 min at 37°C, washed, and immediately analyzed on a BD LSRII. During flow analysis, both Hoechst 33342 and Pyronin Y signal were displayed under linear mode. The carrier cells, B cells, were then a control to define the G0/G1 DNA content (2N).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Side population staining to identify HSCs. Hoechst-stained cells are displayed for red and blue fluorescence simultaneously, and SP cells are identified as a small population of cells (~0.02% of WT 4-month old C57Bl/6 whole mouse bone marrow) off to the side, as indicated by the gate. These cells can be gated to the lineage profile, where most of them will be negative if SP staining is done properly and the cytometer is properly adjusted. This SP and Lineage-negative gate is then displayed for c-Kit and Sca-1 staining, where most of the cells should appear positive. These SP-KLS cells will then be largely CD34^{-/low}, Flk-2⁻, and CD48⁻, but heterogeneous for CD150 staining. With aging (24 months), the frequency of SP cells steadily increases and the percentage of CD150⁺ cells also increases among the SP^{KSL} population.

Figure 2.

Comparison of different HSC isolation schemes demonstrating their highly overlapping nature. (A) The standard SP gating scheme with sequential gates applied. About 40% of the SP-KSL cells are CD150⁺. (B) Starting with whole bone marrow, around 8% of whole bone marrow is considered “lineage-marker negative (Lin⁻)”. When these Lin⁻ cells are displayed for c-Kit and Sca-1, about 4-5% are double positive. This is the so-called “KSL” population that is enriched for stem and progenitor cells. About 7% of these KSL cells are CD150⁺ and CD48⁻ (hallmarks of the long-term HSC), but almost all of those cells are SP cells. (C) Starting again from the KSL scheme, around 10% of KSL are Flk2-negative and CD34-negative (hallmarks of the long-term HSC). Again, almost all of these cells display the SP phenotype.

Figure 3.

Isolation of fetal liver HSCs. Lineage depleted (not including Mac1) fetal liver cells that have been gated through FSC/SSC and PI for live-dead discrimination are then gated on a lineage plot to exclude any remaining lineage positive cells, then to a c-Kit vs. Sca-1 plot with the double positives being further gated for CD48-/CD150+, and finally EPCR+.

Figure 4.

Separation of hematopoietic progenitor populations by flow cytometry. (A) Gating scheme for the LT-HSC, ST-HSC and multipotent progenitors (MPP) (B) Gating scheme for the common myeloid progenitor (CMP), megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs). (C) Gating scheme for the common lymphoid progenitor (CLP). (D) Gating scheme for lymphoid myeloid progenitors (LMPP).

Figure 5.

Rapid flow cytometric analysis of peripheral blood of mice transplanted with HSCs transduced with MSCV-GFP retrovirus. For this analysis, red blood cells are depleted or lysed, and the remainder gated out on a FSC/SSC plot. Then viable white blood cells are gated and displayed on a CD45.2-APC versus GFP dot-plot. Progeny of donor HSC (CD45.2) can be discriminated from recipient cells (CD45.1) by CD45 alleles, and the donor HSCs that were successfully transduced to over-express a test gene are GFP⁺. The CD45.2⁺ / GFP⁺ population can then be gated to a PeCy7 versus Pacific Blue dot-plot to analyze distribution of the blood lineages. Myeloid cells are labeled with Gr1 and Mac-1 conjugated to PeCy7. T-cells are labeled with CD4 and CD8 antibodies conjugated to Pacific Blue (Pac-Blue). By labeling B cells with both B220-PeCy7 and B220-Pac-Blue, all major hematopoietic lineages can be displayed simultaneously on the same plot. The B cells are the double positive population, the myeloid cells (Gr-1⁺, Mac-1⁺) are the PeCy7⁺PacBlue⁻ population, while the T cells (CD4⁺, CD8⁺) are the PeCy7⁻ PacBlue⁺ population. If we were analyzing transplanted mice in which the GFP was not used to follow retroviral marking, we typically use CD45.1-FITC to track the recipient cells in addition to the donor cells.

Figure 6.

Analysis of HSCs turnover by BrdU labeling using flow cytometry. (A) HSCs from mice injected with BrdU are purified from Sca-1-enriched bone marrow (increasing the proportion of SP cells 10-fold) and then fixed and permeabilized overnight. (B) Reanalysis of the sorted cells following intracellular staining for BrdU. A PE versus PeCy5 dot-plot allows for discrimination between sorted HSCs and carrier B cells (the majority of carrier cells are lined up against the x-axis). The HSCs can then be gated to either a stem cells marker versus BrdU dot-plot or to a histogram to determine BrdU incorporation.

Figure 7.

Cell cycle reanalysis of purified SP^{KLS} cells by Ki-67 and Pyronin Y. (A) Using the carrier cell technique, SP^{KLS} cells were purified then fixed and permeabilized for Ki-67 staining. On the reanalysis, HSCs (Sca-1⁺B220⁻) were easily identified from carrier cells (Sca-1⁻B220⁺) and gated to show a distribution of Ki-67 versus DNA content using propidium iodide. This plot allows discrimination of the various stages of cell cycle of the HSC population. (B) Analysis of HSC cell cycle status by Pyronin Y staining. As above, SP^{KLS} were sorted into carrier cells and then both populations were stained for Pyronin Y analysis. On reanalysis, HSCs are gated away from carrier cells to a Hoechst versus Pyronin Y plot which shows the different stages of cell cycle. The use of this assay is clearly demonstrated when comparing normal HSCs to those which have been stimulated with the chemotherapeutic agent 5-fluorouracil (5-FU) which brings them out of quiescence and into cell activation programs. In normal HSCs, the vast majority are resting in the G0 stage, while six days after 5-FU stimulation a much higher proportion are actively engaged in the cell cycle in S/G2-M.

Table 1

Cell surface phenotypes of various hematopoietic stem and progenitor cell populations.

Marker Phenotype	Cell Type	Reference
KLS	Hematopoietic stem and progenitor cells	[19]
SP ^{KLS}	Long-term HSCs (LT-HSC)	[3]
KLS Flk2 ⁻ CD34 ⁻	Long-term HSCs	[1]
KLS CD150 ⁺ CD48 ⁻	(Adult) Long-term HSCs	[2]
KLS CD150 ⁺ CD48 ⁻	(Fetal liver) HSCs	[25]
CD45mid Lin-Rhodamine-low SP	Long-term HSCs	[4]
KLS Flk-2 ⁺ CD34 ⁺	Short-term HSC (ST-HSC) and multipotent progenitors (MPP)	[20]
Lin ⁻ Il7ra ⁺ c-Kit ⁺ Sca-1 ⁺	Common lymphoid progenitors (CLP)	[32]
Lin ⁻ Il7ra ⁻ c-Kit ⁺ Sca-1 ⁻	Myeloid progenitors	[33]
Lin ⁻ Il7ra ⁻ c-Kit ⁺ Sca-1 ⁻ CD34 ⁺ CD16/32 ⁻	Common myeloid progenitor (CMP)	[33]
Lin ⁻ Il7ra ⁻ c-Kit ⁺ Sca-1 ⁻ CD34 ⁻ CD16/32 ⁻	Megakaryocyte-erythrocyte (MEP)	[33]
Lin ⁻ Il7ra ⁻ c-Kit ⁺ Sca-1 ⁻ CD34 ⁺ CD16/32 ⁺	Granulocyte-macrophage progenitors (GMP)	[33]
KLS EPCR ⁺	Fetal liver HSCs	[26]
Lin ⁻ CD45 ⁺ EPCR ⁺ CD48 ⁻ CD150 ⁺	Fetal liver HSCs	[38]