Purification and Characterization of Heterogeneous Pluripotent Hematopoietic Stem Cell Populations Expressing High Levels of c-kit Receptor

By Donald Odic, Roxanne Fischer, Shin-Ichi Nishikawa, Arthur W. Nienhuis, and David M. Bodine

Mouse pluripotent hematopoietic stem cells (PHSC) were fractionated based on size and density using counterflow centrifugal elutriation (CCE). These heterogeneous PHSC populations were further enriched by subtraction of cells with lineage-specific markers (Lin−) followed by positive sorting for c-kit expression. The cells were characterized with functional and biochemical properties. We defined a subpopulation of c-kit−positive cells that expressed high numbers of c-kit receptors (c-kitER). One hundred c-kitER cells from either low- or higher-density fractions were sufficient to repopulate the lymphohematopoietic system in WBB6F1-W/W (W/W) recipients, whereas no PHSC were found in cells with low (c-kitDUL) or no (c-kitNEG) c-kit expression. Lin− c-kitER cells were separated into RhoDULL and RhoER subsets based on their ability to efflux rhodamine 123 (Rho). The PHSC were concentrated in Lin− c-kitER RhoDULL cells and the number of Lin− c-kitER RhoER cells correlated directly with the number of day 12 colony-forming unit-spleen (CFU-S12) in each fraction. We were not able to enrich further for PHSC using monoclonal antibodies to the cell-surface markers AA4.1 or CD4, which have been used by others to isolate PHSC. The small, low-density Lin− c-kitER subset contained PHSC and few CFU-S and progenitors. The purified PHSC obtained using this protocol were assayed in vivo for their physiologic function and biochemical properties and in vitro for RNA expression of the flk-2 gene. These methods provide a 40- to 400-fold enrichment. More recent stem cell purification protocols involve immunobead subtraction of lineage-positive cells (Lin+) followed by FACS sorting based on monoclonal antibody (MoAb) labeling of stem cell surface markers. The phenotype Thy1.1 Lin− Sca-1 (Ly6A/E) identifies a population containing PHSC and day 12 colony-forming unit-spleen (CFU-S12). Although mice of both Ly6a and Ly6b haplotypes express Ly6A/E molecules on peripheral blood cells, only Ly6b mice express Ly6A/E on PHSC. The combination of Thy1.1 and Ly6A/E is found in only one congenic strain of mice. The c-kit receptor, with its cross-species distribution, has now become a principal marker for the isolation of stem cells.

Materials and Methods

Mice. Three to 4-week-old female C57BL/6J mice were used as donors. WBB6F1-+/+ mice were used as recipients in the CFU-S assay and WBB6F1-W/W (W/W) mice were used as recipients in

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the PHSC assay. All mice were obtained from Jackson Laboratories (Bar Harbor, ME).

CCE. Cell fractions were collected as reported previously.6,7 Briefly, a single cell suspension of 2 to 4 x 10⁸ donor cells was introduced into the separation chamber of a JE-6B elutriation system (Beckman Instruments, Palo Alto, CA) at a flow rate of 15 mL/min (FR15) with a rotor speed of 3,000 RPM (1,260g). Cells were collected in 200-mL aliquots at FR15 (discarded), FR25, FR30, FR35, and rotor-off (R/O).

MoAb staining and flow cytometry. Cells from FR25, FR30, and FR35 were incubated with a mixture of rat MoAb directed against the following murine hematopoietic cell lineage-specific surface markers: B lymphocytes (B220), T lymphocytes (CD4 and CD8), granulocytes (Gr-1), and myelomonocytic cells (Mac-1) (Purchased from Caltag Laboratories, San Francisco, CA). In some experiments, the anti-erythroid MoAb TER-119 (a gift from Dr 'Tatsuro' Kina, Kyoto University, Kyoto, Japan) was added to the MoAb cocktail. Antibody-labeled Lin⁺ cells were removed using magnetic immunobeads (Advanced Magnetics, Cambridge, MA). This lineage-depletion procedure reduced the number of cells in each fraction by greater than 75%. The remaining Lin⁻ cells were stained for flow cytometry with a biotin-conjugated anti-c-kit MoAb (Ack-4)⁸⁻¹¹ and subsequently stained with streptavidin-phyceroerythin (SA-PE) (SA-PE). The cells were sorted using an EPICS Elite cell sorter (Couter, Hialeah, FL) at a rate of 2,000 cells/s into subsets with high (c-kitBR) and low (c-kitDULL) c-kit expression. Sorted cells were reanalyzed by FACS and dead cells were excluded by propidium iodide staining. This protocol provided average yields of approximately 2 x 10⁴, 1 x 10³, and 2 x 10² c-kitBR cells from FR25, FR30, and FR35 Lin⁺, respectively, at a purity of 70% to 95%.

The MoAb AA4.1-FITC (a gift from Dr Saul J. Sharkis, Johns Hopkins Oncology Center, Baltimore, MD) was used alone or in combination with Ack-4/biotin/SA-PE to isolate AA4.1⁺, AA4.1⁻, c-kitPOS AA4.1⁺ and c-kitNEG AA4.1⁻ cells for PHSC analysis. In other experiments, anti-CD4 MoAb was omitted from the MoAb cocktail used for lineage subtraction and the Lin⁻ cells were then stained with anti-CD45 MoAb-fluorescein isothiocyanate (FITC) and anti-c-kit MoAb and sorted for c-kitPOS CD4⁺ and c-kitNEG CD4⁻ populations. These subsets were assayed for PHSC activity.

Rhodamine 123 staining. Rho was added at a volume of 0.1 mL (0.1 µg) per 1 x 10⁶ elutriated Lin⁻ cells in 0.9 mL 5% fetal calf serum (FCS) at 37°C for 20 minutes. The cells were then rinsed twice in 5% FCS, resuspended, and incubated for a period of 8 to 10 hours at 37°C to permit Rhodamine 123 staining. The Lin⁻ fractions were then stained with Ack-4, an anti-c-kit MoAb, and separated by magnetic immunobead subtraction. The Lin⁻ fractions were then stained with anti-CD45 MoAb-fluorescein isothiocyanate (FITC) and anti-c-kit MoAb and sorted for c-kitPOS CD4⁺ and c-kitNEG CD4⁻ populations. These subsets were assayed for PHSC activity.

CFU-S₁₂ assay. The CFU-S₁₂ assay was performed by the method of Till and McCulloch.³¹ WW6B6F₁-+/+ recipients were given 900 rad total body radiation using a 137Cs gamma irradiator (Atomic Energy of Canada, Ottawa). Within 1 to 4 hours, the mice were injected with purified bone marrow cells via the tail vein. On day 12 posttransplantation, the spleens were removed and fixed in 10% ethanol/acetic acid/formalin; 20:1;1) and the CFU-S₁₂ colonies were counted.

Long-term repopulation assay. Purified c-kitBR cells (as few as 100), c-kitDULL cells (as many as 2 x 10⁵), and c-kitNEG cells (as many as 5 x 10⁴) were administered to W/W recipients as a single injection via the tail vein. At 1- to 4-week intervals posttransplantation, the peripheral blood cells were monitored for increases in donor hemoglobin by cellulose acetate electrophoresis.³⁶ After 4 months, DNA was extracted from the thymus and bone marrow for Southern analysis to assay for repopulation of nucleated erythroid, myeloid, and lymphoid cells. The C57BL/6J donors are homozygous for single hemoglobin (Hbbα/Hbbα, 10-kb EcoRI fragment), and the W/W recipients are heterozygous for single/diffuse hemoglobin (50% Hbbα/50% Hbbβ, 10-kb and 7-kb EcoRI fragments).³³ Bone marrow and thymus DNA was digested with EcoRI and probed with a 611-bp PvuII/HaeIII fragment from the second intervening sequence of the β⁺ gene.

mRNA analysis. RNA was extracted from unfractionated, partially purified, and highly purified bone marrow populations using Vanadyl Ribonucleoside Complex (BRL Life Technologies, Gaithersburg, MD) as described.³⁴ To generate sufficient amounts of RNA and to control for variation from preparation to preparation, RNA extracted from two populations of Lin⁻ c-kitPOS and Lin⁻ c-kitNEG cells was pooled for each assay. Five micrograms of total RNA from each population was treated with DNase I (Promega, Madison, WI) and divided into two aliquots. One aliquot was treated with reverse transcriptase using random oligonucleotides as described by the manufacturer (US Biochemicals, Cleveland, OH). The other aliquot was treated with the identical reagents, except that the reverse transcriptase was omitted. The equivalent of 1-µg samples from the two reverse-transcriptase reactions were added to similar polymerase chain reactions (PCRs) containing the β₂-microglobulin primers described previously.³⁵ The amplification parameters were 94°C for 30 seconds, 50°C for 1 minute, and 72°C for 2 minutes for 25 cycles. Polyacrylamide gel electrophoresis was used to resolve the 141-bp flk-2-specific product and the 232-bp β₂-microglobulin product.

RESULTS

Separation and enrichment of c-kit⁻ expressing bone marrow stem cells. Donor bone marrow cells (2 to 4 x 10⁸) were separated into small and larger cells by CCE at FR25, FR30, and FR35, respectively. Cells expressing lineage markers were removed by magnetic immunobead subtraction. The Lin⁻ fractions were then stained with Ack-4, an anti-c-kit MoAb, and separated by FACS into c-kitPOS and c-kitNEG cells (Fig 1). Background was set at 1% of isotype labeling and cells were defined as c-kitPOS if they were at a fluorescence level greater than background. This c-kitPOS subset was subdivided into c-kitDULL and c-kitBR. The c-kitBR cells showed a fluorescence signal fivefold to 10-fold greater than background. The average number of Lin⁻ c-kitPOS cells ranged from 2.8% in FR25 to 18.2% in FR35, and the c-kitBR subset ranged from 0.1% in FR25 to 5.1% in FR35.

CFU-S₁₂ in c-kit⁻ expressing bone marrow cells. The CFU-S₁₂ and FR35 Lin⁻ c-kitPOS and c-kitNEG cells were assayed for CFU-S₁₂, as were the FR35 Lin⁻ c-kitBR, c-kitDULL, and c-kitNEG cells (Table 1). FR35 Lin⁻ c-kitBR cells contained an average of 79 CFU-S₁₂ (< 2.7% of all c-kitBR CFU-S₁₂) and none were found in the c-kitNEG subset. In FR35 Lin⁻ and FR35 Lin⁺, many CFU-S₁₂ were present in the c-kitBR population, while few or none were present among the c-kitNEG cells. FR35 Lin⁻ c-kitDULL cells contained CFU-S₁₂, but at a greatly reduced number and frequency compared with FR35 Lin⁻ c-kitBR. This distribution of CFU-S₁₂ among c-kitPOS cells in elutriated fractions is consistent with the distribution obtained earlier by Jones et al³⁶ and ourselves' using elutriated marrow fractions not further enriched by flow cytometry.

Long-term repopulation studies using c-kit⁺ enriched PHSC. Normal bone marrow PHSC have a competitive
advantage over W/W<sup>+</sup> PHSC and will repopulate unirradiated W/W<sup>+</sup> mice within 6 to 8 weeks posttransplantation. We define long-term repopulation of W/W<sup>+</sup> recipients as 100% donor hemoglobin in peripheral blood and greater than 90% donor β-globin allele in bone marrow and thymus at 4 months posttransplantation. Enriched populations of primitive hematopoietic cells were injected in limiting numbers into W/W<sup>+</sup> mice to assay for long-term repopulation. 

One hundred FR25 Lin<sup>−</sup> c-<i>kit</i><sup>B</sup> cells were sufficient to totally repopulate the red blood cells in four of 10 W/W<sup>+</sup> recipients and 200 cells repopulated two of two recipients (Fig 2). The hemoglobin in recipients not repopulated with donor cells remained 50% single/50% diffuse at all time intervals. There was no evidence of transient or partial repopulation. Likewise, 100 c-<i>kit</i><sup>B</sup> cells from the FR30 Lin<sup>−</sup> c-<i>kit</i><sup>B</sup> subset were able to repopulate four of 12 W/W<sup>+</sup> mice and 200 cells from this fraction repopulated two of three recipients. One of eight W/W<sup>+</sup> mice was repopulated with 100 cells from the FR35 Lin<sup>−</sup> subset. When 1,000 c-<i>kit</i><sup>B</sup> cells of any fraction were injected, 100% of the recipients were repopulated. However, 13 recipients of 2 × 10<sup>3</sup> to 2.5 × 10<sup>4</sup> c-<i>kit</i><sup>D</sup> cells from FR35 Lin<sup>−</sup> and 14 recipients of as many as 5 × 10<sup>4</sup> c-<i>kit</i><sup>DEG</sup> cells in FR25, FR30, or FR35 did not show any donor-type globin at time points up to and including 4 months posttransplantation. In other experiments, we showed that PHSC are present in c-<i>kit</i><sup>B</sup> cells. On the y-axis log scale, the c-<i>kit</i><sup>DEG</sup> cells have approximately fivefold to 10-fold more PE fluorescence (c-<i>kit</i> receptors) than the highest 1% isotype background. The increase in percent c-<i>kit</i><sup>DEG</sup> cells from FR25 Lin<sup>−</sup> to FR35 Lin<sup>−</sup> correlates with an increase in the frequency and absolute number of CFU-S<sub>12</sub> in these fractions.

Mice that converted to 100% donor hemoglobin after a transplant of 200 cells from FR25 or FR30 Lin<sup>−</sup> c-<i>kit</i><sup>B</sup> subsets were assayed for lymphoid and myeloid repopulation by Southern analysis of the β-globin genes (Fig 3). The donor β<sup>single</sup> genes reside on 10-kb EcoRI fragments, while the compound heterozygous W/W<sup>+</sup> recipient has 14-kb β<sup>minor</sup> (which does not hybridize well to our probe), 10-kb β<sup>single</sup> and 7-kb β<sup>major</sup> bands. Thymus and bone marrow from repopulated mice contained 90% to 100% donor 10-kb
Table 1. Subpopulations of Bone Marrow Cells Purified by Elutriation and Flow Cytometry (FACS)

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Average No. of Cells</th>
<th>CFU-S/10^6 Cells Injected</th>
<th>Estimated Absolute No. of CFU-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR25 Lin− c-kitBR</td>
<td>1.5 ± 0.39 10^7 (6)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FR25 Lin− c-kitNEG</td>
<td>1.47 ± 0.67 10^7</td>
<td>0.0 ± 0.0 (6)</td>
<td>0</td>
</tr>
<tr>
<td>FR25 Lin− c-kitDULL</td>
<td>2.7 ± 10^6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FR25 Lin− c-kitBR 5'</td>
<td>2.0 ± 10^6</td>
<td>39.5 ± 14.8 (8)</td>
<td>79</td>
</tr>
<tr>
<td>FR30 Lin− c-kitBR</td>
<td>5.0 ± 2.1 10^6 (7)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FR30 Lin− c-kitNEG</td>
<td>1.18 ± 0.35 10^7</td>
<td>0.31 ± 0.35 (4)</td>
<td>365</td>
</tr>
<tr>
<td>FR30 Lin− c-kitDULL</td>
<td>1.1 ± 10^8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FR30 Lin− c-kitBR 5'</td>
<td>1.3 ± 10^5</td>
<td>132 ± 100.3 (19)</td>
<td>1,382</td>
</tr>
<tr>
<td>FR35 Lin− c-kitBR</td>
<td>432 ± 2.1 10^6 (8)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FR35 Lin− c-kitNEG</td>
<td>1.1 ± 0.64 10^7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FR35 Lin− c-kitDULL</td>
<td>9.3 ± 10^6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FR35 Lin− c-kitDULL</td>
<td>1.5 ± 10^6</td>
<td>8.1 ± 10.3 (8)</td>
<td>1,216</td>
</tr>
<tr>
<td>FR35 Lin− c-kitDULL</td>
<td>2.1 ± 10^6</td>
<td>156.5 ± 43.4 (15)</td>
<td>3,327</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

The number of trials shown in parentheses was averaged to determine the average number of total and Lin− cells elutriated at FR25, FR30, and FR35. The average numbers of c-kitPOS, c-kitNEG, and c-kitDULL cells in each fraction were calculated by multiplying the average percentage of each cell type (see Fig 1) by the average number of Lin− cells. Numerical values are given as ± SD.

The number of spleens indicated in parentheses was collected from mice injected with between 100 and 4,000 Lin− c-kitBR cells, 200 to 2,500 10^6 cells, or 9 X 10^4 Lin− c-kitNEG cells.

| Enrichment of PHSC on the basis of Rho efflux. Cells from FR25, FR30, and FR35 Lin− fractions were stained with anti-c-kit MoAb and Rho and sorted into c-kitBR RhoDULL and c-kitBR RhoBR populations. There were CFU-S12 in both Lin− c-kitBR RhoDULL and Lin− c-kitBR RhoBR populations, but they were concentrated in RhoBR populations (data not shown). The representative ratios of RhoDULL to RhoBR cells in FR25, FR30, and FR35 Lin− c-kitBR populations were 2.5:1, 1:1:1, and 1:2:6, respectively (Fig 4). This observation was predicted, since the number of CFU-S12 increased in the c-kitBR population from FR25 to FR35. An injection of 100 Lin− c-kitBR RhoDULL cells from FR25, FR30, and FR35 repopulated a total of five of nine mice (Table 3). The FR35 Lin− c-kitBR RhoDULL cell population showed a fivefold enrichment of the PHSC activity over the FR35 Lin− c-kitBR cells (two of three vs eight mice repopulated with 100 cells), presumably due to removal of RhoBR CFU-S12 and/or other progenitors from the original FR35 Lin− c-kitBR population. Five of six recipients of 1,000 Lin− c-kitBR RhoDULL cells from FR25, FR30, and FR35 were converted to 100% Hbb'. Only one of nine recipients of 100 Lin− c-kitBR RhoBR cells from FR25, FR30, and FR35 subsets were repopulated with Hbb', whereas 1,000 of these cells converted three of six recipients. These data indicate that neither anti-AA4.1 MoAb nor anti-CD4 MoAb can be used to further enrich the Lin− c-kitBR populations.

Expression of AA4.1 and CD4 on PHSC. The FR25, FR30, and FR35 Lin− c-kitPOS populations were also analyzed for expression of AA4.1 and CD4 surface antigens (Table 2). PHSC in FR25 were present in the Lin− AA4.1− subset, but were not found in AA4.1+ or Lin− AA4.1+ subsets, even when as many as 3 X 10^5 cells were injected per recipient. In FR35 subsets, PHSC were present in cell populations that were AA4.1− Lin− AA4.1+, and Lin− AA4.1−. This suggests that the PHSC in FR25 and FR35 have a different expression pattern for this marker. When these results were extended to include two-color sorting for c-kit and AA4.1, all 19 recipients of cells in the subset FR35 Lin− c-kitPOS AA4.1− converted to 100% Hbb', while zero of nine mice that received FR35 Lin− c-kitNEG AA4.1− cells were repopulated. We also analyzed expression of CD4 using anti-CD4 MoAb and anti-c-kit MoAb. Repopulation of recipient mice was observed only in c-kitPOS populations regardless of whether they expressed CD4. There were no CD4+ PHSC in the FR25 Lin− c-kitPOS subset, whereas there were CD4+ PHSC in FR30 Lin− c-kitPOS cells. These data indicate that neither anti-AA4.1 MoAb nor anti-CD4 MoAb can be used to further enrich the Lin− c-kitBR populations.

Fig 2. Long-term repopulating cells in subpopulations of purified cells isolated on the basis of c-kit expression. PHSC were found in c-kitPOS cells of each Lin− subset. The bars indicate the percentage of mice repopulated with donor Hbb+/number of mice injected. In this assay, the criterion for repopulation is 100% donor Hbb' in recipient red blood cells. One hundred c-kitPOS cells from FR26 Lin− and FR30 Lin− subsets repopulated nearly 50% of recipients (4/10 and 4/12, respectively), while 200 cells from these fractions repopulated four of five recipients. The lower ratio of PHSC (1:800 cells) in the FR35 Lin− c-kitPOS subset may result from a dilution of the c-kitPOS PHSC with numerous c-kitNEG CFU-S, and/ or other progenitors observed in FR35 Lin− (Table 1).
cate that Lin⁻ c-kit⁺ BR RhoDULL cells have a 10-fold greater repopulating ability than Lin⁻ c-kit⁺ BR RhoBR cells. The c-kit⁺ BR RhoDULL PHSC may express P-glycoprotein, an efflux pump that transports Rho from the cell.

**Table 2. Enrichment of PHSC by Flow Cytometry**

<table>
<thead>
<tr>
<th>Sample*</th>
<th>No. of Cells Injected/</th>
<th>No. of Mice Repopulated/No. of Mice Assayed</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR25 AA4.1⁺</td>
<td>1.7 x 10⁵</td>
<td>0/4</td>
</tr>
<tr>
<td>FR25 Lin⁻ AA4.1⁺</td>
<td>3 x 10⁵</td>
<td>0/3</td>
</tr>
<tr>
<td>FR25 Lin⁻ AA4.1⁻</td>
<td>3 x 10⁵</td>
<td>4/5</td>
</tr>
<tr>
<td>FR35 AA4.1⁺</td>
<td>1 x 10⁵</td>
<td>3/3</td>
</tr>
<tr>
<td>FR35 Lin⁻ AA4.1⁺</td>
<td>3 x 10⁵</td>
<td>3/3</td>
</tr>
<tr>
<td>FR35 Lin⁻ AA4.1⁻</td>
<td>3 x 10⁵</td>
<td>3/3</td>
</tr>
<tr>
<td>FR35 Lin⁻ c-kitNEG AA4.1⁺</td>
<td>5 x 10³ to 4 x 10⁴</td>
<td>0/9</td>
</tr>
<tr>
<td>FR35 Lin⁻ c-kitNEG AA4.1⁻</td>
<td>5 x 10³ to 1 x 10⁵</td>
<td>19/19</td>
</tr>
<tr>
<td>FR35 Lin⁻ c-kit⁺ RhoDULL CD4⁺</td>
<td>2 x 10⁵ to 2 x 10⁶</td>
<td>0/4</td>
</tr>
<tr>
<td>FR35 Lin⁻ c-kit⁻ RhoDULL CD4⁺</td>
<td>2 x 10⁴ to 4 x 10⁴</td>
<td>4/4</td>
</tr>
<tr>
<td>FR30 Lin⁻ c-kitNEG CD4⁺</td>
<td>2 x 10⁴ to 4 x 10²</td>
<td>3/3</td>
</tr>
<tr>
<td>FR30 Lin⁻ c-kitNEG CD4⁻</td>
<td>2 x 10⁴ to 2 x 10⁴</td>
<td>10/11</td>
</tr>
</tbody>
</table>

* CCE fractions of bone marrow with Lin⁻ cells subtracted. For the experiments involved with the subsets FR25 Lin⁻ c-kit⁺ RhoDULL CD4⁺ and FR30 Lin⁻ c-kit⁻ RhoDULL CD4⁻, anti-CD4 MoAb was omitted from the MoAb cocktail used to subtract Lin⁻ cells.

† Mice received a single injection of sorted cells within the ranges given.

‡ Repopulation was assayed at 4 months postransplantation by analysis of hemoglobin and DNA for donor contribution.

Southern blot analysis of β-globin genes in the thymus and bone marrow of W/W⁺ recipients injected with 100 RhoDULL cells indicated an uneven pattern of repopulation (Table 2). One hundred cells from FR25 Lin⁻ c-kit⁺ BR RhoDULL or FR35 Lin⁻ c-kit⁺ BR RhoDULL subsets repopulated the thymus in one of three and two of three recipients, respectively, whereas bone marrow was not converted in any of six recipients. Although there is no clear explanation for this observation, we may not have allowed sufficient time for myeloid conversion in this limiting dilution study. In contrast, 100 and 1,000 donor cells from the FR30 Lin⁻ c-kit⁺ BR RhoDULL subset repopulated both thymus and marrow in eight of nine W/W⁺ mice. The high dose of 1,000 cells from the FR30 Lin⁻ c-kit⁺ BR RhoBR subset repopulated both thymus and marrow in all recipients. In contrast, cells from FR25 and FR35 Lin⁻ c-kit⁺ BR RhoBR subsets repopulated the thymus in one of seven and bone marrow in one of 10 recipients.

**Gene expression in purified populations.** The flk 2 gene, a member of the tyrosine kinase receptor family, has been reported to be a marker for the most primitive fetal liver hematopoietic cells. To test whether flk-2 expression might also be a marker for adult bone marrow PHSC, RNA was purified from several populations of bone marrow cells in various stages of purity (Fig 5). Using reverse-transcriptase PCR, we detected low levels of flk-2 mRNA in unfractionated bone marrow. Higher levels of flk-2 mRNA were seen in FR30, FR35, and R/O cells, in FR30 Lin⁻ and FR35
STEM CELLS EXPRESS HIGH LEVELS OF c-kit

Lin·, and in FR35 Lin· c-kitNEG, c-kitDULL, and c-kitBR

cells. In contrast, RNA isolated from cells in FR25 Lin·
c-kitNEG and c-kitDULL contained low levels of flk-2 mRNA,
and no flk-2 mRNA was detected in the FR25 Lin· c-kitBR
subpopulation, a subpopulation enriched for
intrinsic to
hematopoiesis, to subdivide FR25, FR30, and FR35 Lin·
c-kitDULL cells was estimated by scanning densitometry
at 12% of the β2-microglobulin mRNA level, whereas FR25
Lin· c-kitBR cells had no flk-2 mRNA while showing 35% of
the β2-microglobulin mRNA seen in FR25 Lin· c-kitDULL
cells.

DISCUSSION

The W locus encodes the c-kit proto-oncogene, a tyrosine
kinase receptor24,33 for SCF.36-38 Mice that are compound
heterozygous for W express pleiotropic abnormalities that
include erythrocyte maturational defects.30,31 Transplantation
experiments show that the effects of c-kit mutations are
intrinsic to PHSC30 and that W/Wmice have a normal
environment for hematopoiesis. This feature allows
the direct injection of limiting numbers of PHSC and avoids
the effects of radiation and the ensuing neutropenic period.
Several studies describe MoAbs to c-kit that have been used
to purify human22,23 and mouse19-21 progenitors and stem
cells. Two of these MoAbs recognize different epitopes on
the extracellular domain of the mouse c-kit receptor. One,
Ack-2, blocks blood cell formation when administered in
vivo. We used the MoAb, Ack-4, which is not antagonistic
to hematopoiesis, to subdivide FR25, FR30, and FR35 Lin·
bone marrow cells into c-kitBR, c-kitDULL, and c-kitNEG
subsets.

Using the W/Wm model, we were able to repopulate the
thymus and bone marrow of approximately 50% of recipients
given 100 to 200 FR25 or FR30 Lin· c-kitBR cells. This
represents a 500- to 1,000-fold enrichment of
PHSC from a population of unfractionated bone marrow.
Nonirradiated W/Wmice, used in our study, did not require the coinjection
of radioprotective cells to supplement the FR25, FR30, and
FR35 Lin· c-kitPOS PHSC subsets.

The PHSC fractionation procedure used here enabled us
to extend the observation of Okada et al21 who showed that 100 c-kitPOS cells from unfractionated, sorted marrow can
reconstitute lethally irradiated mice provided the recipients
were also coinjected with radioprotective cells. Furthermore,
the c-kitPOS cells used by Okada et al21 may have included numerous CFU-S12 and other progenitors, whereas our FR25 Lin· c-kitBR cells contained PHSC with
few or no contaminating CFU-S12 (one CFU-S12 per 253
FR25 Lin· c-kitBR cells, see Table 1) or progenitor cells.6
The c-kit Sca-1 double-positive PHSC isolated by Okada et
al21 were in the c-kitBR fraction as defined in our study.

Several other markers have been used to fractionate bone
marrow cells. Wineman et al have isolated murine bone marrow PHSC based on CD4 expression. In our study, reanalysis of the Lin- cell fractions consistently showed fewer than 1% to 2% CD4+ cells. Nevertheless, to exclude the possibility that a minor fraction of PHSC exists in CD4+ cells, we tested Lin- cells obtained with and without anti-CD4 MoAb added to the MoAb cocktail. No CD4+ PHSC were present in FR25 Lin- c-kitPOS cells, our purest population of PHSC. However, CD4+ PHSC were present in the FR30 Lin- c-kitPOS subset. Our results agree with the finding of Wineman et al in that we have shown a subpopulation of PHSC in the FR30 Lin- c-kitPOS subset that are CD4+.

The AA4.1 antigen was initially used to identify pre-B cells in hematopoietic tissue. Subsequently, it was identified as a marker for myeloid-erythroid progenitors (CFU-GEMM), day 12 to 16 CFU-S, and long-term repopulating stem cells in a 0.5% to 1% subpopulation of fetal liver hematopoietic cells. We observed an equivocal pattern of AA4.1 antigen expression on PHSC from mouse bone marrow. PHSC were present in all AA4.1- subsets tested and in some of the AA4.1+ subsets. This observation represents a clear difference between PHSC in fetal liver, which are all AA4.1+, and adult bone marrow.

The flk-2 gene, like c-kit, is a member of the tyrosine kinase receptor family. Its cDNA was isolated from a population of AA4.1+ fetal liver cells highly enriched for PHSC and CFU-S. The expression pattern of flk-2 mRNA predicted that it was expressed in the most primitive fetal liver hematopoietic cells. Although flk-2 mRNA was abundant in RNA from the FR35 subsets enriched for both CFU-S12 and PHSC (Lin- c-kitRR) or CFU-S12 alone (Lin- c-kitDLU), we were unable to detect flk-2 mRNA in RNA extracted from FR25 Lin- c-kitRR bone marrow cells that were highly enriched for PHSC but depleted of CFU-S12. These data argue that flk-2 mRNA is not expressed in the fraction of PHSC present in the subset elutriated at FR25. This may represent a fundamental difference between FR25 Lin- c-kitRR PHSC and those from fetal liver. We suggest that AA4.1 and/or flk-2 are specific markers on PHSC in cell cycle. Fetal liver PHSC are AA4.1+ and flk-2+ and are likely to be in a state of exponential cell growth. In contrast, many PHSC in adult bone marrow are expected to be in a resting G0 phase. These may be the small PHSC in FR25 that are AA4.1- and flk-2-. The larger AA4.1+ flk-2+ cells in FR35 may include PHSC in cell cycle.

Perhaps the most widely used protocol to date for mouse stem-cell enrichment involves MoAbs to Sca-1 and Thy-1. Studies with this model have been used to demonstrate successfully that as few as 30 Thy1.1 Lin- Sca-1+ cells can reconstitute irradiated mice if coinjected with radioprotective cells. Mice were scored as reconstituted if greater than 1% of their circulating B cells, T cells, and myeloid cells were donor-derived. Since 100 to 200 c-kitRR cells can repopulate the entire thymus and bone marrow in W/W recipients, we regard our PHSC population to be at least as pure as the Thy1.1 Lin- Sca-1+ PHSC subset. This conclusion is supported by the observation that PHSC in elutriated Lin- c-kitRR subsets can repopulate the lymphohematopoietic...
tic system in W/W* mice against a background of competing endogenous PHSC.

The protocol that we developed here has several advantages over previously reported models. First, we are able to isolate a high expression c-kit subset that contains PHSC with relatively few contaminating CFU-S*2 and other progenitors. Second, the W/W* mice used by us do not require irradiation; thus, purified PHSC can be assayed free of injected radioprotective cells. Third, the W/W* model provides an opportunity to trace the progeny of small numbers of PHSC (theoretically a single PHSC) for a 4-month interval during which the recipient lymphomyeloid tissues become 100% repopulated. Finally, our protocol can be used on all strains of mice for PHSC purification and provides an assay to study the biologic and functional characteristics of a single or relatively few transplanted PHSC.

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Purification and characterization of heterogeneous pluripotent hematopoietic stem cell populations expressing high levels of c-kit receptor

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