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Heterogeneity and hierarchy of hematopoietic stem cells

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Hematopoietic stem cells (HSCs) are a more heterogeneous population than previously thought. Extensive analysis of reconstitution kinetics after transplantation allows a new classifications of HSCs based on lineage balance. Previously unrecognized classes of HSCs, such as myeloid- and lymphoid-biased HSCs, have emerged. However, varying nomenclature has been used to describe these cells, promoting confusion in the field. To establish a common nomenclature, we propose a reclassification of short-, intermediate-, and long-term (ST, IT, and LT) HSCs defined as: ST $<$ 6 months, IT $>$ 6 months, and LT $>$ 12. We observe that myeloid-biased HSCs or α cells overlap with LT-HSCs, whereas lymphoid-biased HSCs or γ/δ cells overlap with ST-HSCs, suggesting that HSC lifespan is linked to cell differentiation. We also suggest that HSC heterogeneity prompts reconsideration of long-term (>4 months) multilineage reconstitution as the gold standard for HSC detection. In this review, we discuss relationships among ST-, IT-, and LT-HSCs relevant to stem cell heterogeneity, hierarchical organization, and differentiation pathways. © **2014 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Hematopoietic stem cells (HSCs) are defined as cells with self-renewal and differentiation potential [1]. Accumulated data show that HSCs are a heterogeneous population in multiple aspects, including their degree of self-renewal [2,3], differentiation manner [4,5], and lifespan [6–8]. Retroviral marking studies indicate that HSCs clonally give rise to all blood lineages and self-renew (a finding that represents definitive proof for the existence of HSCs in mouse bone marrow) [9–11]. Moreover, marking techniques have been used to demonstrate various patterns of reconstitution kinetics after HSC transplantation. Interestingly, some clones preferentially reconstitute a lymphoid lineage, whereas others preferentially reconstitute a myeloid one [12].

Lineage reconstitution kinetics have been examined extensively in mice transplanted with cultured bone marrow cells or with limiting doses of bone marrow cells freshly obtained from adult mice [13–15]. These studies suggest the presence of myeloid-biased HSCs (My-bi HSCs), lymphoid-biased HSCs (Ly-bi HSCs), and balanced HSCs (Bala HSCs). On the other hand, α, β, γ, and δ cells have been defined by others [16,17]. The presence of all these HSCs has been verified by single-cell transplantation [16,18–20]. Both types of classification are defined based on myeloid and lymphoid reconstitution ratios, but the criteria used to make these classifications differ fundamentally from one another (discussed later).

In this study, we propose a third classification, LT-, IT-, and ST-HSCs, based on reconstitution time periods [21]. We then examine the relationship of the three classification systems and discuss how different HSC classes are related to one another in the hematopoietic hierarchy. These

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comparisons support that HSC lifespan is tightly associated with lineage contribution [13-17].

In the prevailing bifurcation model [22], following loss of self-renewal potential HSCs give rise to multipotent progenitors (MPPs), which commit to either myeloid or lymphoid lineages exclusively. According to this model, this is the first step in lineage commitment. However, MPPs or their progenitor equivalents have not been identified at the single-cell level. Other studies suggest that loss of lymphoid differentiation potential could occur as one of the first lineage commitment steps [23,24]. It is time to consider a more comprehensive differentiation model. We propose a new differentiation model consisting of LT-, IT-, and ST-HSCs.

HSC classifications

My-bi, Bala, and Ly-bi HSCs

Muller-Sieburg et al. [13,14] have defined My-bi, Bala, and Ly-bi HSCs based on the ratio of lymphoid to myeloid cells (the L/M ratio). The proportions of lymphoid to myeloid cells are calculated among test-donor-derived cells (Supplementary Figure E1, online only, available at www.exphem.org); thus, (% lymphoid cells) + (% myeloid cells) = 100. In this classification, long-term reconstitution is assessed 20 weeks or more after transplantation [13–15]. Transplanted cells are designated My-bi HSCs when the L/M ratio is less than 3 and Ly-bi HSCs when it exceeds 10. Cells are considered Bala HSCs when the L/M ratio exceeds 3 but is less than 10 [13–15].

These types of HSCs were detected basically using in vivo limiting dilution analysis [13–15]. Later, a different group reported that Ly-HSCs and My-HSCs are enriched in the upper and lower portions of SP, respectively, and successfully accomplished single-cell reconstitution with these HSCs [18]. Platelet-biased HSCs have also been reported as a My-bi subclass potentially residing at the apex of the hematopoietic hierarchy [25].

Figure 1 shows typical reconstitution patterns seen following transplantation of single My-bi, Bala, and Ly-bi HSCs. My-bi HSCs reconstitute the myeloid lineage after varying latencies, followed by gradual reconstitution of the lymphoid lineage (Fig. 1A). Thus, the myeloid lineage is more significantly reconstituted at early stages of reconstitution. In contrast, Ly-bi HSCs show both myeloid and lymphoid lineage reconstitution from early stages (Fig. 1C). Ly-bi HSCs reconstitute the myeloid lineage to a less extent than the lymphoid lineage. Myeloid reconstitution is often detectable for only a few months, but lymphoid reconstitution can persist relatively longer. Bala HSCs reconstitute the lymphoid lineage soon after the myeloid lineage (Fig. 1B). The proportions of myeloid and lymphoid lineage cells resemble those seen in the peripheral blood of normal mice [13–15]. Many investigators consider Bala HSCs to be typical HSCs, which might account for why the presence of My-bi HSCs and Ly-bi HSCs has been overlooked [4].

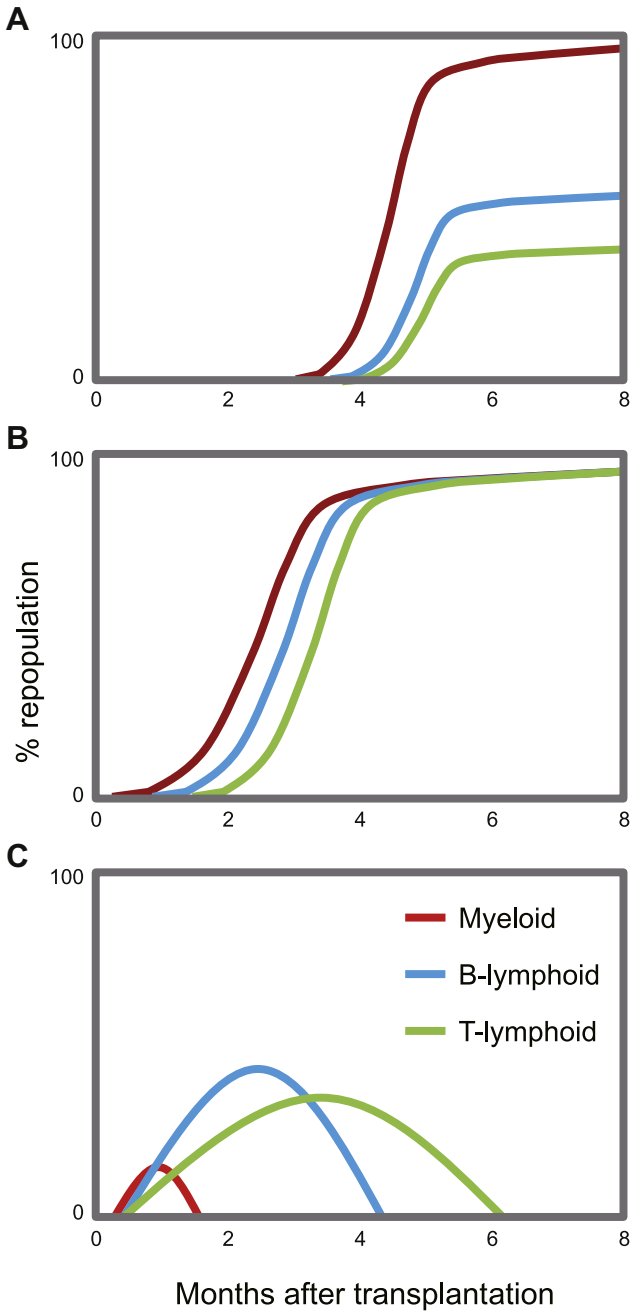

Figure 1. Reconstitution kinetics of My-bi, Bala, and Ly-bi HSCs. Shown are typical reconstitution patterns seen following single-cell transplantation of My-bi **(A)**, Bala **(B)**, and Ly-bi **(C)** HSCs, based on published data [19].

α, β, and γ/δ cells

Eaves et al. have defined α, β, γ, and δ cells as the percentage of myeloid chimerism relative to that of lymphoid chimerism (the M/L ratio) [16,17]. The M/L ratio is not simply the reciprocal of the L/M ratio described by

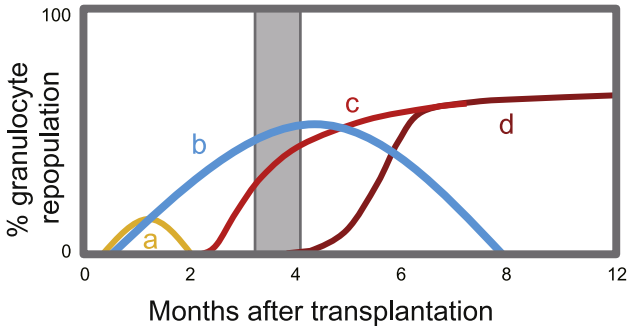

Figure 2. Roles of ST-, IT-, and LT-HSCs in bone marrow transplantation. After bone marrow transplantation, total chimerism occurs because of several types of repopulating cells. In this model, total chimerism is represented by granulocyte reconstitution. (a) After ST-HSC transplantation, a small single wave is observed. (b) After IT-HSC transplantation, a single, larger wave is observed. (c, d) LT-HSC transplantation produces a sigmoid curve. (d) Reconstitution from latent HSCs has a delayed onset. Column in grey is an example of the time window for analysis.

Muller-Sieburg et al. [13-15], because competitor cell contribution is factored in to the M/L ratio. Thus, the percentage of myeloid chimerism is defined as: (% Test cell–derived cells in the granulocyte-macrophage lineage) × 100 / (% Test cell–derived cells + % Competitor cell-derived cells in the granulocyte-macrophage lineage). (See Supplementary Figure E1, online only, available at www.exphem.org.) Percentages of B and T lymphoid cells are defined similarly. The percentage of lymphoid chimerism is defined as (% B lymphoid chimerism) + (% T lymphoid chimerism). In this system, long-term reconstitution is assessed 16 weeks or more after transplantation [16,17].

Single-donor cells are designated α cells when the M/L ratio exceeds 2, and γ or δ cells when it is less than 0.25. When myeloid chimerism exceeds 1%, cells are designated γ cells; when it is less than 1.0%, they are designated δ cells [16,17]. Single-donor cells are designated β cells when the M/L ratio exceeds 0.25 but is less than 2. α and β cells are transplantable into secondary recipient mice, but γ and δ cells are not. It has been reported that γ/δ cells are enriched in $CD150^{low/negative}CD34^{-}KSL$ cells, while α and β cells are enriched in $CD150^{high}$ and $CD150^{med}$ $CD34^{-}KSL$ cells [19,26].

ST-, IT-, and LT-HSCs

Researchers have long recognized the concept of ST-HSCs and LT-HSCs [6–8]. Recently, an intermediate-term, (IT)-HSC, which contributes to reconstitution up to 8 months after transplantation, has been used [20]. IT-HSCs express integrin α2 (CD49b) among $Rho123^{low}$, $CD34^{-}Kit^{+}Sca\text{-}1^{+}lineage^{-}$ (CD34-KSL) cells. Given these findings, we propose the use of ST-, IT-, and LT-HSCs as a classification based on reconstitution time period (Fig. 2) [21]. We suggest the following definitions. Only granulocyte reconstitution is considered as a parameter of this classification, not reconstitution of other lineages such as erythrocytes, B cells, and T cells, because granulocytes are extremely short-lived and their reconstitution directly reflects HSC activity [12,16]. ST would be defined as less than 6 months (note that this is much longer than the previous definition of 1–2 months) [6,7]. Granulocyte reconstitution levels would decrease by 6 months following ST-HSC transplantation. IT would be defined as less than 12 months. Granulocyte reconstitution levels would decrease by 12 months after IT-HSC transplantation. LT would be defined as greater than 12 months. Granulocyte reconstitution would not decrease until 12 months or later after LT-HSC transplantation. Secondary transplantation could be performed within 12 months, such as at 5–6 months after transplantation, but the same criteria would apply [19,21]. For example, if granulocyte reconstitution began to decrease by 6 months after secondary transplantation, this HSC would be designated an IT-HSC. Generally, ST-HSCs do not show any reconstitution activity after secondary transplantation, whereas reconstitution levels do not change after secondary transplantation with LT-HSCs [21].

Relationship of the three HSC classifications

My-bi/Bala/Ly-bi and α/β/γ/δ HSC classifications are based on similar concepts. Thus, α, β, and γ/δ cells likely correspond to My-bi HSCs, Bala HSCs, and Ly-bi HSCs, respectively. To assess this correspondence more precisely, we compared these classifications using published data of transplantation with 30 single HSCs [19]. Those comparisons are shown by Venn diagram analysis in Figure 3. All possible relationships among classifications are also shown in Supplementary Figure E2 (online only, available at www.exphem.org). Ten α cells were included in a total of 14 My-bi HSCs (Fig. 3A), and four Bala HSCs were included in a total of 7 β cells (Fig. 3B). Ly-bi HSCs overlap primarily with γ cells (Fig. 3C). These data support the idea that both classifications, despite the fact that different criteria are used to define them, identify similar classes of HSCs.

Interestingly, 7 of 8 LT-HSCs were also classified as My-bi HSCs; 5 of those 8 were classified as α cells and 2 as β cells (Supplementary Figure E2). Most LT-HSCs thus reconstitute a myeloid lineage prior to the lymphoid lineage. Of 10 IT-HSCs, 6 were classified as My-bi HSCs, 3 as Bala HSCs, and 1 as Ly-bi HSC. Of those 10 IT-HSCs, 4 were classified as α, 5 as β cells, and 1 as a γ cell. These data suggest that IT-HSCs exhibit varying reconstitution patterns. Moreover, we found that an almost identical population of cells is identified by Ly-bi HSCs, γ cells, and ST-HSCs (Fig. 3C).

Relationship between My-bi and Ly-bi HSCs or between α and γ cells

Whether My-bi HSCs can give rise to Ly-bi HSCs remains controversial [14,16,17]. In mice, after transplantation of a

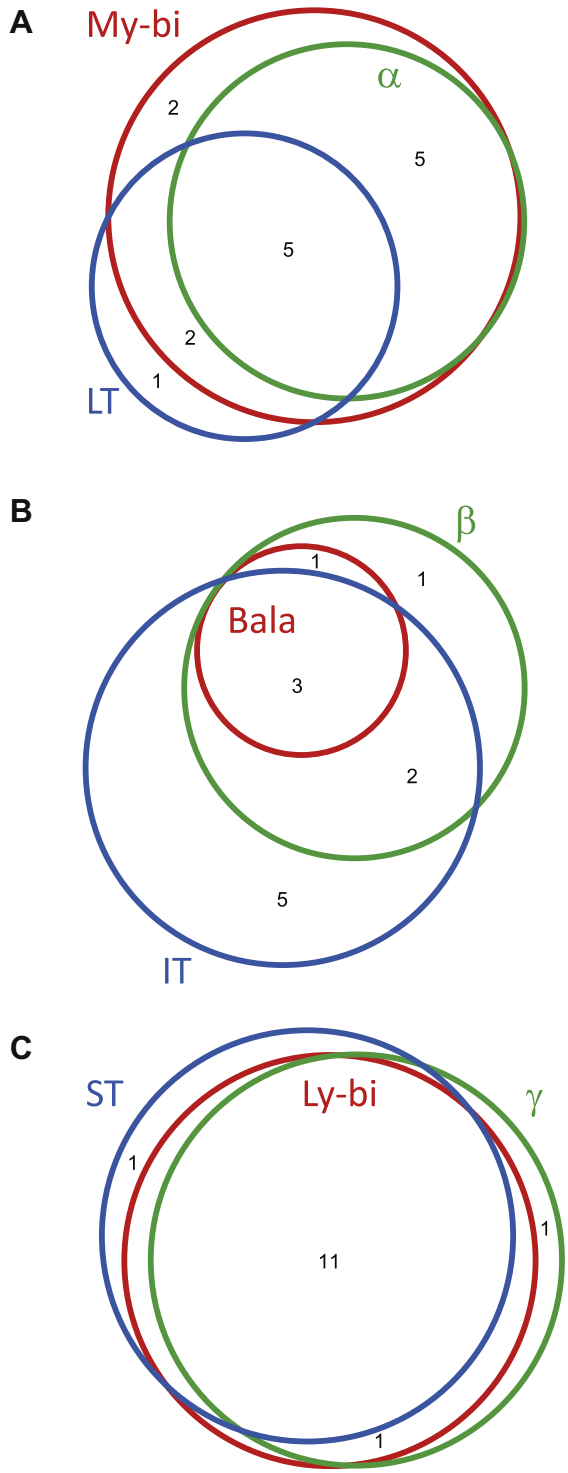

Figure 3. Comparison of different HSC classifications. Thirty single cells were transplanted and resultant reconstitution data were classified based on three different criteria: 14 My-bi HSCs, four Bala HSC, and 12 Ly-bi HSCs were identified; 10 α cells, 7 β cells, and 13 γ cells were identified; and 8 LT-HSCs, 10 IT-HSC, and 12 ST-HSCs were identified. Venn diagram indicates relationships among **(A)** My-bi, α, and LT-HSCs cells; **(B)** Bala, β, and IT-HSCs; and **(C)** Ly-bi, γ, and ST-HSCs. See Supplemental Figure E2 for more detail (online only, available at www.exphem.org).

portion of bone marrow cells from primary into multiple secondary recipients, reconstitution patterns similar to those seen in the primary transplantation are observed, suggesting that My-bi and Ly-bi HSCs use intrinsic differentiation programs and do not undergo interconversion [14,15].

It is technically difficult to detect a small number of γ/δ cells in the presence of a large number of α or β cells. To address this issue, investigators have transplanted either a small number of purified cells or single purified cells from the bone marrow of primary recipients into secondary lethally irradiated mice. Interestingly, production of γ/δ cells by α or β cells was detected in some cases [16,17], suggesting that some α or β cells can differentiate into a lymphoid lineage via γ/δ cells. Following transplantation of single α or β cells, the entire hematopoietic system can be reconstituted for more than 1 year. When whole bone marrow is reconstituted for a long time, it is difficult to think that a particular population is not reconstituted. Therefore, it is unlikely that only γ/δ cells are missing from bone marrow cells reconstituted with α/β cells.

Revisiting criteria for HSC detection

One important question is whether we can detect all HSC classes using criteria commonly used in competitive repopulation.

Repopulating cells in bone marrow reconstitution

When a sufficient number of bone marrow cells (e.g., 1×10^6 per mouse) is transplanted into lethally irradiated mice, the peripheral blood, which represents the entire hematopoietic system, is fully reconstituted over time. In addition to stem cells, a variety of progenitors, including those exhibiting radioprotection activity (e.g., colony-forming units in spleen [27]), are present in transplanted bone marrow, enabling recipients to survive and show complete hematopoietic reconstitution. Figure 2 shows a typical reconstitution pattern after bone marrow transplantation. Results of single-cell transplantation suggest that a large wave of reconstitution occurs via several kinds of repopulating cells with different kinetics [16,19]. Colony-forming units in spleen play a role in rescuing lethally irradiated mice, likely for the initial 1–3 weeks after transplantation [8]. Short-term repopulating cells likely play a role at 3–6 weeks after transplantation [7], and multilineage reconstitution is relayed over months by ST-, IT-, and LT-HSCs. It should be emphasized that all these repopulating cells play essential roles in the process of long-term hematopoietic reconstitution at distinct times.

Competitive repopulation

Competitive repopulation, an approach originally developed by Micklem et al. [28], is used because cotransplantation of competitor cells ensures long-term survival of lethally irradiated mice regardless of whether test donor cells (or test cells) have radioprotection activity or long-term reconstitution potential. Moreover, competitive repopulation permits quantitation of repopulating activity in test donor cells

compared with competitor cells. Both repopulating units (RU) [29] and competitive repopulating units (CRU) [30] have been defined based on competitive repopulation assays. The amount of relative repopulating activity in HSCs can be expressed in RU, while the number of HSCs can be expressed in CRU. The mean activity per stem cell, a number useful to compare HSC qualities, is defined as RU/CRU [31].

The current gold standard for HSC detection

Long-term (≥16 weeks) multilineage reconstitution is the gold standard to detect HSCs. Specifically, greater than 1% of total chimerism in peripheral leukocytes 4 months after transplantation with detectable myeloid, B lymphoid, and T lymphoid lineage reconstitution is the acceptable criteria to detect HSCs [16,32]. However, we suggest that this standard could be reconsidered given that Ly-bi HSCs cannot be detected by this criterion. All myeloid, B lymphoid, and T lymphoid lineage reconstitution after transplantation might not be detected at 4 months, but instead be detected sequentially, such as myeloid reconstitution at 1–2 months and B and T lymphoid reconstitution at 1–4 months. Latent HSCs, a particular type of LT-HSCs, also cannot be detected as multilineage repopulating cells, because they exhibit only a low level of myeloid reconstitution 4 months after transplantation [16,19]. However, latent HSCs do exhibit significant reconstitution of all myeloid, B lymphoid, and T lymphoid lineages later or even after secondary transplantation [19].

A new standard to detect all HSC classes

As noted, data derived from single–time point analysis are not sufficient to detect all ST-, IT-, and LT-HSCs, given that various lineages are reconstituted with different dynamics (Figs. 1 and 2). As illustrated in Figure 2, if recipient mice are analyzed in a narrow time window, some HSC classes may be missed. Ideally, the peripheral blood of recipients should be analyzed as long as animals survive. Ideally, the peripheral blood of recipient mice should be analyzed for as long as animals survive, an approach favored by Harrison [33]. However, practical reasons dictate that recipients be analyzed a minimum of three times, for example at 1–2, 4–6, and 8–12 months after transplantation. Alternatively, mice could be analyzed at 1–2 months and then at 4–6 months after primary transplantation, and 4–6 months after secondary transplantation. Of special note is that only granulocyte reconstitution but tri-lineage reconstitution is essential for HSC detection in the new criteria.

HSC differentiation models

Bifurcation model (Fig. 4A)

Weissman's group has identified common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) in adult bone marrow [22,34] and fetal liver [35,36]. CLPs give rise to B cells, T cells, and natural killer (NK) cells but not granulocytes, macrophages, erythrocytes, or platelets, whereas CMPs give rise to granulocytes, macrophages, erythrocytes, and platelets but not B cells, T cells, or NK cells. Thus, CLPs and CMPs are mutually exclusive populations, suggesting that MPPs are the common progenitors of both. In this model (Fig. 4A), HSCs give rise to MPPs or their equivalent progenitors following loss of self-renewal potential as MPPs maintain all differentiation potentials. This model was proposed over a decade ago [22], but, at the clonal level, MPPs have not been identified experimentally, and the relationship of MPPs to CLPs or CMPs is yet to be clarified.

Myeloid-based model (Fig. 4B)

Katsura 's group analyzed fetal liver cells using an in vitro assay they developed known as a *multilineage progenitor assay* [37]. Fetal thymic organ culture [38] was modified by adding cytokines (SCF, IL-3, IL-7, and EPO) and providing a higher percentage of oxygen to detect B cell progenitors, granulocytes, macrophages, and erythroblasts, in addition to T cell progenitors [37,39]. Progenitors such as granulocyte/macrophage/B cell/T cell (MyBT) progenitors, MyB progenitors, and MyT progenitors were detected [23,37,39], whereas BT progenitors were not. Because erythroblast progenitors were not detected among MyBT progenitors, a myeloid-based model (Fig. 4B) was proposed in which HSCs give rise to MyE or MyBT progenitors and MyBT progenitors then give rise to either MyB or MyT progenitors [39,40]. HSCs were not distinguishable from multipotent progenitors in this system because HSCs cannot be detected by in vitro assays. Megakaryocytes have not been examined in this system. Progenitors in adult bone marrow have not been compared with those in fetal liver by this system.

LMPP model (Fig. 4C)

Jacobsen's group identified lymphoid-primed MPPs (LMPPs) that give rise to granulocyte/macrophage and B/T cell lineages but not the megakaryocyte/erythrocyte lineage [24,41]. Myeloid potential of single LMPPs was detected with in vitro colony assays, whereas B and T cell potentials were detected with coculture with OP9 and OP9/Delta-like 1 stromal cells plus cytokines, respectively. From these analyses, the authors proposed a model combining elements of the bifurcation and myeloid-based models [24]. In this model (Fig. 4C), megakaryocyte/erythrocyte progenitors likely branch from HSCs, resulting in emergence of LMPPs. This first step of HSC differentiation is similar to that proposed in the myeloid-based model. However, lineage tracing studies suggest that LMPPs also differentiate into an ME lineage [42,43]. More extensive studies are required to clarify ME differentiation pathways. LMPPs reportedly give rise to either granulocyte and macrophage progenitors or CLPs [24], a pathway reminiscent of the bifurcation model.

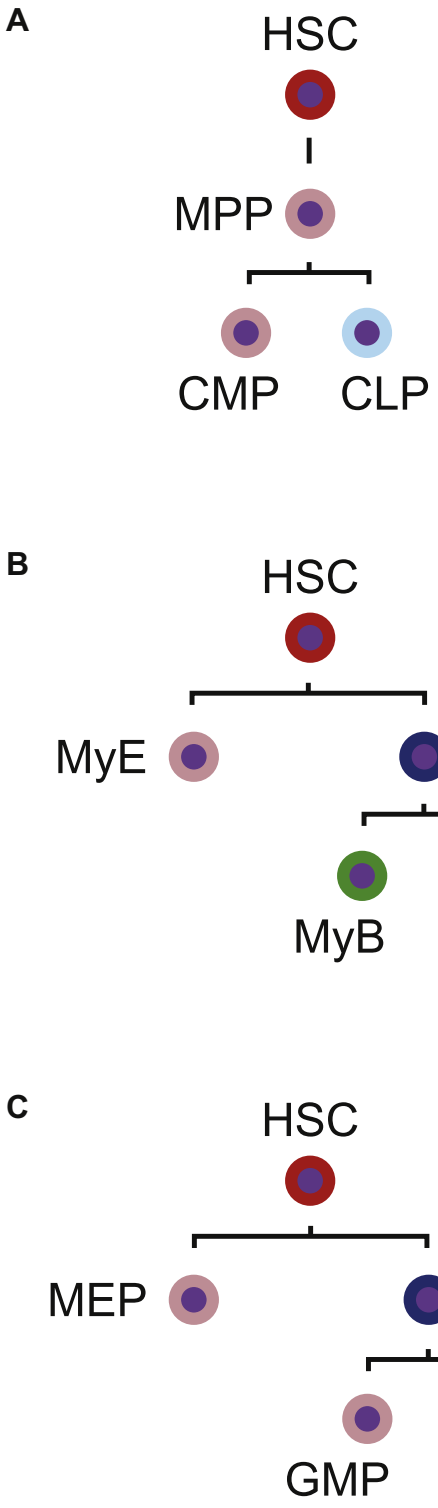

Figure 4. HSC differentiation models. Shown are the **(A)** bifurcation model [22], **(B)** myeloid-based model [40], and **(C)** LMPP model [24]. CLP = common lymphoid progenitor; CMP = common myeloid progenitor; GMP = and granulocyte and macrophage progenitor; LMPP = lymphoid-primed multipotent progenitor; MEP = megakaryocyte and erythrocyte progenitor; MPP = multipotent progenitor; MyB = myeloid progenitor with B cell potential; MyBT = myeloid progenitor with B cell and T cell potential; MyE = myeloid progenitor with erythroid potential; MyT = myeloid progenitor with T cell potential.

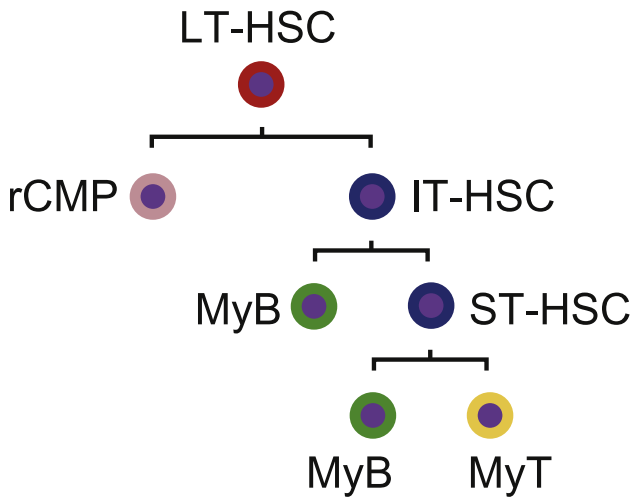

Figure 5. New differentiation model. The authors' current understanding of HSC differentiation pathways is illustrated. LT-HSCs give rise to rCMPs and IT- or ST-HSCs. IT-HSCs may represent an intermediate state between LT-HSCs and ST-HSCs. ST-HSCs give rise to myeloid and B cell progenitors (MyB) or myeloid and T cell progenitors (MyT).

A new HSC differentiation model

Investigators in the field assume that MPPs give rise to CMPs, CLPs, MyBT progenitors, or LMPPs [40,44,45]. However, no clonal study has provided evidence for these pathways, and it is possible that progenitors can be generated without MPPs. The paired daughter cell (PDC) assay is one of the few methods available to address this issue [32,46,47]. In this approach, after a cultured HSC divides into two daughter cells, each daughter cell is separated by a micromanipulator and transplanted with competitor cells. It was recently reported that the megakaryocyte lineage is one of the first blood cell lineages reconstituted by HSCs [21,42]. Megakaryocyte lineage-specific repopulating cells are often found in highly purified HSC populations [21]. Expression of megakaryocyte markers is sometimes detected in the HSC population [25,48], suggesting that these cells are developmentally related to HSCs. To address whether they are, PDC assays using single HSCs were performed [21,32], and emergence of pairs of LT- or ST-HSC and megakaryocyte progenitors, as well as pairs of ST-HSCs and CMPs with repopulating potential (rCMPs)—which differ from previously defined "classical CMPs" [21,22]—was observed. These data strongly suggest that cells of the myeloid or megakaryocyte lineage are generated directly from HSCs via asymmetric division. Accordingly, the myeloid bypass model has been proposed [21].

Considering the position of ST-, IT-, and LT-HSCs in the myeloid bypass model, we now propose a new differentiation model (Fig. 5). LT-HSCs give rise to either rCMPs or IT- and ST-HSCs. rCMPs can be replaced by megakaryocyte progenitors with repopulating potential such that megakaryocyte progenitors are generated from HSCs. Recently,

the bifurcation model has been revised [49]. Now, differences among various models are becoming less apparent.

In our new model, ST-HSCs give rise to B or T cell progenitors with myeloid potential (MyB and MyT progenitors). CLP function in lymphopoiesis remains uncertain, because these cells are extremely rare in bone marrow [50] and have never been detected in single-cell transplantation [19,21]. On the other hand, we have detected single MyB or MyT progenitors (Ema, unpublished data, 2004). Thus, lymphoid differentiation pathways based on the Kawamoto and Katsura model [40] are included in this model. Further work is required to define lymphoid differentiation pathways. In particular, a role of MyT progenitors, if any, needs to be clarified.

Our model predicts that the myeloid compartment is established earlier than the lymphoid compartment. Accordingly, in bone marrow, the myeloid compartment becomes larger than the lymphoid compartment. Similarly, the B lymphoid compartment is larger than the T lymphoid compartment, consistent with the fact that bone marrow is the site of myelopoiesis and B lymphopoiesis but not of T lymphopoiesis. Several candidate thymus-seeding progenitors have been reported [51,52]. It is now critical to determine whether lineage commitment (lineage restriction) occurs before progenitors migrate into the thymus (i.e., whether ST-HSCs or MyT progenitors as shown in Fig. 5 home to the thymus). To address this issue, circulating HSCs, progenitors, or both must be identified.

Future challenges

G0 length in HSCs

HSCs reportedly enter the cell cycle once every month [53–55]. Dormant HSCs reportedly enter the cell cycle at 5-month intervals [56]. Thus, an intriguing question is whether the length of G_0 differs among LT-, IT-, and ST-HSCs. Dormant HSCs may be present among LT-HSCs and their quiescent state cell-intrinsically regulated. Nevertheless, molecular mechanisms distinguishing LT-HSCs from ST-HSCs remain important to characterize, because they might reveal what controls HSC lifespan. Whether IT-HSCs serve as a transition from LT-HSCs to ST-HSCs should also be determined.

Ex vivo HSC expansion

It is difficult to induce in vitro self-renewal in HSCs, possibly because of their heterogeneity [57]. If heterogeneity arises from developmental processes, a specific class of HSCs responsible for their in vivo expansion may be useful for ex vivo expansion and manipulation of HSCs as needed. Nevertheless, LT-HSCs can be used to produce a large number of myeloid progenitors in vitro, an approach that can be applied to prevent severe bacterial infection in cancer patients after intensive chemotherapy or irradiation [58].

In vivo tracking of HSCs

HSCs have not been marked successfully using an HSC-specific reporter. Even if such tracing were possible, it might remain difficult to track single HSCs in vivo under physiologic circumstances, because their engraftment in nonirradiated mice is detectable only after transplantation of a large number of HSCs [59,60]. If a small number of donor-derived mature cells in peripheral blood is detectable, one might be able to analyze HSC dynamics in nonirradiated settings. Recently, barcode analysis in conjunction with next-generation sequencing has been applied to in vivo clonal analysis of mouse HSCs after transplantation [61,62]. The barcode, composed of a short random sequence (~30 nt), is integrated into the HSC genome using retroviral or lentiviral vectors. There remain technical obstacles to this approach, such as unavoidable in vitro manipulation of HSCs for transduction, difficulties in analyzing massive amounts of data from repeated sequencing of multiple blood lineages, and a lack of information about red blood cells or platelets, which lack genomic DNA. However, it may be possible to track rare clones among a large number of normal HSCs using such technology. Moreover, the method should enable the study of human repopulating cells in immunodeficient mice at the clonal level [63]. It is important to understand the degree of heterogeneity in human HSCs and the contribution of this heterogeneity to development of the hierarchical organization of human hematopoiesis.

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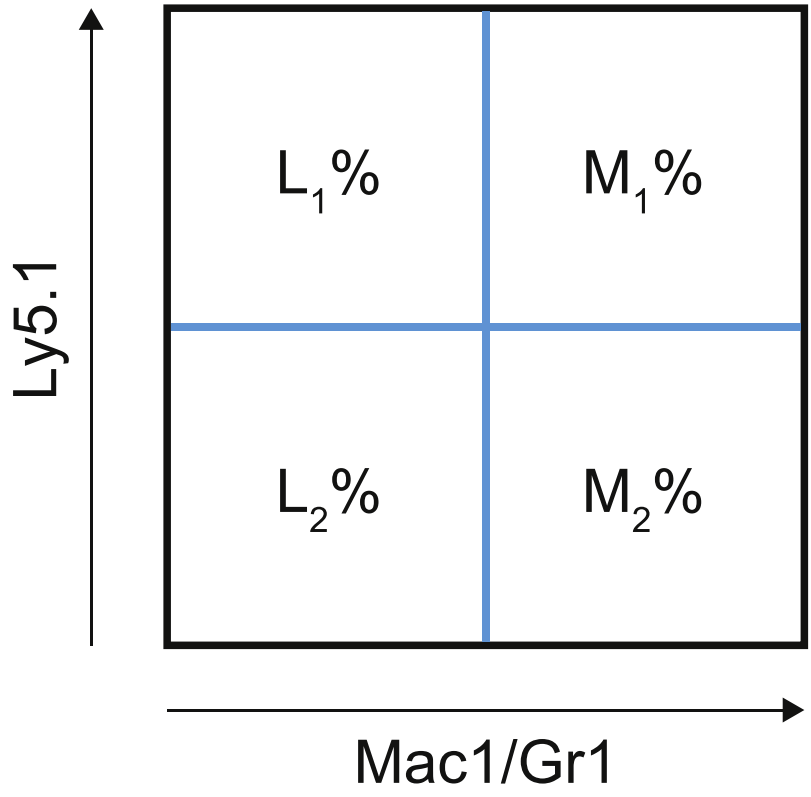

Muller-Sieburg Criteria

Calculated 20 wks after transplanatation

$L/M=L_1/M_1$

My-bi HSC: L/M < 3

Bala HSC: 3< L/M < 10

Ly-bi HSC: 10<L/M

Eaves Criteria

Calculated 16 wks after transplanatation

$$M/L = \frac{M1/(M1+M2)}{L1/(L1+L2)}$$

$$= \%M/\%L$$

$$\Rightarrow \%M/(\%B + \%T)$$

α cells: 2 < M/L

β cells: 0.25 < M/L < 2

γ cells: M/L < 0.25

Supplementary Figure E1. Calculation of lymphoid to myeloid (L/M) and M/L ratios. Flow cytometry data are shown. Ly5.1-positive cells were used as test donor cells. Myeloid cells were detected by anti–Mac-1 and anti–Gr-1 antibodies. The L/M ratio was calculated as L_1/M_1. The M/L ratio was calculated as (% Myeloid chimerism) / (% Lymphoid chimerism). *% Myeloid chimerism* is defined as 100 M_1 / (M_1 + M_2). *% Lymphoid chimerism* is defined as 100 L_1 / (L_1 + L_2). These L_1, L_2, L_3 are not actually measured. Instead, 100 B_1 / (B_1 + B_2) and 100 T_1 / (T_1 + T_2) are calculated for % B cells and % T cell, respectively (B_1, B_2, B_3, T_1, T_1, and T_1 are not shown in this figure), and % L is replaced by (% B cells + % T cells) [16].

A

$$\frac{10\ \alpha + 3\ \beta + 1\ \gamma}{14\ \text{My-bi}} \qquad \frac{4\ \beta}{4\ \text{Bala}} \qquad \frac{12\ \gamma}{12\ \text{Ly-bi}}$$

$$\frac{7\ \text{LT} + 6\ \text{IT} + 1\ \text{ST}}{14\ \text{My-bi}} \qquad \frac{3\ \text{IT} + 1\ \text{LT}}{4\ \text{Bala}} \qquad \frac{11\ \text{ST} + 1\ \text{IT}}{12\ \text{Ly-bi}}$$

B

$$\frac{10\ \text{My-bi}}{10\ \alpha} \qquad \frac{3\ \text{My-bi} + 4\ \text{Bala}}{7\ \beta} \qquad \frac{1\ \text{My-bi} + 12\ \text{Ly-bi}}{13\ \gamma}$$

$$\frac{5\ \text{LT} + 4\ \text{IT} + 1\ \text{ST}}{10\ \alpha} \qquad \frac{2\ \text{LT} + 5\ \text{IT}}{7\ \beta} \qquad \frac{1\ \text{LT} + 1\ \text{LT} + 11\ \text{ST}}{13\ \gamma}$$

C

$$\frac{7\ \text{My-bi} + 1\ \text{Bala}}{8\ \text{LT}} \qquad \frac{6\ \text{My-bi} + 3\ \text{Bala} + 1\ \text{Ly-bi}}{10\ \text{IT}} \qquad \frac{1\ \text{My-bi} + 11\ \text{Lybi}}{12\ \text{ST}}$$

$$\frac{5\ \alpha + 2\ \beta + 1\ \gamma}{8\ \text{LT}} \qquad \frac{4\ \alpha + 5\ \beta + 1\ \gamma}{10\ \text{IT}} \qquad \frac{1\ \alpha + 11\ \gamma}{12\ \text{ST}}$$

Supplementary Figure E2. Relationships of HSCs classified by three systems. All relationships of My-bi, Bala, and Ly-bi HSCs, α, β, and γ/δ cells, and LT-, IT-, and ST-HSCs are shown. **(A)** My-bi, Bala, Ly-bi HSCs (denominators) were reclassified as α, β, and γ/δ cells or LT-, IT-, and ST-HSCs (numerators). **(B)** α, β, and γ/δ cells (denominators) were reclassified as My-bi, Bala, Ly-bi HSCs or LT-, IT-, and ST-HSCs (numerators). **(C)** LT-, IT-, and ST-HSCs (denominators) were reclassified as My-bi, Bala, Ly-bi HSCs or α, β, and γ/δ cells (numerators).