Impaired Steel Factor Responsiveness Differentially Affects the Detection and Long-Term Maintenance of Fetal Liver Hematopoietic Stem Cells In Vivo

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The results of previous studies have shown that the development of hematopoiesis during fetal life can occur in the absence of Steel factor (SF) signaling. On the other hand, impairment of this mechanism can severely compromise the ability of cells from adult bone marrow to regenerate hematopoiesis on their transplantation into myeloablated recipients. This apparent paradox could result from changes during ontogeny in the responsiveness of hematopoietic stem cells to regulators that may substitute for SF as well as from differences in the availability of such factors during embryogenesis and in the myeloablated adult. To investigate these possibilities, we studied the effect of W41 and W42 mutations on the numbers, phenotype, and posttransplant self-renewal behavior of primitive hematopoietic cells present in the fetal liver (FL) of 14.5-day-old mouse embryos. In W41/W41 FL, day-12 spleen colony-forming units and long-term culture-initiating cells appeared both quantitatively and qualitatively similar to their counterparts in the FL of +/+ embryos. W41/W41 FL also contained near normal numbers of transplantable lymphomyeloid stem cells with competitive reconstituting ability in myeloablated adult +/+ recipients (as assessed for up to at least 16 weeks posttransplant). Moreover, both the original phenotype of these W41/W41 competitive repopulating units (CRUs) and their clonal posttransplant output of mature progeny were normal. Similarly, when myeloablated adult +/+ mice were cotransplanted with 5 × 10^6 +/+ FL cells and a sevenfold to 70-fold excess of W41/W41 FL CRUs, the contribution of the +/+ FL CRUs to the circulating white blood cell count present 5 weeks later was markedly reduced as compared with that of mice that received only +/+ FL cells. However, over the next 3 months, the proportion of mature white blood cells that were derived from +/+ precursors increased significantly (P < .002) in all groups (to >30%), indicating that the ability to sustain hematopoiesis beyond 5 weeks is more SF-dependent than the ability to initially reconstitute both lymphoid and myeloid compartments. Cells from individual FL of W41/+ matings also showed an initial ability (at 7 to 8 weeks posttransplant) to competitively repopulate both lymphoid and myeloid compartments of myeloablated +/+ adult recipients. However, in contrast to recipients of normal or W41/W41 FL cells, the repopulation obtained with the W42 mutant stem cells was transient. Secondary transplants confirmed the inability of the W42 mutant cells to regenerate or even maintain a population of transplantable stem cells. Taken together with previous results from studies of CRUs in adult W mice, these findings support the concept of changes in the way hematopoietic stem cells at different stages of development respond to the stimulatory conditions evoked in the myeloablated recipient. In addition, they provide the first definitive evidence that SF is a limiting physiological regulator of sustained hematopoietic stem cell self-renewal in vivo.

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In the adult, a relatively constant output of large numbers of mature blood cells is maintained by the terminal differentiation of cells produced over many cell divisions from a small number of transplantable stem cells with unrestricted hematopoietic differentiation potential and extensive self-renewal ability. Paradoxically, analysis of hematopoietic cell types in the developing embryo suggests the appearance of these populations in reverse order. Thus, cells with limited potencies and detectable in vitro as colony-forming cells (CFCs) are the first known progenitors to appear in the yolk sac at 8 days postcoitum (dpc). Cells with spleen colony-forming activity (CFU-S) become detectable within the next 24 hours, but cells with transplantable long-term lymphomyeloid repopulating potential have not been shown before 10 to 11 dpc. The number of these latter cells present in the embryo is thought to expand initially in the fetal liver (FL), with subsequent colonization of other sites, the bone marrow (BM) ultimately becoming the primary site of hematopoietic activity after birth. As yet, very little is known about the role of specific ligand-receptor interactions on the development of these early cell populations in vivo, most likely because of redundancies in their activities. Nevertheless, in vitro studies have identified a number of factors, including Steel factor (SF), that are able to contribute to stimulating the proliferation and differentiation of primitive hematopoietic cells in vitro. This has suggested a potential contributing, if not exclusive, role of SF in regulating these cell populations in vivo. SF and its cognate receptor, c-kit, are both detectable in the yolk sac as early as 8 to 9 dpc and c-kit continues to be expressed on virtually all hematopoietic progenitors throughout embryonic and adult life. Transplantation studies of cells from and into mice carrying functional mutations in the c-kit and SF genes (W and SI loci, respectively) provided early evidence that the SF-c-kit signaling pathway plays a role in the recruitment of adult hematopoietic stem cells in vivo. In these studies, it was shown that cells from W/W mice fail to form macroscopic spleen colonies and are deficient in their ability to regenerate the CFC compartment in normal recipients, with...
the converse being found when normal cells are transplanted into S1/S1 recipients. In addition, it was found that blood cell production in untreated W/W mice can be gradually and permanently replaced by an injection of normal BM cells. It has also been shown that administration of a neutralizing anti-c-kit antibody can decrease CFC numbers in the developing fetus and both CFC and CFU-S in the adult. We have recently extended these observations in studies of adult BM cells from W/W or W/W+ mice. These mice have an SF receptor with detectably impaired tyrosine kinase activity, although not sufficient to significantly affect the generation in the BM of a normal compartment of SF-CFS or their ability to be detected in irradiated recipients. Nevertheless, BM cells from adult W/W or W/W+ mice are compromised in their ability to regenerate hematopoiesis when transplanted into myeloablated +/- recipients. These findings have provided more direct evidence that SF can play an important role in activating the most primitive hematopoietic cells present in the adult.

In vitro, SF alone is sufficient to support the survival of adult repopulating stem cells in the absence of cell division, but a net expansion of these cells has not been obtained even when proliferation did occur. In addition, the generation of at least some primitive hematopoietic cell populations (Kit−Sca−1Thy−1Lin− cells and CFU-S) during the first 2 weeks of embryonic life is minimally affected in mice that cannot produce SF. The apparent discrepancy between these latter observations and the pronounced effects of impaired SF-mediated signaling in the adult could be explained either by a difference in sensitivity of fetal and adult hematopoietic stem cells to SF (and possibly other cytokines) or by a difference in the types and/or concentrations of other cytokines that are capable of stimulating stem proliferation and differentiation in fetal and adult tissues. In favor of the first hypothesis are numerous examples of differences between the proliferative and differentiative behavior of fetal and adult stem cells even when the expression of these functional attributes is stimulated under identical conditions. Similarly, differences in the expression of certain phenotypic markers, eg, AA4.1 and Mac-1, by fetal and adult stem cells are well documented. On the other hand, recent experiments have also suggested that other factors, such as thrombopoietin, may be capable of stimulating the proliferation of cells with long-term in vivo repopulating potential. To investigate possible differences in the SF dependence of fetal and adult stem cells, we have undertaken a series of experiments in which the numbers, phenotype, and different measures of repopulating potential of transplantable FL stem cells from W/W, W/W+, or W/W+ embryos have been compared with those of their congenic +/- counterparts. The results provide further evidence of functional differences between stem cells present in the FL and in the adult and also suggest an important but variable role of SF in regulating stem cell self-renewal at different stages of development.

MATERIALS AND METHODS

Animals. C57BL/6 (Ly5.2), C57BL/6:Pept3b (Ly5.1), C57BL/6-W/W (Ly5.2), and C57BL/6-W/W+ (Ly5.2) mice were originally obtained from commercial stocks and Dr J. Barker, respectively, at the Jackson Laboratory (Bar Harbor, ME). These mice were bred and maintained with sterilized food, water, and bedding in microisolator units at the Joint Animal Facility of the British Columbia Cancer Research Center. Irradiated animals were additionally provided with acidified water (pH 3). To generate mice of defined gestational ages, appropriate genotypes were placed together in the late afternoon, and the males then removed 16 to 18 hours later. Embryos were considered to be 0.5 dpc in females in which a vaginal plug was detected. In any individual experiment, embryos were isolated from at least two pregnant females on 14.5 dpc. +/- (Ly5.1/Ly5.2) FL were produced by the mating of C57BL/6 and C57BL/6:Pept3b mice. W/W+ mice are fertile; therefore, homozygous males and females could be mated together to yield embryos that were exclusively of this genotype. W/W mice rarely survive beyond 15 dpc and, therefore, had to be produced from matings of W/W+ parents (see below).

Cell suspensions of FL cells were prepared from groups of 10 mice or individual FL as indicated as previously described. The procedure for isolating adult BM cells has also been described. The monoclonal antibodies (MoAbs) and procedures used for phenotype analysis and sorting of FL cell suspensions have also been described previously. Briefly, FL cells at a concentration of 5 to 8 x 10^6 cell/mL were first incubated on ice for 30 minutes with 3 µg/mL of 2.4G2 (an anti-Fe receptor antibody), followed by incubation with a Sca-1 MoAb (E13-161.7) conjugated with cyanine-5 succinimidyl ester and fluorescein isothiocyanate-conjugated lineage-specific MoAbs (anti-Thy1.2, anti-GR-1, anti-B220, and anti-Ly1). Cells were then washed twice in Hank’s Balanced Salt Solution containing 2% fetal bovine serum (HF) at 4°C and propidium iodide (PT; Sigma Chemicals, St Louis, MO) at 1 µg/mL added to the final wash. Cells were sorted on a FACStar (Becton Dickinson, San Jose, CA). Where specified, some cells were also incubated with a biotinylated anti-c-kit antibody (anti-CD119, clone 2B8; Pharmingen, San Diego, CA), washed with HF, and then treated with 1 µg/mL of phycoerythrin conjugated to streptavidin (SA-RPE; Southern Biotechnology Associates, Birmingham, AL), Sca-1, and Lin MoAb before final washes.

Assays for long-term culture-initiating cells (LTC-ICs). The LTC-IC content of the cell suspensions was determined as previously described. Briefly, Sca-1−Lin− cells derived from W/W mice were resuspended in methylcellulose medium (StemCell Technologies, Vancouver, British Columbia, Canada) to freshly dissolved hydrocortisone sodium hemisuccinate (Sigma Chemicals) was added to give a final concentration of 10 µM. Varying cell numbers (8 to 12 replicates each) were then plated on irradiated pre-established mouse BM feeders or on confluent layers of S17 cells in 96-well plates, and the cultures then were maintained for 4 weeks at 33°C with weekly replacement of half of the medium. At the end of the incubation period, both adherent and nonadherent cells from each well were harvested, and the contents of each well then assayed individually for the presence of myeloid CFCs by plating the cells in methylcellulose medium supplemented with 2% pokeweed mitogen-stimulated mouse spleen cell-conditioned medium and 3 U/mL human erythropoietin as a source of growth factors (StemCell). LTC-IC frequencies were calculated by the method of maximum likelihood from the proportion of negative wells (ie, wells that contained no CFCs) measured for each dilution of cells assayed.

CFU-S assays. C57BL/6 recipients were irradiated with 850 cGy of 137 Cs γ-rays at a dose rate of 96 cGy/min, and then each was injected intravenously with 10^5 test cells. Animals were killed 12 days later, and spleens were removed and fixed in Telylene’s solution before counting all macroscopically visible surface colonies.

Assays of repopulating cells. For initial experiments with FL
cells, groups of 6 to 8 recipient animals (C57BL/6 or C57BL/6Pep3b, as indicated) were irradiated with 900 cGy of 137 Cs γ-rays at a dose rate of 96 cGy/min and were then injected intravenously with specified numbers of purified or unfractionated FL cells together with 10^7 BM cells from normal adult +/+ mice of the same genotype as the recipients. Peripheral blood (PB) was obtained 5 to 7 weeks and 12 to 24 weeks later, and the proportions of donor-derived nucleated cells were quantitated. Anti-Ly5.1 MoAb (A20-1-7) was used to detect +/+ FL donor-derived cells in C57BL/6/Ly5.2 recipients and anti-Ly5.2 MoAb (AL14A4) was used to detect the progeny of W41/W41 FL cells in C57BL/6:Pep3b (Ly5.1) recipients.22 Red blood cells were first lysed with NH4Cl, and the nucleated cells were incubated with biotinylated anti-GR-1 MoAb for 30 minutes on ice. The cells were then washed once in HF and incubated for another 30 minutes with 1 μg/mL of the appropriate fluorescein isothiocyanate-labeled anti-Ly5 MoAb and 1 μg/mL of phycoerythrin conjugated to streptavidin. After 2 more washes in HF and PI, the samples were analyzed on a FACSsort (Becton Dickinson). Animals were considered to be reconstituted (positive) only if their blood contained ≥5% test-cell–derived myeloid cells (Gr-1+) and lymphoid cells (low orthogonal light scattering characteristics, Gr-1-). Competitive repopulating unit (CRU)44 frequencies were then calculated by the method of maximum likelihood from the proportion of negative mice measured in each group of mice that received a given dilution of test cells.45

In the studies in which 5 × 10^4 +/+ (Ly5.1/Ly5.2) FL cells were transplanted either alone or together with varying numbers of W41/ W41 (Ly5.2) FL cells into irradiated C57BL/6/Ly5.2 recipients, the proportion of cells that were derived from the injected +/+ FL (Ly5.1+) cells in the PB of the reconstituted recipients was then determined after varying intervals as described above.

In experiments with FL cells obtained from embryos produced by crosses of W41/+ (Ly5.2) parents (only 25% of which would be W41/W41), each FL was handled individually, and the recipients of each FL were kept separate throughout the duration of the experiment. In the initial experiments, 2 × 10^4 cells from individual FL were injected together with 10^5 +/+ (Ly5.1) adult BM cells into groups of 3 to 4 irradiated +/+ (Ly5.1) recipients. In the second experiment, either 2% or 20% of the cells from an individual FL were injected together with 10^5 +/+ (Ly5.1) adult BM cells into groups of 3 irradiated +/+ recipients. In both experiments, the proportion of FL-derived cells in the PB was then measured as described above.

In the second experiment, groups of primary recipients that had each been transplanted with 20% of an FL were selected at 24 weeks posttransplant; the mice were killed, and the cells from the femurs and tibias of each group were pooled. A dose of cells equivalent to 2%, 10%, or the entire contents of a primary recipient’s femur were then injected together with 10^5 normal adult +/+ (Ly5.1) BM cells into irradiated (900 cGy) secondary C57BL/6:Pep3b (Ly5.1) recipients. PB from these secondary recipients was subsequently analyzed for the appearance of Ly5.2- cells as described above.

RESULTS

W41/W41 FL contains a normal number of Sca-1+ Lin- cells. The single nucleotide change that characterizes the W41 allele causes a significant impairment of the in vitro tyrosine kinase activity of the SF receptor,21 which is sufficient to give an anemic phenotype in the homozygous mouse.24 As is shown in Fig 1, the pattern of expression of c-kit on Sca-1+ 14.5-dpc W41/W41 FL cells was found to be similar to that typical of Sca-1- +/+ FL cells of the same gestational age, although the average level of expression of c-kit was approximately twofold lower in the W41/W41 population. In the same analy-

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c-kit

W41/W41 FL contains a near normal LTC-IC population. The LTC-IC content of the Sca-1+ Lin- cell population of 14.5 dpc W41/W41 FL was determined using either primary irradiated BM adherent layers or S17 cells as feeders. As shown in Table 1, the use of S17 feeders allowed more LTC-IC to be detected. However, regardless of the feeder used, the number of LTC-ICs detected in the W41/W41 FL appeared to be at least half that detectable in +/+ FL. There was also no difference in the numbers of CFSCs produced per LTC-IC of either genotype (data not shown).

W41/W41 FL contains a near normal CFU-S population. Irradiated congenic +/+ recipients were injected with 10^7 unseparated 14.5-dpc W41/W41 FL or +/+ cells, and the average numbers of macroscopic colonies visible on the spleen 12 days later were then compared. As also shown in Table 1, the frequency of day-12 CFU-S thus detected was the same for both types of FL cells; however, because of the slightly reduced cellularity of the W41/W41 FL, the total...
number of day-12 CFU-S in these embryos was decreased. Of interest, the spleen colonies generated from the W41/W41 FL cells were morphologically indistinguishable both in size and appearance from those produced by +/- day-12 CFU-S.

W41/W41 FL contains a near normal population of cells detectable as CRUs. Initial measurements of the lymphomyeloid stem cell content of W41/W41 FL made use of the CRU assay. This assay was originally developed for cells from normal mice and has been shown to detect and quantitate the frequency of cells with an ability to regenerate both lymphoid and myeloid cell compartments in lethally irradiated recipients in the presence of a minimal competitor population. Initially, this was 2 x 10^5 BM cells whose reconstituting capacity had been compromised by prior serial transplantation. Later, it was found that 10^5 normal adult BM cells could be used as a substitute competitor population without affecting the frequency of CRUs detected in the test population. To quantitate CRUs in the FL of 14.5-dpc W41/W41 embryos, varying doses of unseparated or Sca-1^-Lin^- cells were injected together with 10^5 congenic +/- (Ly5.1) cells into irradiated +/- (Ly5.1) recipients, and the W41/W41 (Ly5.2)-derived peripheral white blood cell (WBC) counts were assessed after 6 to 8 weeks and again after 16 to 20 weeks, as described in Materials and Methods. The results for W41/W41 CRUs (Table 1) parallel those obtained for LTC-ICs and day-12 CFU-S; i.e., the frequency of CRUs in the unseparated W41/W41 FL measured at both times of assessment was similar to the frequency of CRUs in suspensions of +/- FL, but the total CRU population in the W41/W41 FL was slightly reduced. CRU assays of the Sca-1^-Lin^- FL cells isolated from embryos of both genotypes also indicated a similar degree of CRU purification in this fraction, although the frequency of CRUs in the Sca-1^-Lin^- fraction of W41/W41 FL was slightly (approximately twofold) less.

The use of a limiting dilution design in these experiments made it possible to also examine the peripheral WBC output from a single CRU. Figure 2 shows the proportion of +/- or W41/W41-derived PB cells in individual positive recipients from groups in which \( \geq 37\% \) of the mice were negative. On average, such recipients would have been injected with 1 CRU, and \( \leq 27\% \) of these mice would have received \( \geq 2 \) CRUs. It can be seen that the output of mature cells for up to at least 16 weeks in these recipients of limiting numbers of W41/W41 FL CRUs was similar to that obtained with +/- FL CRUs. Thus, FL stem cells with only partial responsiveness to SF are not significantly compromised in their ability to reconstitute irradiated adult +/- recipients in the presence of \( 10^5 \) normal adult BM +/- competitors.

W41/W41 FL CRUs have equivalent short-term but less competitive long-term repopulating ability by comparison with CRUs in +/- FL. Because +/- FL CRUs can reconstitute irradiated recipients to higher levels and show greater self-renewal than their counterparts from +/- adult BM, it was possible that the adult BM competitor cells used to allow the quantitation of the CRUs present in W41/W41 FL would not have allowed a potential deficiency in their repopulating ability to be revealed. Therefore, a second series of experiments was undertaken in which the extent and time dependence of lymphomyeloid repopulation by transplanted W41/W41 FL cells was assessed by evaluating their ability to compete at varying ratios with a constant number of +/- FL cells. In these experiments, +/- (Ly5.2) recipients were injected with 5 x 10^5 +/- (Ly5.1/Ly5.2) FL cells (=3 CRUs) with or without W41/W41 (Ly5.2) FL cells. The number of W41/W41 FL cells injected varied from 2.5 x 10^5 to 2.5 x 10^6 cells per recipient (i.e., \( \approx 200 \) to 200 W41/W41 CRUs per recipient). The proportion of injected +/- FL-derived (Ly5.1^-) cells in the PB was determined 5, 8, and \( \approx 16 \) weeks later. Using this combination, a distinction between W41/W41 FL-derived cells and host-derived cells could not be made. However, as can be seen from the results shown in Fig 3, the mice that received 5 x 10^5 +/- FL cells exclusively showed high levels of reconstitution by these

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Population Assessed</th>
<th>Total No. (x10^5)</th>
<th>LTC-ICs</th>
<th>CFU-S</th>
<th>CRUs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>BM Feeder</td>
<td>Sca-1^-Lin^-</td>
<td>BD Feeder</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Unseparated</td>
<td>190 +/- 20</td>
<td>ND</td>
<td>ND</td>
<td>10 +/- 3</td>
</tr>
<tr>
<td>W41/W41</td>
<td>Unseparated</td>
<td>85 +/- 12</td>
<td>ND</td>
<td>ND</td>
<td>9 +/- 3</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Sca-1^-Lin^-</td>
<td>0.23</td>
<td>1/58</td>
<td>1/27</td>
<td>450</td>
</tr>
<tr>
<td>W41/W41</td>
<td>Sca-1^-Lin^-</td>
<td>0.25</td>
<td>1/84</td>
<td>1/50</td>
<td>300</td>
</tr>
</tbody>
</table>

The values shown represent the mean +/- SEM for total cells, Sca-1^-Lin^- cells, LTC-ICs, and day-12 CFU-S values and the range defined by +/- SEM for CRU values derived from assessment of recipients at 16 to 20 weeks posttransplant. Similar CRU frequencies were obtained from repopulation data obtained at 5 to 8 weeks posttransplant (data not shown). Results for CFU-S were pooled from two experiments in which a total of \( \approx 10 \) mice were used as CFU-S recipients. Results for W41/W41 CRUs and LTC-ICs were obtained in the same three experiments in which the total number of mice used as recipients in the CRU assays is shown in parentheses below the calculated frequency values. Values for CRUs in +/- FL are based on data published previously (n = 3) and on additional data (n = 2) generated during the course of the present experiments. Values for LTC-ICs in +/- FL were pooled from a total of four experiments, in two of which CRU measurements were also performed.

Abbreviation: ND, not done.
+/+ FL-derived cells at 5 weeks posttransplant, and this was sustained for the duration of the experiments. Because all mice were transplanted with 5 × 10^4 +/+ FL cells, it seems unlikely that any effects on their contribution to the hematopoietic recovery observed in other groups would be caused by changes in the less competitive population of residual stem cells present in the host. Not surprisingly, however, in mice that received +/+ FL cells together with a 7-, 15-, or 70-fold excess of W41/W41 FL CRUs, the concentration of mature cells of +/+ FL origin present in the PB at 5 weeks was reduced in proportion to the number of W41/W41 cells injected. However, the competitive hematopoietic activity of the W41/W41 FL cells in these recipients also appeared to be transient because, by 8 weeks posttransplant, the contribution of +/+ FL-derived cells to the production of mature WBC had significantly (P < .002) increased and continued to increase over the next 8 weeks. Thus, by 16 weeks, the average maximal contribution of W41/W41 cells to the mature WBC compartment was less than 35% in recipients of a 7- to 15-fold excess of W41/W41 CRUs and was less than 65% in recipients of a 70-fold excess of these cells.

Greater effects of the W42 mutation on the maintenance of FL-derived CRUs than on their detection. The results obtained with W41/W41 FL cells suggested that the initial activation and differentiation of FL CRUs in vivo may be less sensitive to an impaired responsiveness to SF than the process of CRU self-renewal. As a more direct test of this hypothesis, we analyzed the lymphomyeloid repopulating potential of cells present in the FL of embryos produced from the mating of W42/+ animals. The W42 mutation (also a single amino acid substitution) causes a complete (rather than a partial) abrogation of the tyrosine kinase activity of the SF receptor.22 In the present experiments, individual 14.5-dpc embryos were classified phenotypically according to their paleness and the decreased cellularity of their FL. These phenotypic properties would be expected to reflect the
increasingly defective erythropoiesis that is characteristic of W42/− and W42/ W42 embryos. (Unfortunately, all attempts to confirm the genotypes of these animals using genomic DNA and polymerase chain reaction-based strategies proved unsuccessful.) In the first experiment undertaken with these mice, five groups of 3 to 4 +/- (Ly5.1) mice each were injected with cells from one of five different FL. Four of these FL were small FL from pale embryos and one was a normal appearing FL from a normal appearing embryo. Each recipient was injected with 2 × 10^6 (Ly5.2) FL cells plus 10^5 BM cells from +/- (Ly5.1) adults. In the second experiment, FL from pale embryos and with an average cellularity of 4 (±1) × 10^6 cells, and one FL from a normal appearing embryo and containing 6 × 10^6 cells were again transplanted into small groups of recipients. In this experiment, each recipient +/- (Ly5.1) mouse was injected with either 2% or 20% of a single (Ly5.2) FL together with 10^5 +/- (Ly5.1) adult BM cells. As shown in Table 2, some mice in all 12 groups of recipients from both experiments were repopulated with FL-derived (Ly5.2−) cells when assessed 7 to 8 weeks posttransplant. However, within another 8 to 16 weeks, the number of circulating FL-derived (Ly5.2−) WBCs and/or the number of FL-derived granulocytes (Ly5.2− Gr-1+ cells), in particular, had decreased to very low or undetectable levels in most of the recipients of cells from abnormal FL. This selective failure of sustained granulocyte production may be caused, at least in part, by a greater dependence of the production of these cells (as compared with that of lymphoid cells) on SF–c-kit signaling16 and their shorter half-life. At 24 weeks posttransplant, three representative groups of recipients from the second experiment (designated A, B, or C), each of which had been initially transplanted with 20% of the cells from one of three different FL (2 abnormal, 1 normal), were then selected. To allow a comparison to be made of the contribution of the originally injected FL cells to the CRU compartment regenerated in these primary recipients, their femoral and tibial BM cells were pooled, and cell numbers equivalent to 4 (±1) × 10^6 cells were detectable after 7 weeks in 2 of the 8 second-
Table 3. Differential Repopulation of Secondary Recipients of Cells Derived From Primary Recipients of Different FL Obtained From Individual Embryos Produced by Matings of W^{42}+/+ Mice

<table>
<thead>
<tr>
<th>Source of Cells Transplanted*</th>
<th>Size of Transplant (in 1^7 Femur Equivalents)</th>
<th>Proportion of Positive 2^nd Recipients (% of FL-derived cells in positive mice)(^{f})</th>
<th>Week 7</th>
<th>Week 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2%</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>2/8 (12,20)</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2%</td>
<td>1/8 (32)</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>2/8 (7,8)</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>4/8 (13,15,22,22)</td>
<td>4/8 (7,9,15,26)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2%</td>
<td>7/7 (15-87)</td>
<td>6/7 (9-60)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>7/7 (46-78)</td>
<td>7/7 (47-73)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>7/7 (65-68)</td>
<td>7/7 (46-80)</td>
<td></td>
</tr>
</tbody>
</table>

* For details, see Table 2 notes.
† Positive recipients are as defined in Table 2. Values in parentheses represent the percent of WBCs in the individual positive mice with the unique genotype of the FL cells originally injected into the primary recipients. In the case of the secondary recipients transplanted with cells from group C primary recipients where all secondary recipients were positive, the range of these values in the group is shown in parentheses.

all secondary animals were repopulated with both myeloid and lymphoid progeny of FL-C origin (Fig 4).

DISCUSSION

c-Kit (the SF receptor) is detectable on hematopoietic cells in the FL of mice 10 to 11 dpc.\(^{12,19}\) Thereafter, it continues to be expressed on CFCs as well as on more primitive cell types, including transplantable stem cells, throughout fetal development\(^{28,36}\) and adult life.\(^{45,46}\) Ample evidence indicates that SF, in conjunction with other cytokines, can promote the expansion and differentiation of primitive hematopoietic cells in vitro.\(^{11}\) However, the physiological importance of this cytokine as a regulator of hematopoiesis in vivo, particularly at the level of the stem cell compartment during normal ontogeny, has been less clear. The present studies were designed to address this question through a comparison of the effects of two naturally occurring mutations of c-kit (W\(^{41}\) and W\(^{42}\)) on a number of endpoints of stem cell competence, as shown by cells present in the FL 14.5 dpc. The W\(^{41}\) and W\(^{42}\) mutations are both single but distinct nucleotide substitutions in the coding region of the c-kit gene that, in the homozygous state, result in partial\(^{21}\) and complete impairment,\(^{22}\) respectively, of the tyrosine kinase activity of the SF receptor and, hence, of its functional integrity.

In the present studies, we have shown that nearly normalized populations of several phenotypically and functionally defined primitive uncommitted hematopoietic cell types are generated during the first 2 weeks of embryogenesis in mice that are homozygous for the W\(^{41}\) mutation. This includes total Sca-1^−Lin^− cells, day-12 CFU-S, LTC-ICs and CRUs. The results for day-12 CFU-S in fetal mice were anticipated given the previous findings that near normal numbers of day-
12 CFU-S are generated in fetal S1/S1 mice in which SF-induced signaling is eliminated because of an absence of SF production. In addition, we had previously shown that near normal numbers of Sca-1+Lin- WGA- cells, day-12 CFU-S, and LTC-ICs are present in the BM of adult W41/W41 mice. Thus, SF does not appear to be a limiting regulator for the generation of these cell types at any stage during normal ontogeny. On the other hand, the present demonstration of near normal numbers and function of CRUs in the FL of W42/W41 embryos is quite different from the marked deficiency of cells with this transplantable activity detectable in the BM of adult mice of the same genotype. Thus, either an impaired responsiveness to SF must limit the continued generation of cells with transplantable hematopoietic activity, or the responsiveness of such cells to other factors that stimulate their proliferation in the myeloablated adult mouse must decrease between fetal and adult life. We have suggested that LTC-IC and CRU assays may identify the same cells, although they may rely on an ability of these cells to respond to different factors. If this suggestion is true, it would favor the latter explanation. However, we have also recently shown that the response mechanisms that allow LTC-ICs and CRUs to be detected can be biologically dissociated not only in terms of a dependence on SF/c-kit-mediated signaling, but also as a consequence of their maintenance in vitro under LTC conditions. If the former explanation were correct, then it would be expected that the self-renewal function of CRUs might be more dependent on SF-induced signaling than would the ability of CRUs to generate sufficient progeny for them to be ultimately detected in the circulation as mature WBCs. On the other hand, if the latter explanation were correct, then it might be expected that fetal +/- CRUs would be as effective in competing against fetal W42/W41 CRUs, as shown previously for adult +/- CRUs competing against adult W42/W41 CRUs.

To test these various predictions, additional experiments were undertaken. Studies in which W41/W41 and +/- FL cells were cotransplanted indicated that the initial hematopoietic activity of W41/W41 CRUs in myeloablated mice is approximately the same as that when these cells are cotransplanted with +/- cells from adult BM. This result indicates that there is a difference between the CRUs in the FL and in adult BM of W41/W41 mice. An explanation that could account for their different behavior would be a change in their responsiveness to factors other than SF that must be produced in myeloablated mice and contribute to the activation of transplanted CRUs. However, in the same experiments, we also showed that, after the initial phase of hematopoietic recovery, the FL +/- cells showed a competitive advantage that was not shown by adult +/- cells. Thus, impaired responsiveness to SF appears to have had a greater impact on the ability of FL CRUs to sustain their activity in vivo rather than on their initial activation and production of mature progeny. Such a result would be compatible with a mechanism of CRU regulation in which exposure of CRUs to SF, in conjunction with other factors, could influence their probability of executing self-renewal divisions.

To further investigate the possibility that SF might regulate CRU self-renewal in vivo, the effect of the W42 mutation on this function was examined. All 11 individually tested FL from embryos produced from matings of W42/+ parents (in which 25% of the embryos were expected to be W42/W42 and 50% were expected to be W42/+) were found to contain some CRUs. This included 9 FL that, on the basis of their pale appearance and reduced cellularity, were assumed to be either W42/W42 or W42+. These findings are consistent with the concept that SF stimulation is not absolutely required to enable these cells to express their ability to rapidly regenerate multiple lymphoid and myeloid compartments in vivo. However, in contrast to +/- or W41/W41 FL CRUs, the W42 mutant FL CRUs showed only transient hematopoietic activity in primary recipients that were cotransplanted with +/- adult BM cells.

Thus, our findings would suggest a model of hematopoietic stem cell regulation in vivo that involves exposure of these cells to multiple synergistic factors including SF. The environment in the myeloablated mouse appears to use SF as one of its dominant stem cell stimulators, whereas SF may not be a limiting regulator during early ontogeny. However, in addition to these environmental differences, the stem cells themselves appear to change during ontogeny with respect to their relative dependence on interactions with SF (later stem cells being increasingly SF-dependent). Finally, regardless of the developmental status of the stem cells being stimulated, the strength of the SF-induced signaling achieved may influence their subsequent self-renewal or differentiation behavior.

Previous experiments have shown that SF alone can act on very primitive cells, although greater effects can clearly be obtained when the same cells are exposed to SF in combination with other factors. Very recently, we have found that near maximal amplification of CFCs in serum-free cultures of CD34+CD38- human BM cells could be achieved when the cells were incubated in much lower concentrations of the same SF-containing cytokine cocktail than that required for optimizing the amplification of LTC-ICs is present. It is also interesting to note that mice that can only produce soluble SF (S1/S1 mice) show an impairment of their hematopoietic response mechanisms that allow LTC-IC and CRU assays may identify the same W42/W42 CRUs that can be detected in the circulation as mature WBCs. On the other hand, the present demonstration of near normal numbers and function of CRUs in the FL of W42/W41 embryos is quite different from the marked deficiency of cells with this transplantable activity detectable in the BM of adult mice of the same genotype. Thus, either an impaired responsiveness to SF must limit the continued generation of cells with transplantable hematopoietic activity, or the responsiveness of such cells to other factors that stimulate their proliferation in the myeloablated adult mouse must decrease between fetal and adult life. We have suggested that LTC-IC and CRU assays may identify the same cells, although they may rely on an ability of these cells to respond to different factors. If this suggestion is true, it would favor the latter explanation. However, we have also recently shown that the response mechanisms that allow LTC-ICs and CRUs to be detected can be biologically dissociated not only in terms of a dependence on SF/c-kit-mediated signaling, but also as a consequence of their maintenance in vitro under LTC conditions. If the former explanation were correct, then it would be expected that the self-renewal function of CRUs might be more dependent on SF-induced signaling than would the ability of CRUs to generate sufficient progeny for them to be ultimately detected in the circulation as mature WBCs. On the other hand, if the latter explanation were correct, then it might be expected that fetal +/- CRUs would be as effective in competing against fetal W42/W41 CRUs, as shown previously for adult +/- CRUs competing against adult W42/W41 CRUs.

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