Evidence of Both Ontogeny and Transplant Dose-Regulated Expansion of Hematopoietic Stem Cells In Vivo

By Robert Pawliuk, Connie Eaves, and R. Keith Humphries

Recent assessment of the long-term repopulating activity of defined subsets of hematopoietic cells has offered new insights into the characteristics of the transplantable stem cells of this system; however, as yet, there is very little known about mechanisms that regulate their self-renewal in vivo. We have now exploited the ability to quantitate these cells using the competitive repopulating unit (CRU) assay to identify the role of both intrinsic (ontological) and extrinsic (transplanted dose-related) variables that may contribute to the regulation of CRU recovery in vivo. Ly5.1 donor cells derived from day-14.5 fetal liver (FL) or the bone marrow (BM) of adult mice injected 4 days previously with 5-fluorouracil were transplanted at doses estimated to contain 10, 100, or 1,000 long-term CRU into irradiated congenic Ly5.2 adult recipient mice. Eight to 12 months after transplantation, there was a complete recovery of BM cellularity and in vitro clonogenic progenitor numbers and a nearly full recovery of day-12 colony-forming unit-spleen numbers irrespective of the number or origin of cells initially transplanted. In contrast, regeneration of Ly5.1+ donor-derived CRU was incomplete in all cases and was dependent on both the origin and dose of the transplant, with FL being markedly superior to that of adult BM. As a result, the final recovery of the adult marrow CRU compartment ranged from 15% to 62% and from 1% to 18% of the normal value in recipients of FL and adult BM transplantation, respectively, with an accompanying maximum CRU amplification of 150-fold for recipients of FL cells and 15-fold for recipients of adult BM cells. Interestingly, the extent of CRU expansion from either source was inversely related to the number of CRU transplanted. These data suggest that recovery of mature blood cell production in vivo may activate negative feedback regulatory mechanisms to prematurely limit stem cell self-renewal ability. Proviral integration analysis of mice receiving retrovirally transduced BM cells confirmed regeneration of totipotent lymphomyeloid repopulating cells and provided evidence for a greater than 300-fold clonal amplification of a single transplanted stem cell. These results highlight the differential regenerative capacities of CRU from fetal and adult sources that likely reflect intrinsic, genetically defined determinants of CRU expansion but whose contribution to the magnitude of stem cell amplification ultimately obtained in vivo is also strongly influenced by the initial number of CRU transplanted. Such findings set the stage for attempts to enhance CRU regeneration by administration of agents that may enable full expression of regenerative potential through the expression of intracellular gene products that may alter intrinsic regenerative capacity.

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zygous for Ly5.2 and PepC3Fl mice, are Ly5.1/Ly5.2 heterozygotes. All animals were housed in microisolator cages and provided with sterile food and acidified sterile water.

Transplantation and quantitation of CRU. FLs were removed from day-14.5 embryos obtained from timed matings of C57Bl/6Ly-Pep3b male and C3H/HeJ female mice. Cells were suspended in α medium containing 5% fetal calf serum (FCS); StemCell Technologies, Vancouver, British Columbia, Canada) by repeated gentle aspiration through 5-mL, 2-mL, and 1-mL pipettes, followed by 18- and 21-gauge needles. BM cells from male or female PepC3Fl, mice injected 4 days previously with 5-fluorouracil (5-FU; 150 mg/kg body weight) were flushed from femoral shafts with α medium and 5% FCS and a single-cell suspension was similarly obtained. Cells were counted using a standard hemocytometer. Cell survival was greater than 98% as determined by trypan blue exclusion. FL cells or post-5-FU BM cells from Lys.11 donors were injected in combination with a life-sparing dose of 10^8 BM cells from normal 8- to 12-week-old B6C3Fl, (Ly5.2+) mice11 into the tail vein of recipient B6C3Fl, (Ly5.2+) mice previously irradiated with 950 cGy (110 cGy/min, 10Cs gamma rays).

CRU were measured by injecting groups of lethally irradiated B6C3Fl, (Ly5.2+) recipients in combination with 10^7 syngeneic (Ly5.2+) normal BM cells and assessing the recipients 16 weeks later for the presence of Ly5.1+ lymphoid and myeloid cells in their peripheral blood (PB). For this procedure, 50- to 100-μL samples from tail vein puncture were depleted of erythrocytes by incubating them for 10 minutes on ice in 4 vol of sterile 1 mL NH4Cl, washed in Hank's balanced salt solution containing 2% FCS (HF), and resuspended in HF containing 1 μg/mL of a fluorescein isothiocyanate (FITC)-conjugated anti-Ly5.1 monoclonal antibody (hybridoma A20-1.7 originally from Dr G. Spangrude [Rocky Mountain Laboratory, Hamilton, MT] and purified antibody prepared by Dr P. Lansdorp [Terry Fox Laboratory, Vancouver, British Columbia, Canada]). After 40 minutes on ice, the cells were washed once again in HF and finally in HF containing 1 μg/mL 7-aminactinomycin D (7AAD, Sigma, St Louis, MO) to exclude dead (7AAD+) cells from the analysis by flow cytometry (using a FACScan; Becton Dickinson and Co, San Jose, CA). Recipient mice were considered positive if greater than 0.5% of each of myeloid and lymphoid PB cell population (identified by their unique forward and side scatter profiles, respectively) showed the donor Ly5.1 cell surface phenotype. CRU frequency was calculated by determining the number of negative recipients as a function of the number of test cells injected and applying Poisson statistics using the method of least likelihood.11,12

In one experiment, the original cells transplanted were first transduced with a recombinant retrovirus (JZenCD24tkneo) that contains an tkneo cassette. Construction of the JZenCD24tkneo retrovirus, details of the protocol used for prestimulation (2 days with murine [m] Steel factor, murine interleukin-3 [mIL-3], and human IL-6 [hIL-6]) and infection of murine BM cells (2-day cocultivation in the presence of the same growth factors in addition to 7 μg/mL polybrene) and the anti-CD24 antibody staining procedure have been described previously.13 Transduced cells were selected 48 hours after infection on the basis of their cell surface expression of CD24 using a FACStar® (Becton Dickinson and Co) equipped with a 5-W argon and a 30-mW He-Ne laser. CD24+ cells were collected in sterile endopore vials in α medium with 50% FCS and then transplanted as described above for nontransduced cells.

In vitro colony-forming cell (CFC) assays. Cells to be assayed were suspended at appropriate dilutions in 0.8% methylcellulose in α medium supplemented with 30% FCS, 1% bovine serum albumin (BSA), 10^-4 mol/L β-mercaptoethanol, 3 U/mL human erythropoietin, 2% spleen cell conditioned medium (SCCM), and 10% agar-stimulated human leukocyte conditioned medium (Stem Cell) and plated in 1.1-mL volumes in 35-mm petri dishes (Stem Cell). Cultures were incubated at 37°C in humidified atmosphere of 5% CO2 in air. Colonies derived from colony-forming unit–granulocyte-macrophage (CFU-GM), burst-forming unit–erythroid (BFU-E), or colony-forming unit granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM) were scored in situ after 8 to 14 days of incubation using well-established criteria.10,11

Day-12 colony-forming unit-spleen (CFU-S) assays. Lethally irradiated B6C3Fl, mice (910 to 950 cGy, 110 cGy/min, 10Cs gamma rays) were injected intravenously with 7.5 × 10^8 or 10^9 cells from primary recipients. Twelve days later, animals were killed and macroscopic spleen colonies were counted after fixation in Telleyesnicky’s solution.

Analysis of proviral integrants. DNA was purified from NaDodSO4/proteinase K-digested cells by phenol/chloroform extraction.14 DNA was dialyzed for 16 hours against 1× TE (10 mmol/L Tris, pH 7.5, 1 mmol/L EDTA, pH 8.0) buffer and digested with Xba I or EcoRI (Canadian Life Technologies, Burlington, Ontario, Canada) at 37°C for 12 to 16 hours. After ethanol precipitation, DNA was dissolved in 20 μL of 1× TE buffer, separated on a 0.8% agarose gel, and transferred to a nylon membrane (Zeta-Probe; Bio-Rad, Hercules, CA) by blotting. Membranes were subsequently probed for proviral sequences derived from the JZenCD24tkneo virus by hybridization to a fragment of the neomycin resistance (neoR) gene previously labeled with 3P by random priming.

RESULTS

Overall experimental design. The purpose of these experiments was to examine and compare the kinetics of recovery of every level of hematopoietic cell development in myelosblotted recipients as a function of both the CRU content and the origin of the cells transplanted. Donor cells were obtained from either day-14.5 FLs or the BM of adult mice injected 4 days previously with 150 mg/kg of 5-FU and groups of mice then injected with a range of cell numbers estimated to contain 1,00, 100, or 10 CRU, ie, 10, 1%, and 0.1% of the total marrow CRU content of an average untreated adult mouse or 90%, 9%, or 0.9% of the CRU content of a single-day-14.5 FL.9 All mice also received 10^8 BM cells from an unmanipulated adult Ly5.2 B6C3Fl, donor containing an estimated competing graft of 10 CRU.12 Recipients of these grafts were killed 8 to 12 months after transplantation for the assessment of the test graft (Ly5.1+)-derived contribution both to their reconstituted PB cells as well as their marrow CFC, CFU-S, and CRU populations.

Kinetics of reconstitution of the terminal compartments. Flow cytometric analysis of mature cells in the PB 8 months after transplantation showed extensive reconstitution of both lymphoid and myeloid compartments with Ly5.1+ test cells for all transplant groups (Table 1). The proportion of Ly5.1+ cells contributing to both of these mature compartments was highly consistent between experiments for each transplant dose and made up nearly all (≈80%) of the cells in recipients of FL cells and in recipients of all but the lowest transplant dose of adult BM cells (containing 10 CRU). In these, the proportion of PB cells that were Ly5.1+ was slightly lower, albeit still approximately 50% of the total, reflecting their origin from an equivalent proportion (also ≈50%) of all the CRU transplanted.

Reconstitution of the marrow. Two mice per transplant group were chosen for further analysis of the level of test
of FL cells. For recipients of all other sources or numbers control mice (Table 2). In contrast, for day-12 CFU-S, this equivalent to those found in unmanipulated age-matched primary recipients, irrespective of the number or origin complete, ranging from 45% to 85% of normal values (Table 2).

Although there was a trend towards a greater recovery of this reconstitution attained by this source, recipients in which at least 80% of the test cells, recovery of day-12 CFU-S numbers was incom-

plantation. In an effort to detect the maximum levels of

lymphoid cells in the PB of the secondary CRU assay recipients was evaluated after 16 weeks and used to derive the CRU numbers shown in Fig 1. In most cases, the Ly5.1+ CRU population had regenerated to a level corresponding to only a small proportion of the normal CRU population, and the levels achieved correlated positively with the original dose of Ly5.1+ CRU used to reconstitute the primary recipients. Interestingly, the level of CRU regeneration was consistently higher (P < .01 to < .1) for FL transplants as compared with marrow transplants containing the same original number of CRU. Thus, on a per input CRU basis, CRU amplification in the FL transplants was up to 15 × more effective than with primary BM transplants and only in primary recipients of 1,000 CRU of FL origin did the transplanted CRU recover to a normal sized population within 8 months after transplantation.

Effects of the transplant dose as well as the tissue origin are more dramatically shown by using the same data to calculate the extent of Ly5.1+ CRU amplification in each experimental situation tested. The results of such calculations are shown in Table 3. It can be seen that, for both FL and adult BM transplants, CRU expansion was inversely related to the initial number of CRU transplanted, although the extent of CRU expansion for input FL CRU was greater than that of their counterparts in BM. The maximum CRU expansion observed was 150-fold for FL CRU compared with a maximum CRU expansion of only 15-fold for adult BM transplants.

Regenerative ability of a single CRU assessed using retroviral marking. To gain further insight into CRU regeneration, we used retroviral marking for clonal analysis of self-renewal and lympho-myeloid reconstitution. 5-FU-treated

transplant-derived cells in the marrow 8 months after transplantation. In an effort to detect the maximum levels of reconstitution attained by this source, recipients in which at least 80% of the PB cells were Ly5.1+ were selected. In addition, the BM cells of one of the recipients were also stained with the Ly5.1 antibody and, upon FACS analysis, were found, like the blood, to contain greater than 80% Ly5.1+ (i.e., test transplant-derived) cells (data not shown).

The total marrow cellularity and CFC numbers in all pairs of primary recipients, irrespective of the number or origin of the cells initially transplanted, had regenerated to levels equivalent to those found in unmanipulated age-matched control mice (Table 2). In contrast, for day-12 CFU-S, this was true only for the recipients of the highest transplant dose of FL cells. For recipients of all other sources or numbers of test cells, recovery of day-12 CFU-S numbers was incomplete, ranging from 45% to 85% of normal values (Table 2). Although there was a trend towards a greater recovery of this more primitive compartment in recipients of higher initial transplant doses, the actual differences between the groups were not statistically significant (P < .05).

Reconstitution of the marrow CRU compartment. To compare the accompanying level of regeneration of Ly5.1+ donor-derived stem cells in the marrow of these same pairs of primary recipients, CRU frequencies and hence numbers were also determined. The presence of Ly5.1+ myeloid and lymphoid cells in the PB of the secondary CRU assay recipients was evaluated after 16 weeks and used to derive the CRU numbers shown in Fig 1. In most cases, the Ly5.1+ CRU population had regenerated to a level corresponding to only a small proportion of the normal CRU population, and the levels achieved correlated positively with the original dose of Ly5.1+ CRU used to reconstitute the primary recipients. Interestingly, the level of CRU regeneration was consistently higher (P < .01 to < .1) for FL transplants as compared with marrow transplants containing the same original number of CRU. Thus, on a per input CRU basis, CRU amplification in the FL transplants was up to 15 × more effective than with primary BM transplants and only in primary recipients of 1,000 CRU of FL origin did the transplanted CRU recover to a normal sized population within 8 months after transplantation.

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Table 1. Proportion of Ly5.1+ PB Cells From Primary Recipients Transplanted With Varying Numbers of Ly5.1+ Syngeneic Adult BM- or FL-Derived CRU

<table>
<thead>
<tr>
<th>Source and Transplant</th>
<th>Estimated No. of CRU Transplanted</th>
<th>% Ly5.1+ PB Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Exp 1)</td>
<td>(Exp 2)</td>
</tr>
<tr>
<td>BM 2 x 10⁵</td>
<td>1,000</td>
<td>91 ± 2 (5)</td>
</tr>
<tr>
<td>BM 2 x 10⁶</td>
<td>100</td>
<td>77 ± 4 (5)</td>
</tr>
<tr>
<td>BM 2 x 10⁷</td>
<td>10</td>
<td>42 ± 7 (13)</td>
</tr>
<tr>
<td>FL 1.7 x 10⁷</td>
<td>1,000</td>
<td>91 ± 1 (5)</td>
</tr>
<tr>
<td>FL 1.7 x 10⁸</td>
<td>100</td>
<td>94 ± 0 (3)</td>
</tr>
<tr>
<td>FL 1.7 x 10⁹</td>
<td>10</td>
<td>79 ± 4 (5)</td>
</tr>
</tbody>
</table>

Values shown are the mean ± SEM (number of mice analyzed) of the proportion of Ly5.1+ cells in the circulating WBC populations present in primary transplant recipients analyzed 8 months posttransplantation in two individual experiments.

* The estimation of the number of CRU transplanted is derived from a control value of 1/2,000 (95% CI, 1 in 1,300 to 1 in 5,700) for adult 5-FU BM and 1/17,000 (95% CI, 1 in 11,500 to 1 in 26,000) for FL.

Table 2. Regeneration of Total Cells, CFC, and Day 12 CFU-S Populations in the Femurs of Mice Transplanted With Various Numbers of Adult BM or FL CRU 8 Months Posttransplantation

<table>
<thead>
<tr>
<th>Transplant Source</th>
<th>Estimated No. of CRU Transplanted</th>
<th>Total Nucleated Cells/Femur (+SD)</th>
<th>CFU-C/Femur*(+SD)</th>
<th>Day-12 CFU-S/Femur × 10⁶ (+SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult BM</td>
<td>10</td>
<td>2.1 ± 0.7 x 10⁷</td>
<td>380 ± 61</td>
<td>10 ± 2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.3 ± 0.1 x 10⁷</td>
<td>500 ± 10</td>
<td>16 ± 2</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>2.5 ± 0.5 x 10⁷</td>
<td>480 ± 160</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>FL</td>
<td>10</td>
<td>2.1 ± 0.5 x 10⁷</td>
<td>330 ± 99</td>
<td>12 ± 2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.3 ± 0.1 x 10⁷</td>
<td>390 ± 18</td>
<td>14 ± 1</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>2.4 ± 0.7 x 10⁷</td>
<td>390 ± 38</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>Normal control</td>
<td>2.3 ± 0.1 x 10⁷</td>
<td>480 ± 140</td>
<td>24 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

For each experiment, 2 mice showing the highest proportion of Ly5.1+ cells in the PB were chosen as donors for analysis. The average proportion of Ly5.1+ cells in the PB of chosen mice for each group was as follows: adult marrow: 10 CRU, 81%; 100 CRU, 85%; 1,000 CRU, 94%; FL: 10 CRU, 82%; 100 CRU, 94%; 1,000 CRU, 92%. Normal control animals were 6- to 8-month-old unmanipulated B6C3F1 mice.

* Cells were plated in methylcellulose at 2.7 x 10⁶ cells/mL and colonies were scored 12 days later.

+ A total of 7.5 x 10⁶ to 1 x 10⁷ marrow cells of pooled marrow from the two primary recipients were injected into 5 irradiated (950 cGy) B6C3F1 mice/group. Animals were killed 12 days later and macroscopic spleen colonies were counted. See Table 1 for calculated CRU frequencies in unmanipulated adult BM and FL.
Ly5.1 marrow cells were cocultured with cells producing a retrovirus containing the coding region of the human CD24 cell surface antigen and were then stained with an anti-CD24 antibody and CD24+ cells selected by FACS 48 hours after infection, as described in the Materials and Methods. Of these Ly5.1+ cells (estimated to contain 3.0 ± 1.0 CRU from previous unpublished data), 10^2 were transplanted into Ly5.2+ recipients. All of 13 such recipients showed detectable levels of Ly5.1+ (donor cell-derived) repopulation, with values ranging from 3% to 32% Ly5.1+ PB leukocytes (10 of 13 myeloid/lymphoid repopulation; 3 of 13 lymphoid-restricted repopulation). The hematopoietic tissue of all of these mice also showed the presence of intact provirus at 11 months after transplantation, but in only 3 was CD24 expression detectable in the PB leukocytes (where values of 2% and 20% CD24+ were measured). One of these recipients showing 32% donor-derived Ly5.1+ cells, of which 63% were CD24+, was chosen for further study to quantitate the clonal regeneration of Ly5.1+ CRU. Southern blot analysis of the BM, spleen, and thymus of this mouse showed identical proviral banding patterns and band intensities in all of these tissues, consistent with the repopulation of this primary recipient by a single transduced totipotent repopulating stem cell. When the marrow cells of this mouse were then assayed for their content of Ly5.1+ CRU, 0.2 ± 0.05 Ly5.1+ CRU per 10^5 marrow cells or 50 Ly5.1+ CRU per femur were detected. This represents 3.7% of the CRU population in the femur of a normal adult B6C3F1 mouse. Seventeen of the 19 secondary transplant recipients who showed the presence of Ly5.1+ PB cells were also positive for CD24 expression, with values ranging from 1.5% to 14.2% CD24+ PB leukocytes. Strikingly, the same proviral banding pattern seen in the primary animal was also observed exclusively in the hematopoietic tissues of all of the secondary recipients who were reconstituted with Ly5.1+ cells. This observation indicates a 370-fold amplification of the original transduced CRU during the period of 11 months after it was transplanted into the primary recipient. This result provides formal evidence of CRU self-renewal in vivo and extends the previous findings indicating the extent to which this can occur.

### DISCUSSION

Previous studies have indicated that the capacity of most primitive hematopoietic cells capable of regenerating the entire system cannot be maintained on serial transfer. We now show that this may be at least partially attributable to a common failure of the CRU compartment to be fully regenerated even 8 months after transplantation despite, indeed perhaps because of, a complete recovery of later cell types, including day-12 CFU-S and cells detectable as CFC.

#### Table 3. Expansion of Donor-Derived CRU in Primary Recipients of FL or Adult BM Cells

<table>
<thead>
<tr>
<th>Transplant Source</th>
<th>Estimated No. of CRU Transplanted*</th>
<th>Estimated No. of BM CRU After Expansion (range ± SEM)**</th>
<th>Fold Expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult BM</td>
<td>10</td>
<td>100 (75-150)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1,500 (1,000-2,100)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>1,800 (1,300-2,500)</td>
<td>2</td>
</tr>
<tr>
<td>FL</td>
<td>10</td>
<td>1,500 (1,000-2,100)</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3,100 (2,300-4,200)</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>6,200 (4,800-8,500)</td>
<td>6</td>
</tr>
<tr>
<td>Untransplanted control</td>
<td>—</td>
<td>10,000 (6,300-14,900)</td>
<td>—</td>
</tr>
</tbody>
</table>

* See Table 1 for calculated CRU frequencies in unmanipulated adult BM and FL.

** Results are expressed as the number of CRU per mouse based on the estimate that 2 × 10^7 femoral marrow cells constitute ~10% of the total hematopoietic population of the mouse.
Interestingly, there was, nevertheless, a quantitative relationship between the extent of amplification seen in donor CRU numbers and both the size of the initial transplant and its ontological source.

It has been suggested by several investigators that transplantable stem cells may fail to regenerate the stem cell compartment to normal (nontransplant) levels because of an inherently limited capacity for self-renewal.13,18 According to such a model, the absolute extent of CRU amplification would be anticipated to decrease as the number of stem cells transplanted was decreased, because the number of stem cells with the highest self-renewal potential would also decrease proportionately. On the other hand, it is possible that the transplantation of smaller numbers of marrow cells might place a higher stress on the system, resulting in the production of stimuli that could favor differentiation rather than self-renewal responses. Evidence of decreased stem cell regeneration under conditions that support their proliferation both in vitro,19 in utero,20 and after BM transplantation19,18,21,22 have been reported. However, such studies do not necessarily measure the capacities of the cells tested but rather their response under a given set of molecularly undefined and poorly understood environmental conditions. The present studies, which have used a quantitative assay to provide a direct measurement of the size of the regenerated totipotent, transplantable stem cell compartment, indicate that a higher degree of stem cell amplification is consistently obtained with the smallest number of stem cells transplanted, even though this is insufficient to achieve a comparable level of regeneration of the stem cell population by comparison to its size in unperturbed animals (Table 3 and Fig 1). Thus, the extent to which either FL or adult BM stem cells express their full regenerative potential varies according to how many of them (and/or accompanying marrow cells) are transplanted and this decreases with inoculum size. A possible explanation for this finding would be the activation of negative feedback regulatory mechanisms in vivo that can limit stem cell expansion prematurely, perhaps via the production by mature hematopoietic cells of such factors as macrophage-inhibitory protein-1α (MIP-1α) and transforming growth factor-β (TGF-β) that may selectively decrease the proportion of primitive hematopoietic cells in cycle.23-25 Such factors might thus attenuate or even terminate CRU expansion even though the numbers of these cells might still be significantly below the normal level. It is interesting to speculate that this effect might be promoted by the cotransplantation of large numbers of mature hematopoietic cells or their immediate precursors. Such a possibility is consistent with the observation that the maximum degree of CRU expansion was observed with the smallest transplant dose. Accordingly, it would be anticipated that stem cell expansion might be enhanced when purified stem cells are transplanted to reduce the number of mature cells present during the initial stages of engraftment.

It is important to note that, in these experiments, only the regeneration of donor Ly5.1+ CRU were quantitated. Our results clearly show that the transplanted Ly5.1+ CRU were unable to reconstitute the CRU compartment of primary transplant recipients to levels found in normal adult mice. However, the extent to which 10 Ly5.2+ CRU present in the competitor cell population or those surviving in the host might have contributed to regeneration of the total CRU compartment is not known. It seems highly unlikely that a large reserve of inactive Ly5.2+ CRU would have been present in the primary recipients because the vast majority of all the cells in the marrow and the PB of these mice were Ly5.1+

Results from many previous studies have indicated that the regenerative behavior of hematopoietic cells in a transplant setting is a function of their ontological state. Thus, more than 20 years ago, it was found that a transplant of FL cells would outcompete adult BM after their combined transplant into recipient mice.5 Similarly, 10 years later, it was shown that day-8 CFU-S derived from FL possess a greater capacity for self-renewal as compared with their adult BM counterparts.4 Recently, Rebel et al8 have shown that limiting numbers of FL CRU are able to produce a greater output of mature blood cells in vivo as compared with adult BM. Moreover, when marrow cells from primary recipients of limiting numbers of FL CRU were injected into secondary recipients, a significantly higher percentage of these mice showed donor-derived reconstitution of their lymphoid and myeloid compartments as compared with mice that had received marrow cells from primary recipients of similar numbers of adult BM CRU. These results are thus also highly suggestive of a greater regenerative capability of FL HSC compared with adult BM. However, because of their design, none of these studies could discriminate between quantitative differences in the self-renewal of transplantable HSC and possible differences in the extent of proliferation achieved by their more differentiated progeny. By addressing this question here, it has been possible to establish that FL CRU are indeed superior to their adult BM counterparts both in terms of the relative and the absolute numbers of CRU they will regenerate under similar conditions.

A possible explanation for these differences is that FL CRU possess a greater intrinsically regulated probability for self-renewal when stimulated to divide in the microenvironment of the posttransplant myeloablated mouse. Intriguing evidence of candidate genes that may be involved in the intrinsic control of self-renewal was recently reported by Sauvageau et al26 from studies of the effects of Hox genes on hematopoiesis in vivo. These showed that overexpression of HOXB4, whose expression is normally restricted to the most primitive adult BM cells in the adult,27 resulted in a 50-fold increase in the regeneration of transduced CRU as compared with neo-transduced control cells. However, the possibility that the genes expressed within FL and adult BM HSC influence this self-renewal probability does not preclude other mechanisms that might influence the observed differences in the rates of FL and adult marrow CRU amplification. For example, these may also exhibit differences in the time required to transit one complete cell cycle28 or in the proportion of cells recruited or maintained within the microenvironment of the marrow of the adult mouse.

The use of recombinant retroviruses as genetic tags has been used extensively to track the proliferative and differentiative behavior of individual stem cell clones both in

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In the present study, we used a recombinant retrovirus containing the coding region of the human CD24 cell surface antigen to confirm the totipotentiality of the CRU detected using the CRU assay and to aid in the verification and quantitation of the degree of expansion exhibited by individual CRU in vivo. Only one proviral banding pattern was detected in BM and thymic DNA in the primary and all secondary transplant recipients (Fig 2), which suggests that the regeneration of the stem cell compartment in the primary recipient was monoclonal in nature. This clone regenerated the CRU compartment in the primary recipient was monoclonal in nature. This clone regenerated the CRU compartment to 3.7% of the normal level, which is comparable to that observed in recipients of 10 adult BM CRU. This represents a CRU expansion of 370-fold, which is higher than that observed for any transplant dose of FL or adult BM (Table 3). Differences in estimates of CRU expansion at the level of the whole population versus individual clones likely reflect heterogeneity among the regenerative activity displayed by biologically equivalent individual HSC responding to proliferative stimuli. However, at the same time, the present results confirm the ability of totipotent repopulating stem cells to undergo self-renewal divisions during clonal expansion in vivo and show the enormous regenerative potential that some stem cells may therefore possess.

The results of this study have important implications for BM transplantation efforts. A sufficiently quantitatively or qualitatively impaired pool of hematopoietic stem cells could influence the longevity of a patient’s graft and, furthermore, might reduce tolerance to cytotoxic agents or other circumstances that would impose a proliferative demand on the stem cell pool. This study thus highlights the importance of optimizing stem cell numbers in BM transplants and suggests potential consequences of transplanting different numbers or sources of stem cells in protocols seeking to rescue or, alternatively, to genetically modify the marrow. These studies also set the stage for attempts to enhance CRU regeneration after transplantation by the administration of exogenous agents or the expression of transduced intracellular factors that may enhance the apparent regenerative potential of stem cells expressed under a defined experimental condition in vivo.

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Evidence of both ontogeny and transplant dose-regulated expansion of hematopoietic stem cells in vivo

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