Searching for Hematopoietic Stem Cells: Evidence That Thy-1.1\textsuperscript{+} Lin\textsuperscript{−} Sca-1\textsuperscript{+} Cells Are the Only Stem Cells in C57BL/Ka-Thy-1.1 Bone Marrow

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Summary

Hematopoietic stem cells (HSCs) are defined in mice by three activities: they must rescue lethally irradiated mice (radioprotection), they must self-renew, and they must restore all blood cell lineages permanently. We initially demonstrated that HSCs were contained in a rare (~0.05%) subset of bone marrow cells with the following surface marker profile: Thy-1.1\textsuperscript{+} Lin\textsuperscript{−} Sca-1\textsuperscript{+}. These cells were capable of long-term, multi-lineage reconstitution and radioprotection of lethally irradiated mice with an enrichment that mirrors their representation in bone marrow, namely, 1,000–2,000-fold. However, the experiments reported did not exclude the possibility that stem cell activity may also reside in populations that are Thy-1.1\textsuperscript{−}, Sca-1\textsuperscript{−}, or Lin\textsuperscript{−}. In this article stem cell activity was determined by measuring: (a) radioprotection provided by sorted cells; (b) long-term, multi-lineage reconstitution of these surviving mice; and (c) long-term, multi-lineage reconstitution by donor cells when radioprotection is provided by coinjection of congenic host bone marrow cells. Here we demonstrate that HSC activity was detected in Thy-1.1\textsuperscript{+}, Sca-1\textsuperscript{+}, and Lin\textsuperscript{−} fractions, but not Thy-1.1\textsuperscript{−}, Sca-1\textsuperscript{−}, or Lin\textsuperscript{−} bone marrow cells. We conclude that Thy-1.1\textsuperscript{+} Lin\textsuperscript{−} Sca-1\textsuperscript{+} cells comprise the only adult C57BL/Ka-Thy-1.1 mouse bone marrow subset that contains pluripotent HSCs.

Hematopoietic stem cells (HSCs)\textsuperscript{1} are intriguing because of their ability to perpetuate themselves (self-renewal) and to give rise to progeny that differentiate into all blood cell types. Hematopoiesis commences with clonogenic pluripotent HSCs, which pass through several stages of differentiation, finally producing functionally mature blood cells (reviewed in reference 1). Historically, HSC have also been described as the constituents of bone marrow transplantation essential for saving lethally irradiated hosts, presumably by self-renewal and long-term, multi-lineage blood cell repopulations (2–4).

The concept and definition of HSCs was first developed by Till and McCulloch (5). They developed a quantitative assay for clonogenic bone marrow precursors of myeloid cells; namely, the assay for spleen CFU (CFU-S) (5). While it was initially believed that CFU-S were equivalent to HSCs, it is now accepted that CFU-S are heterogeneous (6–8). The CFU-S activity of a cell inoculum can be used as a quantitative measure of the proliferative and differentiative capacities of several classes of hematopoietic progenitors (8). Early-forming (day 8) CFU-S are derived from more committed progenitors, while late-forming (day 12) CFU-S are derived from more primitive hematopoietic cells (7). Further, a subset of cells, defined as pre-CFU-S, are capable of forming CFU-S after secondary bone marrow transplantation (9, 10). However, the relationships between HSCs and any subsets of CFU-S/pre-CFU-S are unclear.

Radioprotection is defined as the ability of transplanted hematopoietic cells to prevent lethally irradiated animals from hematopoietic death. Although the mechanisms underlying radioprotection are poorly understood, radioprotection often refers to a property of HSC activity. The radioprotective capacity of HSCs has to satisfy two criteria: (a) self-renewal capacity, which leads to long-term blood cell production; and (b) differentiation potential, which generates all blood cell types.

In an attempt to purify HSCs using physical and biological separation methods, Jones et al. used counter-flow centrifugal elutriation to fractionate cells on the basis of size and density (11). In their experiment, one fraction of bone marrow cells (representing 25% of the bone marrow) was relatively depleted of CFU-S and granulocyte-macrophage CFU (CFU-GM), yet contained precursor cells for long-term engraftment.

\textsuperscript{1}Abbreviations used in this paper: CFU-GM, granulocyte/macrophage CFU; CFU-S, spleen CFU; CFU-T, thymic CFU; HSC, hematopoietic stem cell; Lin, lineage markers; PD, radioprotective bone marrow cell dose; Rh-123, rhodamine-123; Sca-1, stem cell antigen-1; WBM, whole bone marrow.
They concluded that long-term engraftment was a property of pre-CFU-S HSCs that could be separated from CFU-S on day 8 and day 12 (11).

This result was in apparent conflict with the findings of Spangrude et al. (12, 13), that HSCs could be identified and enriched according to cell surface phenotype. HSC activity was found in cells that expressed low but significant levels of Thy-1.1 (Thy-1.1b), high levels of stem cell-associated antigen (Sca-1), and undetectable levels of blood cell lineage-specific differentiation markers (Lin). This rare subset of bone marrow cells (~0.05%) was highly enriched by using antibodies in a combination of magnetic bead selection and FACS enrichment of (a) late-forming CFU-S, (b) thymic CFU-S, (c) progenitor activity giving rise to long-term hematolymphoid cultures, and (d) radioprotective activity. Thus these cells were proposed as candidate HSCs (12, 14). Spangrude et al. (12, 13), that HSCs could be identified and enriched according to cell surface phenotype. HSC activity was found in cells that expressed low but significant levels of Thy-1.1 (Thy-1.1b), high levels of stem cell-associated antigen (Sca-1), and undetectable levels of blood cell lineage-specific differentiation markers (Lin). This rare subset of bone marrow cells (~0.05%) was highly enriched by using antibodies in a combination of magnetic bead selection and FACS enrichment of (a) late-forming CFU-S, (b) thymic CFU-S, (c) progenitor activity giving rise to long-term hematolymphoid cultures, and (d) radioprotective activity. Thus these cells were proposed as candidate HSCs (12, 14).

Materials and Methods

**Mouse Strains.** The C57BL/6-Ly-5.1-Pep b (Thy-1.2, Ly-5.1), C57BL/Ka-Thy-1.1 (Thy-1.1, Ly-5.1), C57BL/Ks-Thy-1.1 (Thy-1.1, Ly-5.1), C57BL/10-Wa-Thy-1.1 (Thy-1.2, Ly-5.2), and (C57BL/10-Wa-Thy-1.1 × C57BL/Ks-Thy-1.1)F, mouse strains used in the study were bred and maintained in the mouse facility at Stanford University (Stanford, CA). All mice were regularly maintained on acidified water (pH 2.5).

**Cell Preparation.** Bone marrow cells were obtained by flushing tibias and femurs as described (12, 16). Cells were stained sequentially as follows: (a) cells were first incubated with lineage marker rat antibodies to B220 (RA3-6B2), CD4 (GK-1.5), CD8 (53.6.72), Gr-1 (RB6-8C5), Mac-1(M1/70.15.11.5), and erythrocytes (antibody TER-119); (b) the washed cells were then exposed to phycoerythrin-conjugated goat anti-immunoglobulin (Biomed, Foster City, CA); (c) the washed cells were then incubated in 20% normal rat serum to block free binding sites, followed by addition of biotinylated rat antibody to Sca-1 (antibody E13 161-7) and directly fluorescentconjugated mouse antibody to Thy-1.1 (antibody 19XE5); and (d) the washed cells were then exposed to Texas red-conjugated avidin (Cappel Laboratories, Malvern, PA). The cells were incubated for 20 min on ice for each step, followed by a wash through a FCS cushion. After the final wash, cells were resuspended in HBSS containing 1 µg/ml propidium iodide. The labeled cells were analyzed and sorted with a dual laser FACS® (Becton Dickinson Immunocytometry Systems, Mountain View, CA), modified as described (17), and made available through the FACS® shared user group at Stanford University. Whole bone marrow (WBM) cells were separated on the basis of background levels of fluorescein (Thy-1.1) from intermediate and high levels of fluorescein (Thy-1.1), background low levels of Texas red (Sca-1) from high levels of Texas red (Sca-1), and background and low levels of phycoerythrin (Lin-) from high levels of phycoerythrin (Lin+). Dead cells were excluded from analysis by propidium iodide staining detected by the allophycocyanin channel. After sorting, each bone marrow fraction was reanalyzed by FACS®.

**Radioprotection and Long-Term Reconstitution Assays.** Recipient mice were lethally irradiated (9 Gy) by a 250-kV x-ray machine at 100 rad/min in two split doses (4.5 Gy each) with a 3-h interval. After irradiation, mice were maintained on antibiotic water containing 106 U/liter of polymyxin B sulfate and 1.1 g/liter of neomycin sulfate. The next day, sorted bone marrow subsets or WBM cells were injected (200 µl/mouse) intravenously into the retro-orbital plexus of anesthetized mice. Surviving animals were monitored for 100 d (radioprotection assay).

For the long-term reconstitution assay, sorted donor bone marrow subsets were injected together with 106 host congenic WBM cells to provide radioprotection.

**Peripheral Blood Analysis.** Peripheral blood was obtained from the retro-orbital sinus. Immunofluorescence staining and FACS® analysis were performed as described previously (12, 16). Two-color staining was carried out using monoclonal antibodies specific for lineage marker for B cells (anti-B220), myeloid cells (anti-Gr-1 and anti-Mac-1), or T cells (anti-Thy-1.1) alone with antibodies to congenic markers specific for donor hematopoietic cells (anti-Ly-5.2, antibody A20.1).

**Results**

**Experimental Design.** We wanted to test whether Thy-1.1b Lin− Sca-1− cells were the only cells in the bone marrow that have pluripotent HSC activity. To avoid excluding any bone marrow cell populations from the analysis, we decided to determine how HSC activity was divided among Thy-1.1b versus Thy-1.1− pools, Sca-1− versus Sca-1+ pools, and Lin− versus Lin+ pools. To measure HSC activity we chose two experimental assays: (a) radioprotection, followed by long-term, multi-lineage reconstitution of T cells, B cells, and myelomonocytic cells in surviving mice; and (b) long-term, multi-lineage reconstitution by donor cells when radioprotection is provided by congenic host WBM cells.

If HSCs are rare and express these antigens uniformly, HSCs will be enriched in one fraction and correspondingly depleted in the other. Bone marrow cells from C57BL (Thy-1.1, Ly-5.2) congenic mice were separated into two (~or +) fractions on the basis of expression of Thy-1.1, Sca-1, or Lin (Fig. 1). Frequency analysis of both − and + populations show Thy-1.1− cells (representing 4% of WBM cells) and Thy-1.1b cells (96% of WBM cells), Sca-1− cells (6% of WBM cells) and Sca-1+ cells (94% of WBM cells), or Lin− cells
Figure 1. Separation of bone marrow cells by FACS®. (a) Phenotypic analysis of bone marrow cells from C57BL/6-Tby-1.1 × C57BL/6-Ly-5.2)F1 mice. Density plots of Thy-1.1, Sca-1, and lineage marker stainings are shown. The percentages in the panel indicate negative or positive cell fractions defined by the gates shown (dotted lines). The lineage markers include a panel of rat antibodies against B cells (anti-B220), macrophages (anti-Mac-1), granulocytes (anti-Gr-1), erythrocytes (antibody TER-119), and T cells (anti-CD4 and anti-CD8). (b) Reanalysis of sorted bone marrow cells. Tby-1.1+, Sca-1+, or Lin+ fractions (not shaded) are separated from Thy-1.1-, Sca-1-, or Lin- fractions (shaded), respectively.

Of WBM cells) and Lin+ cells (89% WBM cells) (Fig. 1). Under these sorting conditions, we obtained Thy-1.1- cells (98% pure upon reanalysis), Thy-1.1+ cells (64% pure), Sca-1- cells (98% pure), Sca-1+ (80% pure), Lin- (95% pure), and Lin+ cells (96% pure) (Fig. 1). For technical reasons we could not sort highly purified Thy-1.1+ cells or Sca-1+ cells. Because we assumed that no HSC activity would be found in Thy-1.1- or Sca-1- cells, the contamination of these cell populations should not contribute to radioprotection.

The Ly-5.2 donor sorted cells were injected into C57BL/6-Ly-5.1 hosts so that in reconstituted animals all donor cells and their progeny could be distinguished by a monoclonal antibody specific for Ly-5.2. Furthermore, Thy-1.1 and Thy-1.2 allelic markers allowed us to identify and follow donor-derived T cells in reconstituted animals.

Table 1. Number of Cells Injected for Radioprotection Assay

| Cells injected | Percent cells of bone marrow | No. of cells injected |
|----------------|-------------------------------|-----------------------|
|                | Low cell number | High cell number  |
| WBM            | 100             | 4 × 10^4 | 2 × 10^4  |
| Thy-1.1-       | 96              | 4 × 10^4 | 2 × 10^4  |
| Thy-1.1+       | 4               | 2 × 10^4 | 6–8 × 10^4 |
| Sca-1-         | 94–95           | 4 × 10^4 | 2 × 10^4  |
| Sca-1+         | 5–6             | 3–4 × 10^4 | 1 × 10^4 |
| Lin-           | 82–89           | 3 × 10^4 | 2 × 10^4  |
| Lin+           | 11–18           | 4–5 × 10^3 | 2 × 10^3 |

Figure 2. Protection from lethal irradiation by various fractions of bone marrow cells. Groups of 10 mice per experiment from three separate experiments were lethally irradiated (8 or 9 Gy) and injected with different bone marrow fractions as shown in Fig. 1b. The data were combined from three independent experiments; each experiment had similar survival kinetics. Mice were reconstituted with two different numbers (PD0s and PD9s, low and high, respectively) of WBM cells (a and b), Thy-1.1+ vs. Thy-1.1- cells (c and d), Sca-1+ vs. Sca-1- cells (e and f), and Lin- vs. Lin+ cells (g and h), as described in Table 1. The numbers of mice presented in the figure are as follows: no cells, n = 30; WBM and all bone marrow fractions, n = 20. The groups of mice injected with Thy-1.1-, Sca-1+, and Lin- fractions are shown with solid lines. The groups of mice injected with Thy-1.1+, Sca-1-, and Lin+ fractions are shown with broken lines. The shaded line in each panel represents a group of mice with no cells injected, as an irradiation control. (Although the intended dose of irradiation was 9 Gy, in one experiment mice were irradiated at 8 Gy due to an x-ray machine calibration error.) In the Lin- group (e), a single cage of animals all died on day 7, whereas the control group (no cells injected) did not start dying until later. We have shown the results both including (labeled Lin-, n = 20) and excluding (labeled Lin-, n = 15) data from these animals.

Which Bone Marrow Subsets Mediate Radioprotection of Lethally Irradiated Mice? HSC activity was tested by injecting the sorted bone marrow subsets intravenously into lethally irradiated mice. These separated – versus + pools were examined for their ability to protect lethally irradiated mice from hematopoietic death for at least 100 d (i.e., radioprotection).

To provide the most sensitive assay for enrichment or depletion of radioprotective activity, we transferred these cells in...
numbers corresponding to their representation in WBM predicted to be 50% radioprotective (PD$_{90} = 4 \times 10^5$ WBM cells) and 95–100% radioprotective (PD$_{90} = 2 \times 10^6$ WBM cells). These were designated, respectively, the low dose group and the high dose group; the actual numbers of cells injected is given in Table 1. Fig. 2 shows the percentage of surviving animals given low versus high doses of either WBM or of the FACS$^+$-sorted populations.

When the lethally irradiated mice received no cells, the majority of the mice died 10–18 d (90%) after irradiation. In this study we observed survival of 1 in 30 animals injected with no cells. No animals survived that were injected with the PD$_{90}$ equivalent dose of Thy-1.1$^-$, Sca-1$^-$, or Lin$^+$ cells. It should be noted that mice receiving Thy-1.1$^-$ or Sca-1$^-$ cells, indicated by arrows in Fig. 2, c and e, survived ~6 d longer on average than mice receiving no cells. In contrast, radioprotective activity was detected in the PD$_{90}$ equivalent dose of Thy-1.1$^+$, Sca-1$^+$, or Lin$^-$ cells, indicated by arrows in Fig. 2, d, f, and h, the majority of mice receiving Thy-1.1$^+$, Sca-1$^+$, or Lin$^-$ cells survived ~20 d longer on average than mice receiving no cells. Radioprotection was provided by the PD$_{90}$ equivalent dose of Thy-1.1$^+$ (75%), Sca-1$^+$ (95%), or Lin$^-$ (80%) cells.

It is evident from these experiments that Thy-1.1$^+$, Sca-1$^+$, or Lin$^+$ cell populations, which represent ~82–96% of WBM cells, were significantly depleted for their ability to radioprotect lethally irradiated mice. The survival kinetics provided by Thy-1.1$^+$, Sca-1$^+$, and Lin$^-$ cell populations at both cell doses were similar to those with low or high doses of WBM cells. Thus the radioprotective activity of WBM cells is a property of Thy-1.1$^+$, Sca-1$^+$, and Lin$^-$ cells.

The surviving animals described above must have been radioprotected either by progeny of donor HSCs or by a transient population of circulating donor-derived blood cells followed by recovery of endogenous HSC activity. If long-term radioprotection was provided by progeny from donor HSCs, we should observe long-term, multi-lineage reconstitution by donor cells. We tested the levels of donor-derived, Ly-5-marked peripheral blood cells in each lineage at 100 or 180 d after irradiation and cell injection (Fig. 3). We analyzed the mice injected with the PD$_{90}$ equivalent cell dose. From past studies detection of sustained myelopoiesis is a good indicator of active hematopoiesis from HSCs and progenitor cells (18; Uchida, N., and I. L. Weissman, unpublished observations), because mature granulocytes are short-lived (on average <24 h) once they enter the blood circulation (18; Uchida, N., and I. L. Weissman, unpublished observations). It is clear that consistent long-term, multi-lineage reconstitution occurs only with WBM, Sca-1$^+$, Lin$^-$, or Thy-1.1$^+$ cells (Fig. 3, b, d, f, and h). It should be noted that in the experiment shown in Fig. 3 h, we were only able to inject $6 \times 10^5$ Thy-1.1$^+$ cells into each recipient, a dose that represents only two-thirds of the expected PD$_{90}$ equivalent cell dose.

We also tested the surviving animals that received no cells, or the PD$_{90}$ equivalent dose of Thy-1.1$^-$, Sca-1$^-$, or Lin$^+$ cells. No multi-lineage reconstitution was detected except from one mouse injected with $2 \times 10^5$ Thy-1.1$^-$ cells (Fig. 3, a, c, e, and g). We believe these surviving animals were almost certainly radioprotected due to repopulation by endogenous stem cells that survived the irradiation. As pointed out above, mice receiving Thy-1.1$^-$, Sca-1$^-$, or Lin$^+$ cells survive, on average, a few days longer than mice receiving no cells, implying that these cells might contain short-term engraftment activity. In that way, a few of these mice may have survived.
Table 2. Long-Term Multi-Lineage Reconstitution of Irradiated Ly-5 Congenic Mice with WBM, Sca-1−, Lin+, or Thy-1.1− Cells Plus 10^5 Host-Type WBM

| Donor cell type | No. of cells injected | n    | 4  | 8-10 | 13-14 |
|-----------------|-----------------------|------|----|------|-------|
| WBM             | 4 x 10^4              | 4    | 1  | 4    | 3     |
| Sca-1−          | 4 x 10^4              | 4    | 0  | 0    | 0     |
| Lin+            | 4 x 10^4              | 5    | 0  | 0    | 0     |
| Thy-1.1−        | 4 x 10^4              | 5    | 1  | 1    | 0     |
| Thy-1.1−        | 2 x 10^5              | 5    | 0  | 0    | 0     |

Peripheral blood of the reconstituted mice was analyzed at three time points as indicated. The mice reconstituted with all three lineages (Multi+) were scored if they had detectable levels (>1%) of donor-derived B cell lineage (B), myelomonocytic cell lineage (M/G), and T cell lineage (T) at each time point (B + M/G + T).

Table 3. Long-Term Reconstitution of Irradiated Ly-5 Congenic Mice with WBM, Sca-1−, Lin+, or Thy-1.1+ Cells Plus 10^5 Host-Type WBM

| Donor cell type | No. of cells injected | n    | 4  | 8-10 | 13-14 |
|-----------------|-----------------------|------|----|------|-------|
| WBM             | 4 x 10^4              | 4    | 4  | 4    | 4     |
| Sca-1−          | 4 x 10^4              | 4    | 2  | 0    | 0     |
| Lin+            | 4 x 10^4              | 5    | 4  | 3    | 2     |
| Thy-1.1−        | 4 x 10^4              | 5    | 5  | 4    | 4     |
| Thy-1.1+        | 2 x 10^5              | 5    | 5  | 5    | 5     |

Peripheral blood of the reconstituted mice was analyzed at three time points as indicated. Mice were scored positive if they had detectable levels (>1%) of donor-derived B cell lineage (B), myelomonocytic cell lineage (M/G), or T cell lineage (T) at each time point.
### Table 4. Long-Term Reconstitution of Irradiated Ly-5 Congenic Mice with WBM, Sca-1⁻, Lin⁺, or Thy-1⁻ Cells Plus 10⁵ Host-Type WBM

| Fraction | No. of cells injected | Lineage analyzed | Percent donor cells |
|----------|-----------------------|------------------|---------------------|
| WBM (n = 4) | 4 × 10⁴ | B | 8.8 | 27 | 60 |
| | | M & G | 2.8 | 81 | 96 |
| | | T | <1 | 4.1 | 52 |
| | 4 × 10⁴ | B | 12 | 15 | 36 |
| | | M & G | 5.5 | 38 | 47 |
| | | T | <1 | 7.0 | 16 |
| | 4 × 10⁴ | B | 9.4 | 9.6 | 8.9 |
| | | M & G | 7.8 | 13 | <1 |
| | | T | <1 | 1.5 | 8.6 |

**continued**

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Table 4. (continued)

| Fraction | No. of cells injected | Lineage analyzed | Percent donor cells |
|----------|----------------------|------------------|---------------------|
|          |                      |                  | Week 4 | Week 8 | Week 13-14 |
| Thy-1.1+ (n = 5) | 4 x 10⁴ | B | <1 | <1 | <1 |
|           |          | M & G | <1 | <1 | <1 |
|           |          | T | <1 | <1 | <1 |
|           | 4 x 10⁴ | B | 3.3 | <1 | <1 |
|           |          | M & G | <1 | <1 | <1 |
|           |          | T | <1 | <1 | <1 |
|           | 4 x 10⁴ | B | 17 | 28 | 26 |
|           |          | M & G | 9.6 | 1.2 | <1 |
|           |          | T | 1.1 | 6.8 | 1.4 |
|           | 4 x 10⁴ | B | 6.1 | 1.2 | 1.5 |
|           |          | M & G | <1 | <1 | <1 |
|           |          | T | <1 | <1 | <1 |
|           | 4 x 10⁴ | B | 8.2 | 2.8 | 2.0 |
|           |          | M & G | <1 | <1 | <1 |
|           |          | T | <1 | <1 | <1 |
|           | 4 x 10⁴ | B | 16 | 4.5 | 3.6 |
|           |          | M & G | <1 | <1 | <1 |
|           |          | T | 1.2 | <1 | <1 |
| Thy-1.1+ (n = 5) | 2 x 10⁵ | B | 34 | 13 | 13 |
|           |          | M & G | <1 | 2.2 | 3.4 |
|           |          | T | <1 | <1 | <1 |
|           | 2 x 10⁵ | B | 14 | 5.5 | 5.6 |
|           |          | M & G | <1 | <1 | <1 |
|           |          | T | 1.6 | <1 | <1 |
|           | 2 x 10⁵ | B | 27 | 12 | 13 |
|           |          | M & G | <1 | <1 | 2.0 |
|           |          | T | <1 | <1 | <1 |
|           | 2 x 10⁵ | B | 15 | 2.8 | 3.2 |
|           |          | M & G | <1 | <1 | <1 |
|           |          | T | <1 | <1 | <1 |
|           | 2 x 10⁵ | B | 18 | 16 | 15 |
|           |          | M & G | <1 | <1 | <1 |
|           |          | T | 2.5 | 3.3 | <1 |

WBM or the Thy-1.1+ subset of WBM was used at either the PD₅₀ or PD₉₅ equivalent doses. Donor T cells were detected in the PD₅₀ equivalent dose of WBM (53 ± 19%) and Thy-1.1+ (52 ± 20%). Averages of 73 ± 7 and 69 ± 15% of donor T cells, respectively, were detected in the blood of mice that received the PD₉₅ equivalent dose of WBM and Thy-1.1+ cells, whereas only 4% of donor T cells were detected from the single surviving animal injected with the
PD~5~ equivalent dose of Thy-1~ cells. Thus we could detect radioprotective and T lineage repopulating activities in WBM and Thy-1.1~ cells, but not in Thy-1~ cells.

**Which Bone Marrow Subsets Mediate Long-Term Multi-Lineage Reconstitution in Mice Radioprotected by Host WBM Cells?** The experiments described above show that Thy-1.1~, Sca-1~, and Lin~ cells do not radioprotect lethally irradiated mice. This assay does not exclude the possibility that cells contained in the Thy-1~ , Sca-1~, or Lin~ subsets do indeed have long-term engrafment activity, but this could not be detected if they failed to radioprotect in the short term. Jones et al. reported that they could isolate a bone marrow subset that contained long-term repopulating but not radioprotective activity (11). We wanted to test whether similar activity was present among Thy-1.1~ , Sca-1~, or Lin~ cells. To provide these cells the opportunity to demonstrate long-term engrafment, 1 \times 10^5 host bone marrow cells were coinjected with each donor bone marrow fraction tested. As the short-term engrafment potential of Thy-1.1~ , Sca-1~, and Lin~ cells had already been tested, we limited our examination of possible long-term repopulating ability of Thy-1~ , Sca-1~, and Lin~ cells, and compared it with that of WBM cells. Tables 2, 3, and 4 show the results of long-term reconstruction assays of irradiated Ly-5 congenic hosts given sorted, donor cell populations plus 1 \times 10^5 host-type WBM cells for radioprotection. Under these conditions it is clear that Thy-1~ , Sca-1~, and Lin~ cells could not contribute to long-term, multi-lineage reconstruction (Table 2). An equivalent dose of WBM cells could contribute to significant levels of long-term, multi-lineage reconstruction (Table 4). In three of four mice in this WBM group, 24–96% of peripheral myeloid cells were donor derived 14 wk after transplantation. Some short-term engrafment was observed with Thy-1.1~ , Sca-1~, or Lin~ cells (Tables 3 and 4). Thy-1.1~ cells contained reproducible and significant B cell progenitor activity, while no long-term B cell progenitor activity was detected in Sca-1~ cells (Tables 3 and 4). Thus, these progenitors must be Sca-1~, but may be Lin~ or Lin~ cells. By the two approaches described above we found that radioprotective activity tightly correlated with long-term, multi-lineage repopulating activities.

**Discussion**

*Only Thy-1.1~ Lin~ Sca-1~ Bone Marrow Cells Are HSCs.* In these experiments we searched for HSC activity in all fractions of bone marrow by radioprotection and long-term reconstruction assays. The experimental design allowed us to detect two possible HSC activities: (a) radioprotective and long-term, multi-lineage repopulating, and (b) not radioprotective, but long-term repopulating. Thy-1.1~ cells (representing 96% of WBM), Sca-1~ cells (94–95% of WBM), and Lin~ cells (28–89% of WBM) neither radioprotected nor contributed to long-term, multi-lineage reconstruction. Long-term, multi-lineage reconstruction was a property of Thy-1.1~ (4% of WBM), Sca-1~ (5–6% of WBM), and Lin~ (11–19% of WBM) cells. Thus, Thy-1.1~, Sca-1~, and Lin~ cells, which together represent 99.95% of bone marrow cells, do not possess stem cell activity. Thy-1.1~ Lin~ Sca-1~ cells, but no other cells, appear to be the only pluripotent hematopoietic stem cells in adult C57BL/Ka-Thy-1.1 bone marrow as measured by these assays (radioprotection and long-term, multi-lineage reconstruction).

It is most likely that the same population also has self-renewal activity. Elsewhere we have shown that hosts demonstrating sustained, donor-derived myelopoiesis (>6 mo) contain bone marrow cells that can transfer radioprotection and long-term, multi-lineage hematopoiesis to secondary hosts (15, 18). In these experiments, clonogenic Thy-1.1~ Lin~ Sca-1~ Ly-5 marked single cells (or clonogenic units found at limit dilution) were transferred into Ly-5 congenic hosts along with either 1 \times 10^5 WBM or 100 Thy-1.1~ Lin~ Sca-1~ host cells. Two outcomes were evident when donor-derived cells could be detected in the blood: multi-lineage differentiation that fails to sustain myelopoiesis, and multi-lineage outcomes that sustain myelopoiesis (18; Uchida, N., and I. L. Weissman, unpublished observation). Only those chimeras with sustained donor-derived myelopoiesis contained donor-derived cells that could yield long-term, multi-lineage outcomes after secondary transplantation. Only WBM or Thy-1.1~ Lin~ Sca-1~ cells contain such activity (18; Uchida, N., and I. L. Weissman, unpublished observations). Because virtually all mice given PD~50~ or PD~90~ doses of Thy-1.1~, Lin~, or Sca-1~ cells exhibited sustained myelopoiesis, we infer that self-renewal has also occurred in these cases. Because no mice given Thy-1.1~, Lin~, or Sca-1~ cells (along with host WBM cells) demonstrated donor-derived, long-term, multi-lineage reconstruction, these populations do not contain pre-CFU-S or self-renewing HSCs.

We found no evidence for the existence of cells that augment or inhibit HSC activity, as mixing WBM cells with the sorted populations appeared to neither inhibit the Lin~ fraction (12) nor help the Lin~ fraction. It is interesting to note that Thy-1~ , Sca-1~, and Lin~ cells provided some degree of short-term or lineage-restricted engrafment. For example, the Thy-1~ fraction gave rise to a large number of B cells in one of four mice injected with 2 \times 10^6 cells, and in four of five mice injected with 2 \times 10^5 cells over the full length of assay. This is consistent with the presence of these progenitors in a Thy-1~ fraction reported previously (19).

Jones et al. used counterflow centrifugal elutriation to separate bone marrow cells into four fractions on the basis of size and density (11). Progenitor activities of these cells were assayed by day 8 and day 12 CFU-S and by CFU-GM. They used male-specific DNA probes to trace donor-origin engrafment at days 11 and 60. One fraction of cells that elutriate at 25 ml/min (representing 25% of input cells) was enriched for morphologic lymphocytes and depleted of blast cells. This fraction was significantly depleted for progenitor cells that could produce CFU-GM and CFU-S; 1 \times 10^5 of these cells could not radioprotect lethally irradiated animals, but did contain a long-term progenitor activity (see below). Another fraction, representing ~25% of bone marrow cells, was highly enriched for blast cell types. This fraction contained CFU-GM and CFU-S activities, and could radioprotect 15 of 28 animals given 2 \times 10^5 cells for at least 60 d. Donor-
derived cells in bone marrow of the reconstituted animals, detected by dot blots probed for male-specific DNA, were decreased from 98% of male donor type at day 14 to 11% at day 60.

Interestingly, 2 x 10^4 male donor 25 ml/min cells contained multi-lineage reconstitution activity assayed at day 60 when the irradiated mice were radioprotected by 2 x 10^4 female "blast" fraction cells. While these hosts had no detectable male donor cells in bone marrow at day 14, male donor cells were detected in bone marrow at abundant levels (~50% of normal) at day 60. Jones et al. concluded that two independent classes of bone marrow cells mediate two different phases of hematopoietic recovery in lethally irradiated animals. The "blastic" fraction would contain committed progenitors that provided radioprotection via rapid, but short-term engraftment, while the 25 ml/min fraction contained pre-CFU-S or "true" HSC that could not contribute to radioprotection by early stage myeloerythroid development, but contained cells that could produce delayed, multi-lineage reconstitution activity (11).

Can these results be reconciled with the data presented here and elsewhere describing activities of Thy-1.1^b Lin^-Sca-1^- cells? Thy-1.1^b Lin^-Sca-1^- cells are similar in size to the 25 ml/min fraction, as shown by Spangrude et al. (12). Also, >97% of this cell type are in the G0-G1 phase. Combining these findings, Thy-1.1^b Lin^-Sca-1^- cells should not be included in a fraction enriched for blast cells. Yet they are highly enriched for day 12 CFU-S, in contrast to the 25 ml/min fraction of Jones et al. (11), and are responsible for both long- and short-term reconstitution. It has been shown that several different cell types can contribute to CFU-S (8). Purified Thy-1.1^b Lin^-Sca-1^- cells have low day 8 CFU-S activity and high day 12–13 CFU-S activity (1:10 ratio), while three other FACSS^®-enriched populations give rise to CFU-S with varying ratios of day 8/day 12 activity (8). It is unclear which populations were deleted from the 25 ml/min fraction described by Jones et al. (11). The enrichment or depletion of CFU-S and long-term progenitor activities in fourfold enriched populations is, of course, much different than the highly-enriched activities described in a subset representing only 0.05% of WBM.

Nevertheless, the findings of Jones et al. (11) raise questions concerning the "homogeneity" of the Thy-1.1^b Lin^-Sca-1^- cells. In fact, they appear to be heterogeneous. Spangrude and Johnson showed that Thy-1.1^b Lin^-Sca-1^- cells can be further divided into rhodamine-123 low and high (Rh123^b, Rh123^h) cell types (15). While WBM contain

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We thank Dr. G. Spangrude for stimulating discussions and suggestions, and for sharing unpublished data. We are also grateful to Mr. T. Knaak for efficient cell sorter operation, Mr. L. Hidalgo and the animal facility for excellent animal care, and Ms. L. Jerabek, L. Hu, and M. Hurlbut for their technical support. We also appreciate Drs. J. Friedman, K. Ikuta, S. Sell, P. Sherwood, B. Bee, and D. Vaux for discussions and for reviewing the manuscript.
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