The Journal of Experimental Medicine

Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment

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Hematopoietic stem cells (HSCs) have been extensively characterized based on functional definitions determined by experimental transplantation into lethally irradiated mice. In mice, HSCs are heterogeneous with regard to self-renewal potential, in vitro colony-forming activity, and in vivo behavior. We attempted prospective isolation of HSC subsets with distinct properties among CD34$^{-/low}$ c-Kit^{+}Sca-1^{+}Lin^{-} (CD34^{-}KSL) cells. CD34^{-}KSL cells were divided, based on CD150 expression, into three fractions: CD150^{high}, CD150^{med}, and CD150^{neg} cells. Compared with the other two fractions, CD150^{high} cells were significantly enriched in HSCs, with great self-renewal potential. In vitro colony assays revealed that decreased expression of CD150 was associated with reduced erythroblast/megakaryocyte differentiation potential. All three fractions were regenerated only from CD150^{high} cells in recipient mice. Using single-cell transplantation studies, we found that a fraction of CD150^{high} cells displayed latent and barely detectable myeloid engraftment in primary-recipient mice but progressive and multilineage reconstitution in secondary-recipient mice. These findings highlight the complexity and hierarchy of reconstitution capability, even among HSCs in the most primitive compartment.

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Abbreviations used: APC, allophycocyanin; CD34^{-}KSL, CD34$^{-/low}$ c-Kit^{+}Sca-1^{+}Lin^{-}; HSC, hematopoietic stem cell; LMPP, lymphoid-primed multipotent progenitor; LTRC, long-term repopulating cell; nmEM, neutrophil/macrophage/erythroblast/megakaryocyte.

Long-term multilineage reconstitution of lethally irradiated mouse bone marrow has been used as a gold standard to define the functionality of hematopoietic stem cells (HSCs). Based on this, HSCs have been studied extensively, yielding great contributions to the field of stem cell biology. The development of strategies for prospective isolation of HSCs has made in vivo clonal analysis of HSCs possible over the last decade. Analyses of individual purified HSCs or limiting doses of whole bone marrow transplanted into irradiated animals have revealed marked functional heterogeneity in HSCs with regard to repopulating activity, self-renewal activity, and in vitro colony-forming activity (Abkowitz et al., 2000; Müller-Sieburg et al., 2002, 2004; Uchida et al., 2003; Takano et al., 2004; Ema et al., 2005, 2006; Sieburg et al., 2006; Dykstra et al., 2007). Different patterns in lineage reconstitution by individual HSCs have also been observed (Uchida et al., 2003; Müller-Sieburg et al., 2004; Sieburg et al., 2006; Dykstra et al., 2007). Of interest is that the donor-derived myeloid/lymphoid ratio in reconstituted mice reportedly indicates the degree of self-renewal potential in transplanted HSCs (Müller-Sieburg et al., 2004; Dykstra et al., 2007). If a variety of HSCs exist, HSCs may exhibit and define hierarchical organization within the most primitive hematopoietic compartment. Alternatively, heterogeneity of HSCs may be generated during development of the hematopoietic system and remain fixed thereafter. To further address questions of functional diversity and hierarchy in HSCs, HSC subsets with distinct properties must first be isolated prospectively.

CD34$^{-/low}$ c-Kit^{+}Sca-1^{+}Lin^{-} (CD34^{-}KSL) cells in mouse bone marrow are highly enriched in adult HSCs (Osawa et al., 1996; Sudo et al., 2000; Matsubara et al., 2005; Morita et al., 2006). To identify candidate cell-surface markers that could prospectively identify functionally distinct HSCs, we screened a large number of antibodies and identified those with heterogeneous staining patterns on CD34^{-}KSL cells. These candidates were then tested for functional differences in vivo. In this way, we found that expression of CD150 (Kiel et al., 2005)

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The Rockefeller University Press $30.00
J. Exp. Med. Vol. 207 No. 6 1173-1182
www.jem.org/cgi/doi/10.1084/jem.20091318

could be used to enrich for long-term repopulating cells (LTRCs) with distinct reconstitution kinetics patterns. CD34^{-}KSL cells were subdivided into CD150^{high}, CD150^{med}, and CD150^{neg} fractions, and the functions of these cells were compared at the clonal level using single-cell transplantation and cultures.

While performing this study, we noticed the existence of a very rare type of LTRCs. These cells were named "latent HSCs" and were operationally defined as cells that exhibited significant engraftment only after 12 wk or more in the primary recipient yet showed multilineage reconstitution in the secondary recipient. Given that these cells could fully reconstitute the secondary recipients, they are assumed to have high reconstitution potential. However, they do not satisfy (at the level of primary transplantation) some of the criteria that currently define HSCs.

Our results indicate that differences in reconstitution activity are caused by intrinsic differences among cells and that classes of HSCs with distinct in vivo behavior can be prospectively isolated. We also report the discovery of a very slowly engrafting, myeloid-biased HSC whose full potential can be seen only after secondary transplantation. The existence of latent HSCs requires reconsideration of the concept of HSCs and leads us to propose a revision of the current criteria for HSCs.

RESULTS

Fractions in CD34^{-}KSL cells

As in previous studies (Abkowitz et al., 2000; Müller-Sieburg et al., 2002, 2004; Uchida et al., 2003; Sieburg et al., 2006; Dykstra et al., 2007), our single-cell transplantation experiments yielded data showing that CD34^{-}KSL cells display heterogeneous reconstitution patterns in primary- and secondary-recipient mice (Ema et al., 2005). For instance, there are HSCs that sequentially reconstitute myeloid, B lymphoid, and T lymphoid lineages in primary-recipient mice, followed by multilineage reconstitution that is maintained in secondary-recipient mice; HSCs that predominantly reconstitute myeloid lineage in primary-recipient mice, followed by greater levels of multilineage reconstitution in secondary-recipient mice; and HSCs that predominantly reconstitute lymphoid lineage in primary-recipient mice, followed by markedly reduced levels of reconstitution in secondary-recipient mice. To achieve greater purification of HSCs, and to identify cell-surface markers that distinguish HSCs with heterogeneous reconstitution behaviors, CD34^{-}KSL cells were costained with a comprehensive list of additional antibodies (Table S1) and analyzed by flow cytometry (Fig. 1; and Figs. S1 and S2).

In this study, 118 cell-surface markers listed in Table S1 were examined. Among them, 81 markers appeared not to be expressed on CD34^{-}KSL cells. Most of these were not expressed on CD34^{+}KSL cells either, with some notable exceptions, as shown in Fig. S1. Markers with heterogeneous expression in CD34^{+}KSL cells include CD48 and CD244, members of the Slam family recognized as negative markers for HSCs (Kiel et al., 2005), and CD135, used to isolate lymphoid-primed multipotent progenitors (LMPPs; Adolfsson et al., 2001, 2005). These markers, as well as CD11a (LFA1), CD62L (L-selectin), and CD138 (Syndecan1), can be used for better discrimination of CD34^{-} from CD34^{+} cells within the KSL population. Whether N-cadherin is expressed on HSCs remains somewhat controversial (Zhang et al., 2003; Kiel et al., 2007, 2008; Haug et al., 2008). In our analysis, CD34^{-}KSL cells did not express N-cadherin, as additionally demonstrated in Fig. S1.

On the other hand, 22 markers were expressed on CD34^{-}KSL cells, as depicted in Fig. S2. Although most such markers were expressed on both CD34^{-} and CD34^{+}KSL cells, expression of CD27, CD201, and c-Mpl seemed down-regulated on some CD34^{+}KSL cells. This is consistent with earlier reports in which CD201 and c-Mpl were used as positive markers for HSC purification (Balazs et al., 2006; Yoshihara et al., 2007). Only 15 markers showed variable expression on CD34^{-}KSL cells. We were particularly interested in this group of markers because their use permitted us to test fractions of CD34^{-}KSL cells to determine whether they might represent functionally distinct subsets of HSCs. These candidates were AA4.1, CD1d, CD11b, CD18, CD31, CD38, CD49b, CD51, CD61, CD86, CD103, CD147, CD150, FcγR, and Tie2 (Fig. 1).

Based on expression of these antigens, CD34^{-}KSL cells were sorted into positive and negative fractions by flow cytometry. In the case of CD38, CD34^{-}KSL cells were separated into CD38^{high} and CD38^{med} fractions, which amounted to 22.4 $\pm$ 3.8% and 56.1 $\pm$ 5% of CD34^{-}KSL cells, respectively (n = 5; mean $\pm$ SD); the CD38^{neg} fraction was barely detectable. In the case of CD150, CD34^{-}KSL cells were separated into CD150^{high}, CD150^{med}, and CD150^{neg} fractions (Fig. 1). These represented 26 $\pm$ 5.6%, 24 $\pm$ 2.9%, and 14.6 $\pm$ 3.6% of CD34^{-}KSL cells, respectively (n = 8; mean $\pm$ SD); CD150 expression levels varied widely among CD34^{-}KSL cells.

We next used transplantation to compare the in vivo function of fractions of CD34^{-}KSL cells. Each of a group of lethally irradiated mice received 10 cells from these individual fractions along with 2×10^5 competitor cells. Recipient mice were analyzed between 16 and 20 wk after transplantation. As shown in Fig. 2, long-term (16 wk or more) repopulating activity was detected in both the positive and negative fractions in all cases, suggesting that none of these markers is particularly useful for excluding non-LTRCs from CD34^{-}KSL cells. However, we found significant differences in the percentage of chimerism among fractions separated on the basis of expression of CD38, CD147, CD150, and Tie2 (Fig. 2).

We observed particularly interesting reconstitution kinetics patterns in the cases of CD150 and CD38. CD150^{high}CD34^{-}KSL cells exhibited low levels of reconstitution in the early phase after transplantation, but reconstitution levels gradually increased thereafter (15 out of 19; Fig. 3). CD150^{med}CD34^{-}KSL cells exhibited relatively high levels of reconstitution in the early phase, and these levels were maintained (11 out of 19). CD150^{neg}CD34^{-}KSL cells exhibited low levels of reconstitution in the early phase without change thereafter (9 out of 14),

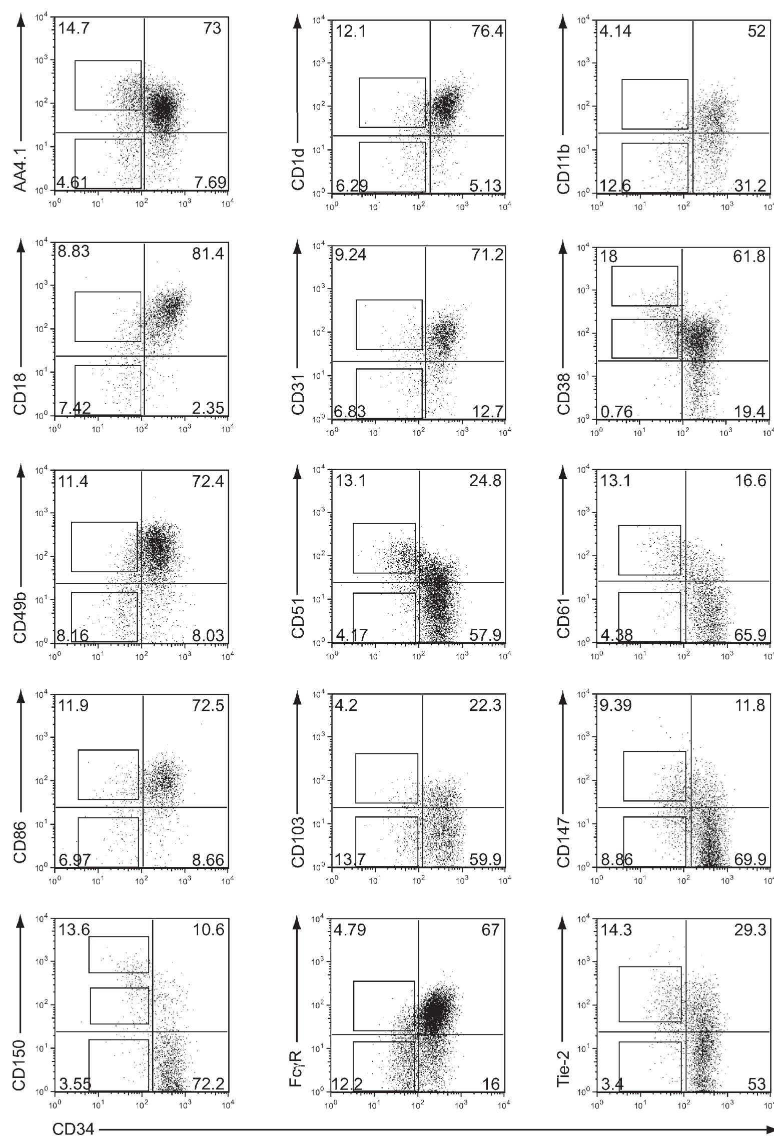


Figure 1. Markers with heterogeneous expression in CD34⁻KSL cells. KSL cells were stained with FITC-conjugated anti-CD34 antibody and additional PE-conjugated antibodies as shown. Shown are flow cytometric profiles for the markers that had heterogeneous expression within CD34⁻KSL cells (percentages are shown). Marker-positive or -negative cells were separated by using the sorting gates (shown as squares). In the case of CD150, CD34⁻KSL cells were separated into CD150^{high}, CD150^{med}, and CD150^{neg} fractions. In the case of CD38, CD34⁻KSL cells were separated into CD38^{high} and CD38^{med} fractions. The data represent four to eight independent experiments.

with the exception of one case in which the level of reconstitution gradually increased. Lineage compositions in reconstituted mice significantly differed between recipients of CD150^{high} and recipients of CD150^{neg}CD34⁻KSL cells ($P < 0.0001$ by the *t* test; Fig. 3). When CD150^{high}CD34⁻KSL cells were transplanted, the myeloid lineage was more reconstituted than the lymphoid lineage. In contrast, when CD150^{neg}CD34⁻KSL cells were transplanted, the lymphoid lineage was more reconstituted than the myeloid lineage. CD150^{med}CD34⁻KSL cells gave rise to a seemingly intermediate pattern between those obtained using CD150^{high} cells and those obtained using CD150^{neg} cells. In addition, the percentages of chimerism 16 wk after transplantation with CD150^{med}CD34⁻KSL cells were significantly greater than those with CD150^{high} or CD150^{neg}CD34⁻KSL cells (Fig. 2). CD38^{high} and CD38^{med}CD34⁻KSL cells exhibited reconstitution kinetics similar to CD150^{high} and CD150^{neg}CD34⁻KSL cells, respectively (unpublished data).

The similarity in reconstitution pattern between CD38^{high} and CD150^{high} CD34⁻KSL cells prompted us to examine simultaneously the expression of these two markers on CD34⁻KSL cells. As shown in Fig. S3, most CD38^{high}CD34⁻KSL cells expressed CD150 strongly. These data let us focus on CD150 as a marker that might permit identification of distinct HSC subsets.

Identification of distinct LTRCs

To compare the frequencies and functional properties of LTRCs within CD150^{high}, CD150^{med}, and CD150^{neg}CD34⁻KSL cells, single-cell transplantation was performed. Cells were individually transplanted into each of a total of 40 lethally irradiated mice together with 2×10^5 competitor cells in two independent experiments. 6–7 mo later, secondary transplantation was performed by injecting 5×10^6 bone marrow cells from reconstituted recipient mice into three to five lethally irradiated mice.

During these experiments, we made an important observation. Some LTRCs showed very low levels of myeloid reconstitution in primary-recipient mice but exhibited much higher reconstitution levels in secondary-recipient mice. In this study, therefore, we considered reconstitution to have been achieved when the percentage of donor chimerism was 0.3% or more at one time point or more after transplantation, regardless of which lineage was reconstituted.

Results of primary and secondary transplantation with single cells are shown in Fig. 4. In the three 40-mouse cohorts that were primary recipients of CD150^{high}, CD150^{med}, and CD150^{neg}CD34⁻KSL cells, LTRC activity was detected in 16, 13, and 13 mice, respectively. The similar rates of reconstitution suggest that LTRCs are equally present among CD150^{high}, CD150^{med}, and CD150^{neg}CD34⁻KSL cells.

Results of secondary transplantation differed remarkably, however. After secondary transplantation, LTRCs were detected in all of the recipient mice transplanted from primary-recipient mice that had initially received CD150^{high}CD34⁻KSL cells (Fig. 4 A). In most recipient mice, hematopoiesis was sustained 5 mo after secondary transplantation. Unfortunately,

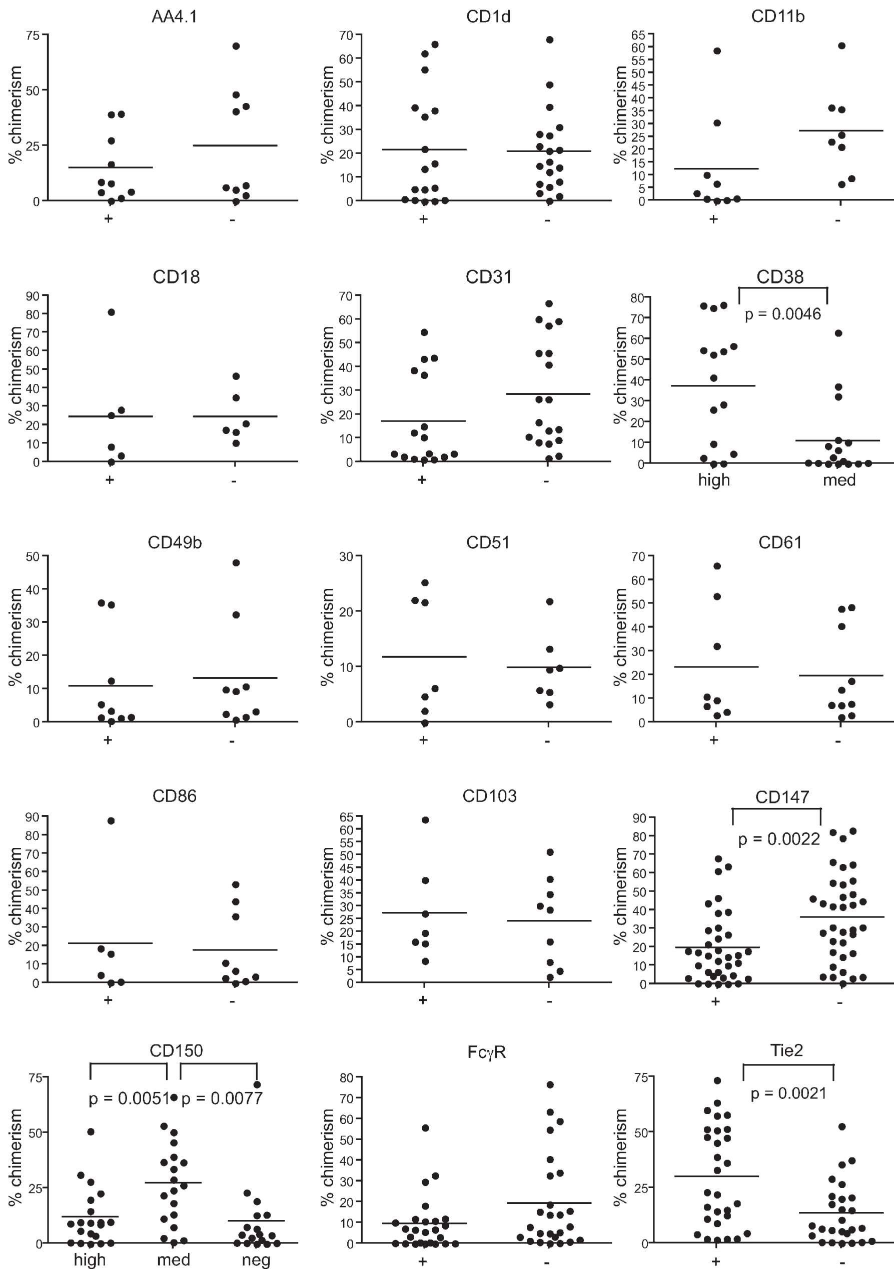


Figure 2. Long-term reconstitution by fractions of CD34⁻KSL cells. CD34⁻KSL cells were subdivided into fractions positive and negative for additional markers. 10 cells from each fraction were transplanted into each member of a group of lethally irradiated mice along with 2×10^5 competitor cells. Recipient mice were analyzed 4–5 mo after transplantation. Chimerism levels for all individuals in each group of mice are shown. Horizontal lines represent means.

three primary-recipient mice in this cohort could not be used as donors for secondary transplantation because they died early.

Among the primary recipients of CD150^{med} and CD150^{neg} CD34$^-$KSL cells, one and two mice died, respectively. LTRCs were detected in 4 out of 13 and in 2 out of 12 cohorts of secondary-recipient mice whose donors had initially received CD150^{med} and CD150^{neg}CD34$^-$KSL cells, respectively. Consistent with data from 10-cell transplantation experiments (Fig. 3), CD150^{high}CD34$^-$KSL cells predominantly reconstituted the myeloid lineage, whereas CD150^{neg}CD34$^-$KSL cells predominantly reconstituted the lymphoid lineage (Fig. 4).

With respect to in vivo reconstitution kinetics, percentages of chimerism progressively increased throughout primary and secondary transplantation in 10 out of 13 cases initiated with single CD150^{high}CD34$^-$KSL cells. When CD150^{med}CD34$^-$KSL cells were transplanted, this progressive increase occurred in only 1 out of 13 cases, and when CD150^{neg}CD34$^-$KSL cells were transplanted, no such instance was observed out of 12 cases. From these data, we concluded that CD150^{high}CD34$^-$KSL cells are greatly enriched in highly self-renewing LTRCs.

Noteworthy is that a latent and a myeloid-limited type of LTRCs existed among CD150^{high}CD34$^-$KSL cells. Cells of the latent type represented 1 out of every 10 CD150^{high}CD34$^-$KSL cells (approximately one in a million total bone marrow cells) and produced virtually undetectable levels of blood cells for several months after transplantation. Beginning at 12 wk or later, these LTRCs effected a low level of myeloid reconstitution (Fig. 4 A, cases #3, #5, #6, and #16). However, these cells showed progressive and robust repopulating activity in secondary-recipient mice. Cells of the myeloid-limited type represented 1 out of 20 CD150^{high}CD34$^-$KSL cells and exhibited a low level of myeloid reconstitution without lymphoid reconstitution during primary and secondary transplantation (Fig. 4 A, cases #7 and #15).

Figs. S4–S6 show reconstitution kinetics in individual secondary-recipient mice of bone marrow cells reconstituted with single CD150^{high}, CD150^{med}, and CD150^{neg} CD34$^-$KSL cells. When high levels of reconstitution took place in primary-recipient mice, high levels of reconstitution were achieved in all secondary-recipient mice (e.g., cases #1 and #2; Fig. S4). As previously reported (Müller-Sieburg et al., 2002; Dykstra et al., 2007), reconstitution patterns were very similar among secondary-recipient mice in each group. However, in the case of latent LTRCs, various levels of reconstitution were observed for secondary-recipient

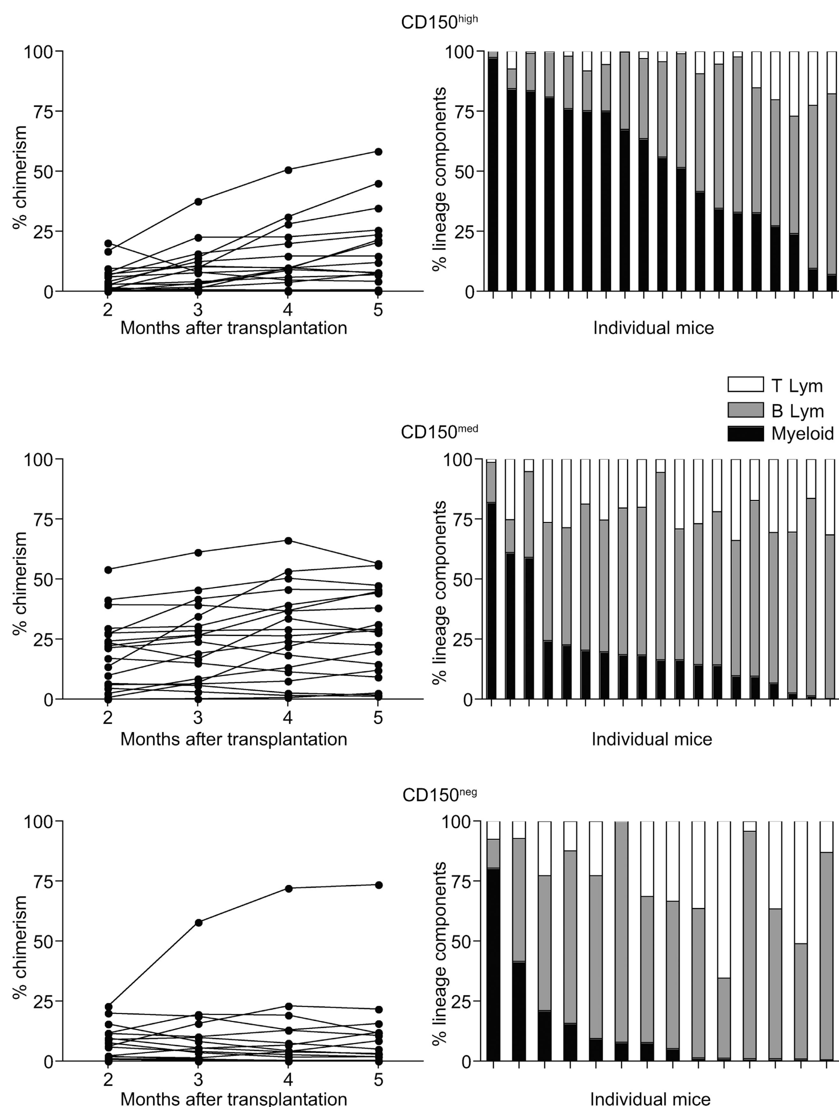


Figure 3. Reconstitution kinetics with CD150^{high}, CD150^{med}, and CD150^{neg} CD34$^-$KSL cells. Each of a group of lethally irradiated mice received 10 CD150^{high} cells, 10 CD150^{med} cells, or 10 CD150^{neg}CD34$^-$KSL cells. (left) The change in the percentage of chimerism over time. Blood of recipient mice was analyzed 2, 3, 4, and 5 mo after transplantation. Each line shows a change in the percentage of chimerism from one recipient mouse. (right) The relative myeloid, B lymphoid, and T lymphoid lineage contributions in reconstituted donor-derived blood cells of individual recipient mice 4 mo after transplantation. Myeloid lineage represents 53.7 ± 27.2% (n = 19), 22 ± 21.8% (n = 19), and 13 ± 21.7% (n = 14; mean ± SD) after transplantation with CD150^{high}, CD150^{med}, and CD150^{neg}CD34$^-$KSL cells, respectively. B lymphoid lineage represents 38.2 ± 21.5% (n = 19), 56.2 ± 17.9% (n = 19), and 60.7 ± 21.5% (n = 14; mean ± SD) after transplantation with CD150^{high}, CD150^{med}, and CD150^{neg}CD34$^-$KSL cells, respectively. T lymphoid lineage represents 8 ± 8.4% (n = 19), 21.8 ± 9.4% (n = 19), and 26.3 ± 19.4% (n = 14; mean ± SD) after transplantation with CD150^{high}, CD150^{med}, and CD150^{neg}CD34$^-$KSL cells, respectively. The proportion of myeloid lineage reconstitution by CD150^{high} CD34$^-$KSL cells was significantly greater than that of CD150^{neg}CD34$^-$KSL cells ($P < 0.0001$).

mice in each group (e.g., cases #6 and #16; Fig. S4). Furthermore, reconstitution patterns differed remarkably among recipient mice. Although only myeloid-lineage reconstitution was detected in primary-recipient mice of latent LTRCs, B and T lymphoid lineages became readily detectable in most secondary-recipient mice.

Dykstra et al. (2007) recently proposed that four types of LTRCs exist based on their observations in single-cell transplantation. According to their classification, α cells show a myeloid/lymphoid contribution ratio of >2:1, β cells show a balanced ratio between 1:4 and 2:1, γ cells show a myeloid/lymphoid contribution ratio of <1:4, and δ cells show a ratio of <1:4, with < 1% myeloid reconstitution. As shown in Fig. S7, 75% of CD150^{high}CD34$^-$KSL LTRCs qualified as α cells and the remaining cells qualified as β cells. On the other hand, 100% of CD150^{neg}CD34$^-$KSL LTRCs qualified as γ or δ cells. Among CD150^{med}CD34$^-$KSL LTRCs, 15% were assigned to each of the α, β, and γ cell categories, with the remaining cells assigned as δ cells. Collectively, these data suggest a continuum of expression of CD150 among CD34$^-$KSL cells, where CD150 expression is tightly associated with repopulation kinetics and with self-renewal potential.

Identification of balanced LTRCs

On single-cell transplantation, CD150^{med} CD34$^-$KSL cells did not show any unique properties as compared with CD150^{high} or CD150^{neg} CD34$^-$KSL cells. Also, the percentage of balanced LTRCs, like β cells, in all single-cell transplants was less than that in previous studies (Ema et al., 2005; Dykstra et al., 2007). In the single-cell experiments described in the previous section, we set a rather narrow gate for the CD150^{med}CD34$^-$KSL cells to see the difference more clearly, as in Fig. 1 (bottom). Although a relatively large number of cells lay between the CD150^{med} and CD150^{high} CD34$^-$KSL fractions, these cells were not sorted. We assumed that balanced LTRCs are mostly in this population. To address the effects of gating, we performed two independent single-cell transplantation experiments. In these experiments, gates were set to sort CD34$^-$KSL cells into four fractions: CD150^{high}, CD150^{int}, CD150^{low}, and CD150^{neg}, as shown in Fig. S8 A. LTRCs were detected in 11, 12, 10, and 9 recipient mice of CD150^{high}, CD150^{int}, CD150^{low}, and CD150^{neg}CD34$^-$KSL cells, respectively. Results from the CD150^{high} and CD150^{neg} transplants were nearly identical to the initial single-cell

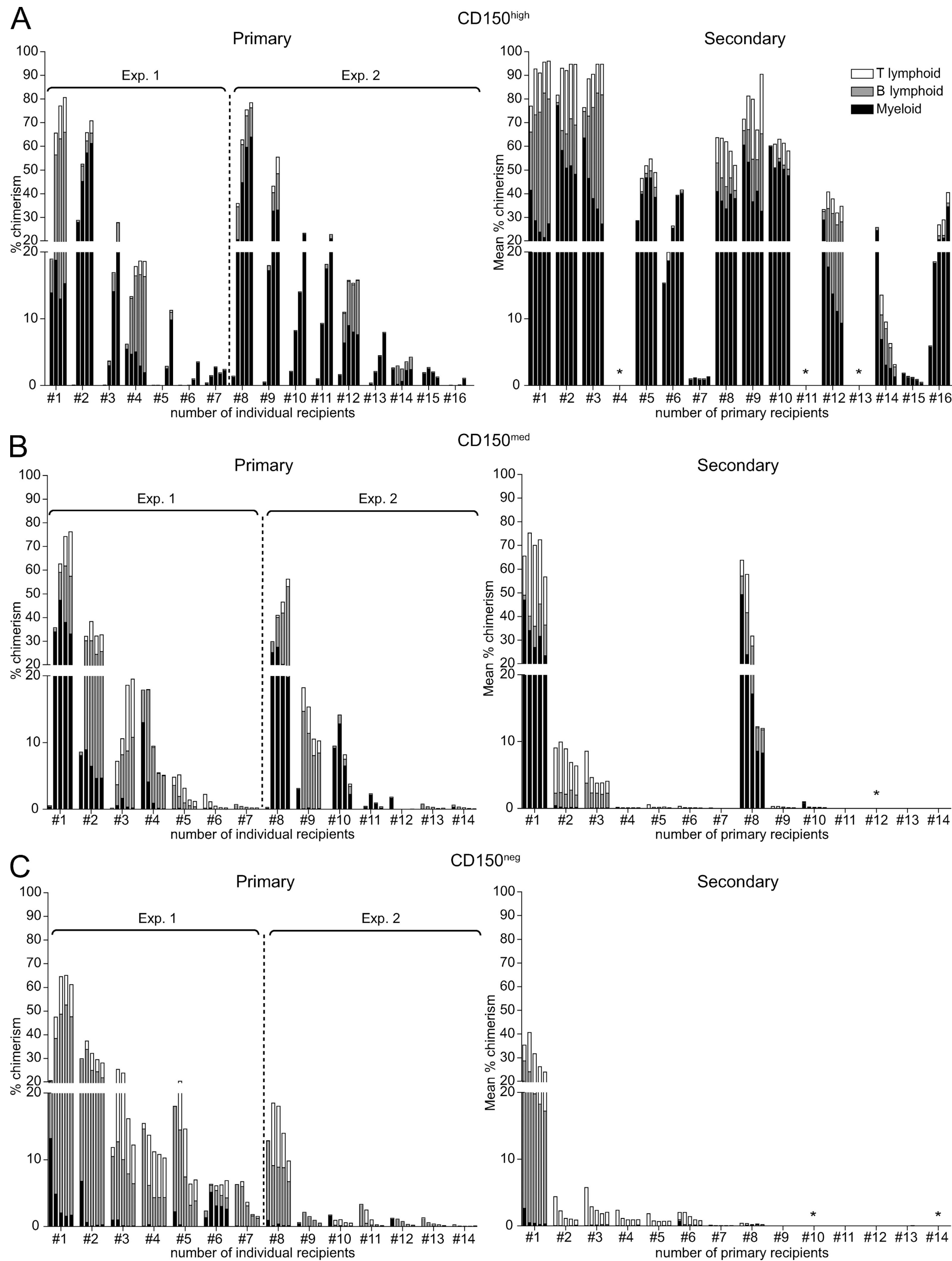


Figure 4. Single-cell transplantation. (A–C) Single CD150^{high}CD34⁻KSL (A), CD150[med]CD34⁻KSL (B), and CD150^{neg}CD34⁻KSL cells (C) were transplanted into 40 lethally irradiated mice together with 2×10^5 competitor cells. Blood of recipient mice was periodically analyzed 1, 2, 3, 4, and 5 mo after transplantation. Secondary transplantation was performed using 5×10^6 reconstituted bone marrow cells of recipient mice. Blood of secondary-recipient mice was periodically analyzed 1, 2, 3, 4, and 5 mo after transplantation. Data from secondary transplantation show the mean percentage of chimerism (n = 3–5). The five sequential bars for each recipient mouse indicate the percentage of chimerism 1, 2, 3, 4, and 5 mo after transplantation. *, mice that died before secondary transplantation.

transplantation experiments (Fig. S7 B). On the other hand, CD150^{int}CD34⁻KSL LTRCs exhibited high levels of chimerism (Fig. S8 B), and the majority (7 out of 12) were classified as β cells, revealing that large numbers of balanced LTRCs were present just beneath the CD150^{high}CD34⁻KSL cells. These data provide further evidence that functionally distinct LTRCs can be identified according to their levels of CD150 expression.

Colony-forming ability in distinct LTRCs

Single-cell liquid cultures were performed to compare in vitro colony-forming abilities among CD150^{high}, CD150^{med}, and CD150^{neg}CD34^{-}KSL cells. Cells were individually cultured (48 cells from each fraction) for 14 d in the presence of stem cell factor, thrombopoietin, IL-3, and erythropoietin.

Frequencies of colony formation were similar among these fractions (Fig. 5). The colony types formed, however, differed markedly. On average, 60% of CD150^{high}CD34^{-}KSL cells formed neutrophil/macrophage/erythroblast/megakaryocyte (nmEM) colonies, whereas only 7% of CD150^{neg}CD34^{-}KSL cells did so. In contrast, 66% of CD150^{neg}CD34^{-}KSL cells formed neutrophil/macrophage colonies, whereas 12% of CD150^{high}CD34^{-}KSL cells did so. CD150^{med}CD34^{-}KSL cells seemed to exhibit activity in between that of CD150^{high} and CD150^{neg}CD34^{-}KSL cells. These results suggest that the loss of erythroblast/megakaryocyte differentiation potential is associated with loss of CD150 expression within CD34^{-}KSL cells.

Hierarchical order in distinct LTRCs

From transplantation data, we predicted that CD150^{high} cells reside in the uppermost portion of the hematopoietic hierarchy. To assess this, we evaluated the reversibility of CD150 expression. Three cohorts of three mice that had been reconstituted with 10 CD150^{high}, CD150^{med}, or CD150^{neg}CD34^{-}KSL cells were analyzed 8 mo after transplantation. Flow cytometric analysis was performed on bone marrow cells from these mice to assess CD150 expression on reconstituted CD34^{-}KSL cells. To distinguish test donor-derived cells from competitor- and recipient-derived cells, only Ly5.1^{+}Ly5.2^{-} cells were gated and analyzed. As shown in Fig. 6, test donor-derived CD150^{high}CD34^{-}KSL cells were detected in each of the three mice transplanted with CD150^{high}CD34^{-}KSL cells. The CD150 expression pattern in the reconstituted CD34^{-}KSL cells mimicked that seen in freshly isolated CD34^{-}KSL cells. Very few CD150^{high}CD34^{-}KSL cells were detected in one out of three mice transplanted with CD150^{med}CD34^{-}KSL cells. Test donor-derived KSL cells were not detected in three mice transplanted with CD150^{neg}CD34^{-}KSL cells (unpublished data). These data support our hypothesis that CD150^{high} cells rank highly among CD34^{-}KSL cells.

DISCUSSION

Single-cell culture and transplantation are powerful assays for the characterization of individual HSCs (Ema et al., 2000, 2005, 2006; Takano et al., 2004; Seita et al., 2007). Our experience with >2,000 single-cell transplantations of CD34^{-}KSL cells leads us to expect engraftment in 20–50% of recipients. Since establishment of this experimental system, we have been searching for markers useful to further enrich for HSCs. During the course of these studies, we have found, even among CD34^{-}KSL cells, extensive heterogeneity in reconstitution activity that could be identified only by clonal analysis. This study challenges received ideas on the prospective isolation and identification of HSC subsets. We identified three distinct subsets of LTRCs with regard to repopulating activity, self-renewal potential, in vitro colony-forming activity, and in vivo kinetics after transplantation.

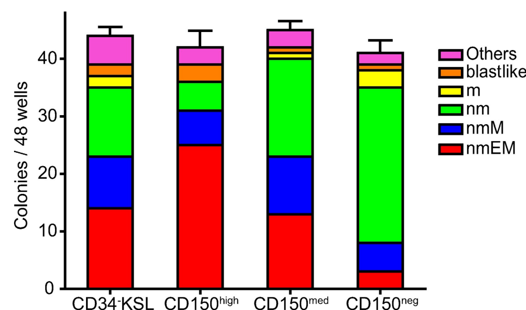


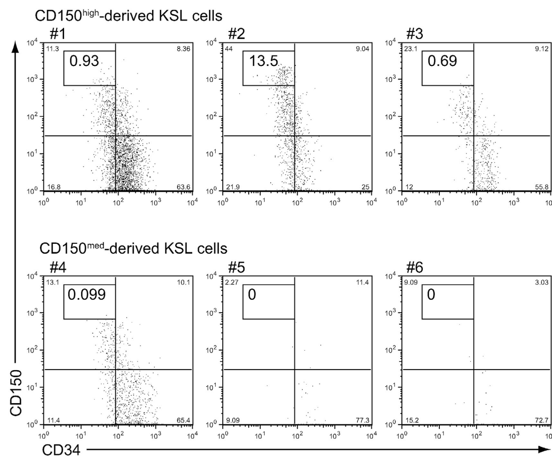
Figure 5. Colony formation by single cells. Single-cell cultures were performed using CD34^{-}KSL, CD150^{high}CD34^{-}KSL, CD150^{med}CD34^{-}KSL, and CD150^{neg}CD34^{-}KSL cells. 48 CD34^{-}KSL, CD150^{high}CD34^{-}KSL, CD150^{med}CD34^{-}KSL, and CD150^{neg}CD34^{-}KSL cells formed 44 ± 3, 42 ± 5, 45 ± 3, and 41 ± 4 colonies, respectively (n = 3; mean ± SD). E, erythroblast; m, macrophage; M, megakaryocyte; n, neutrophil.

CD150^{high}CD34^{-}KSL cells were highly enriched in LTRCs, with greater self-renewal potential than cells in the other two subsets (Figs. 3 and 4). The repopulating activity increased gradually from primary through secondary transplantation. Myeloid reconstitution significantly dominated lymphoid reconstitution. On average, 87.5% of CD150^{high}CD34^{-}KSL cells formed colonies in vitro. More than half of the colonies included all nmEM elements. These data imply an association among high self-renewal potential, myeloid-biased reconstitution, and nmEM differentiation potential.

CD150^{neg}CD34^{-}KSL cells were highly enriched in LTRCs, with limited self-renewal potential (Figs. 3 and 4). The repopulating activity decreased from primary through secondary transplantation. Lymphoid reconstitution significantly dominated myeloid reconstitution. Only limited myeloid reconstitution was observed in some cases. On average, 85% of CD150^{neg}CD34^{-}KSL cells formed colonies in vitro, and 73% of these colonies consisted of only neutrophil/macrophage or macrophage elements. These data imply an association among low self-renewal potential, lymphoid-biased reconstitution, and loss of erythroid and megakaryocytic potential.

Kent et al. (2009) recently reported that HSCs with higher repopulating activity are enriched in the CD150^{+} subset of EPCR^{+}CD48^{-}CD45^{+} bone marrow cells, whereas those in the CD150^{-} subset have limited self-renewal activity. These data all support our hypothesis that CD150 expression levels can be used prospectively to separate distinct types of LTRCs.

In this study, we found latent LTRCs among CD150^{high} CD34^{-}KSL cells that exhibited a very late onset of repopulation (>4 mo after transplant) despite exposure to the "cytokine storm" presumed to rage in the early phase of reconstitution. Cells similar to LTRCs of the latent type have been described by some groups (Ortiz et al., 1999; Dykstra et al., 2007). The existence of such LTRCs challenges the current experimental definition of HSCs: multilineage reconstitution in primary



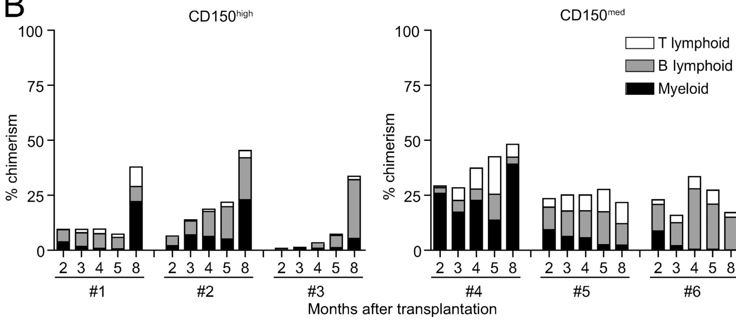


Figure 6. Regeneration of CD150^{high}CD34^{-}KSL cells after transplantation. (A) Three recipient mice reconstituted with 10 CD150^{high} or CD150^{med}CD34^{-}KSL cells were analyzed 8 mo after transplantation. CD34 and CD150 expression by KSL bone marrow cells derived from CD150^{high}CD34^{-}KSL or CD150^{med}CD34^{-}KSL cells is shown (percentages are shown). (B) Reconstitution kinetics of recipient mice described in A.

recipients 12–16 wk after transplantation. Of importance is that, in addition to this late onset of engraftment, these cells do not always show multilineage reconstitution at a particular point after transplantation. Because of these unique properties, these cells are not likely defined as HSCs by current experimental criteria. Furthermore, we have found mice in which engraftment was not observed until 8 mo after transplant. We have also experienced cases in which multilineage engraftment was confirmed only in the secondary recipients, with no engraftment at all in the primary recipients (unpublished data).

In addition, we have found "myeloid-limited LTRCs" among CD150^{high}CD34^{-}KSL cells. These cells exhibited low-level but continuous myeloid reconstitution for at least 40 wk, with no detectable lymphoid differentiation potential. We speculate that they may be downstream of latent HSCs, because latent HSCs exhibit myeloid-predominant reconstitution in primary-recipient mice and express the same markers as those expressed by myeloid-limited LTRCs. CD150^{high}CD34^{-}KSL cells also contain at least two other types of LTRCs. One exhibits balanced reconstitution during primary and secondary transplantations (Fig. 4 A, cases #1 and #12). The other exhibits myeloid-predominant reconstitution, with high repopulating activity and without delayed reconstitution, during primary and secondary transplantations (Fig. 4 A, cases #2, #8, and #10). The use of additional markers may permit distinction of these functionally distinct LTRCs from among the CD150^{high}CD34^{-}KSL population. In addition, there may be other novel types of HSCs that were not detected by the current assay system. Given such heterogeneity even among the most primitive HSC compartment, the criteria for HSC identification must be redefined. Considering the data presented in this paper, multilineage reconstitution in a secondary transplant recipient appears to be a useful baseline definition for conventional HSCs. This definition would probably include HSCs and define myeloid-limited LTRCs. If this definition is applied, 11 CD150^{high}, 2 CD150^{med} and 1 CD150^{neg}CD34^{-}KSL LTRCs in the initial single-cell transplantation experiments are considered as conventional HSCs. However, this definition requires expensive and time-consuming experiments. Therefore, novel assays that permit rapid and efficient detection of all sorts of HSCs need to be developed.

Of interest is how such HSCs with diverse reconstitution capacity and kinetics are generated. We have shown that HSC dormancy occurs via hibernation (Yamazaki et al., 2006). Although latent HSCs appear to be found primarily within CD150^{high}CD34^{-}KSL cells, because they are so infrequent it is as yet unclear whether they differ from other HSCs in their expression of FOXO transcriptional factors, cell-cycle regulators, and other molecules assumed to be involved in hibernation. Alternatively, different niches may differently regulate HSC hibernation and responses to cytokines. Of particular interest will be to determine to what extent the latent HSCs found in this study are relevant to the recently reported infrequently cycling HSCs among CD150^{+}CD34^{-}KSL cells (Wilson et al., 2008; Foudi et al., 2009). Recent reports of cell-cycle regulation by TGF-β in HSCs (Yamazaki et al., 2008; Challen et al., 2010) raise the possibility of its involvement in the unique behavior of latent HSCs. Understanding the mechanism that controls the latent HSC cell cycle may contribute to exploration of the pathogenesis and treatment of some forms of leukemia in which quiescent leukemic stem cells may be highly resistant to current chemotherapy.

As the heterogeneity of HSCs with respect to their differentiation potentials becomes better understood, hematopoietic differentiation pathways from HSCs to mature blood cells also become more complicated than previously thought (Adolfsson et al., 2005; Arinobu et al., 2007). In particular, early differentiation pathways remain poorly understood. Based on our data from this study, we propose a hierarchical

organization model in which CD150^{high} cells with high self-renewal potential give rise to CD150^{med} cells with moderate self-renewal potential and, finally, to CD150^{neg} cells with very limited self-renewal potential (Fig. S9).

When CD150^{high}, CD150^{med}, and CD150^{neg}CD34^{-}KSL LTRCs in secondary recipients were classified based on the schema in Dykstra et al. (2007), it was interesting to note that their classes shifted toward more lymphoid potential than in primary recipients (Fig. S10). The latent HSCs also exhibited varying reconstitution patterns among secondary recipients in the same groups. These data support our hierarchical organization model.

LMPPs have been shown to have B lymphoid, T lymphoid, and neutrophil/macrophage differentiation potentials without erythroblast/megakaryocyte differentiation potential (Adolfsson et al., 2005). In this study, we showed that CD150^{neg} cells have very similar potential but greater repopulating activity. Thus, it seems likely that CD150^{neg} cells give rise to LMPPs. Reduced expression of CD150 is associated with reduction of self-renewal potential and loss of erythroblast/megakaryocyte differentiation potential, leading to neutrophil/macrophage and lymphoid-lineage specification. Although CD150 expression levels can be used to mark self-renewal potential and myeloid predominance, as yet we have no idea why HSCs with high self-renewal activity express high levels of CD150, nor do we know the function of CD150. Our data imply that self-renewal potential, biased differentiation manner, and reconstitution kinetics are predetermined by an intrinsic program within HSCs. Future studies should clarify the physiological and pathological roles of these HSC subsets in the hematopoietic system.

MATERIALS AND METHODS

Mice. C57BL/6 mice congenic for the Ly5 locus (B6-Ly5.1 mice) were bred and maintained at Sankyo Labo Service. B6-Ly5.2 mice were purchased from Japan SLC. All procedures were approved by the Animal Care and Use Committee, Institute of Medical Science, University of Tokyo.

Screening of antibodies. Antibodies used for screening are listed in Table S1. Bone marrow cells were obtained from 8–10-wk-old B6-Ly5.1 mice and were suspended in PBS. Low-density cells (<1.077 g/ml) were isolated by density gradient centrifugation and were stained with each additional antibody for screening. For staining of FITC- or Alexa Fluor 488–conjugated additional antibodies, CD34^{-}KSL cells were identified by PE-conjugated anti–Sca-1 (D7; eBioscience), PE-Cy7–conjugated anti–c-Kit (2B8; eBioscience), Alexa Fluor 647–conjugated anti-CD34 (RAM-34; eBioscience), and an allophycocyanin (APC)-Cy7–conjugated or a biotinylated anti–lineage marker antibody cocktail (anti-CD4, -CD8, -CD11b, -CD45R, -CD127, –Gr-1, and -TER119 antibodies; eBioscience). The biotinylated anti–lineage marker cocktail was developed using streptavidin-conjugated APC-Cy7.

For staining of PE-conjugated antibodies, as when PE-conjugated anti-CD38 (90) or PE-conjugated anti-CD150 (TC15-12F12.2; BioLegend) was used, CD34^{-}KSL cells were identified by FITC-conjugated anti-CD34 (RAM-34), PE-Cy7–conjugated anti–Sca-1, APC-conjugated anti–c-Kit, and APC-Cy7–conjugated or biotinylated anti–lineage marker antibodies. The biotinylated antibodies of interest were developed with streptavidin-PE. PE-conjugated anti–rabbit IgG was used for visualization of unconjugated antibody. To test anti-CD4, -CD8, -CD11b, -CD45R, -CD127, –Gr-1, or -TER119 antibodies, each antibody was excluded from the lineage marker cocktail. To prepare cells for sorting, low-density cells were depleted of lineage marker–positive cells using magnetic beads (Miltenyi Biotec).

Flow cytometric analysis and sorting were performed on a MoFlo (Beckman Coulter) equipped with solid-state (488-nm) and HeNe (633-nm) lasers, or a FACSVantage SE (BD).

Transplantation. Competitive repopulation assays with 2×10^5 whole bone marrow cells from Ly5.1/Ly5.2-F1 mice were performed in B6-Ly5.2 mice lethally irradiated with two doses of 4.75 Gy, delivered 4 h or more apart. 1 or 10 cells were transplanted per recipient mouse. Peripheral blood cells from the recipient mice were analyzed at 1, 2, 3, 4, and 5 mo after transplantation. After erythrocyte lysis, cells were stained with FITC-conjugated anti–Ly5.2, PE-Cy7–conjugated anti-B220, PE-conjugated anti-CD4 and -CD8, APC-conjugated anti–Mac-1 and –Gr-1, and biotinylated anti-Ly5.1 antibodies. The biotinylated antibody was developed with streptavidin–Alexa Fluor 594. Before flow cytometric analysis, cells were suspended in PBS containing 1 µg/ml propidium iodide. Six-color flow cytometric analysis was performed using a FACSVantage SE equipped with argon (488-nm) and dye (599-nm) lasers. The percentage of chimerism was defined as follows: (percentage of Ly5.1^{+} test donor cells) × 100/(percentage of Ly5.1^{+} test donor cells + percentage of F1 competitor cells). If chimerism was 0.3% or more at any time point after transplantation, regardless of which lineage was reconstituted, mice were considered to be reconstituted with test donor cells.

Secondary transplantation was performed by transferring 5×10^6 bone marrow cells from femurs and tibias of the primary-recipient mice into each of three to five lethally irradiated B6-Ly5.2 mice. Peripheral blood cells from the secondary-recipient mice were analyzed at 1, 2, 3, 4, and 5 mo after transplantation.

LTRCs were defined as cells that exhibited 0.3% or more chimerism 16 wk or more after transplantation. Latent HSCs were defined as HSCs that began to exhibit 0.3% or more chimerism beginning at 12 wk or more after primary transplantation and exhibited multilineage reconstitution after secondary transplantation.

Single-cell colony assays. Cells were sorted clonally into 96-well plates containing 200 µl S-clone SF-O3 medium (Sanko Junyaku) supplemented with 10% fetal bovine serum, 1% bovine serum albumin, 10 ng/ml mouse stem cell factor, 10 ng/ml human thrombopoietin, 10 ng/ml mouse IL-3, 1 U/ml human erythropoietin (PeproTech), and 5×10^{-5} M β-mercaptoethanol. After culture for 14 d, colonies were cytocentrifuged onto glass slides and stained with Hemacolor (Merck). Cells comprising colonies were identified by light microscopy using morphological criteria.

Online supplemental material. Fig. S1 shows flow cytometric profiles of the markers that were expressed by CD34^{+}KSL cells but not by CD34^{-}KSL cells. Fig. S2 shows flow cytometric profiles of the markers that were expressed by CD34^{-}KSL cells. Fig. S3 shows coexpression of CD150 and CD38 in CD34^{-}KSL cells. Figs. S4–S6 show the percentage of chimerism of individual secondary-recipient mice. Fig. S7 shows the classification of LTRCs in CD150 subsets. Fig. S8 shows that the CD150^{int}CD34^{-}KSL fraction is enriched in balanced LTRCs. Fig. S9 shows the hierarchal organization model within the primitive HSC compartment. Fig. S10 shows the classification of LTRCs in secondary transplantation. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091318/DC1.

We thank A.S. Knisely for critical review of the manuscript.

This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

The authors have no conflicting financial interests.

Submitted: 17 June 2009
Accepted: 8 April 2010

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