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Research Article

Hematology

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Hematopoietic stem cells proliferate until after birth and show a reversible phase-specific engraftment defect

Michelle B. Bowie,¹ Kristen D. McKnight,¹ David G. Kent,¹ Lindsay McCaffrey,¹ Pamela A. Hoodless,^{1,2} and Connie J. Eaves^{1,2}

¹Terry Fox Laboratory, BC Cancer Agency, Vancouver, British Columbia, Canada.

²Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada.

The regulation of HSC proliferation and engraftment of the BM is an important but poorly understood process, particularly during ontogeny. Here we show that in mice, all HSCs are cycling until 3 weeks after birth. Then, within 1 week, most became quiescent. Prior to 4 weeks of age, the proliferating HSCs with long-term multilineage repopulating activity displayed an engraftment defect when transiting S/G₂/M. During these cell cycle phases, their expression of CXC chemokine ligand 12 (CXCL12; also referred to as stromal cell-derived factor 1 [SDF-1]) transiently increased. The defective engrafting activity of HSCs in S/G₂/M was reversed when cells were allowed to progress into G₁ prior to injection or when the hosts (but not the cells) were pretreated with a CXCL12 antagonist. Interestingly, the enhancing effect of CXCL12 antagonist pretreatment was exclusive to transplants of long-term multilineage repopulating HSCs in S/G₂/M. These results demonstrate what we believe to be a new HSC regulatory checkpoint during development. They also suggest an ability of HSCs to express CXCL12 in a fashion that changes with cell cycle progression and is associated with a defective engraftment that can be overcome by in vivo administration of a CXCL12 antagonist.

Introduction

HSCs are defined as cells with multilineage hematopoietic differentiation potential and sustained self-renewal activity. Operationally, HSCs are detected by their ability to regenerate long-term multilineage hematopoiesis in myeloablated recipients. HSC numbers can be quantified by endpoints that measure this regenerative activity in genetically distinguishable, radioprotected hosts transplanted with limiting numbers of HSCs (1). HSCs are also characterized by extensive heterogeneity. Variability in many HSC properties is dictated by changes in their state of activation, and the consequent changes in these properties are thus reversible. For example, most of the HSCs present in normal adult mice are in a deeply quiescent (G₀) state (2–4) and, in association with this status, they express CD38 but not CD34 or Mac1 (5, 6). These G₀ HSCs also actively exclude certain fluorescent dyes, such as rhodamine-123 (Rho) (7, 8) and Hoechst 33342 (Hst) (9). The latter property underlies the detection of adult mouse HSCs as side population (SP) cells (10). However, when HSCs are activated, they rapidly downregulate expression of CD38 (6, 11), increase expression of CD34 (12) and Mac1 (13, 14), and acquire a Rho^{bright}, non-SP phenotype (15). In association with these changes, some HSCs begin to differentiate and hence permanently lose their long-term repopulating activity, but many do not in spite of their transiently altered phenotype (16). Another property of HSCs that appears to vary reversibly is their ability to exit from the circulation into the

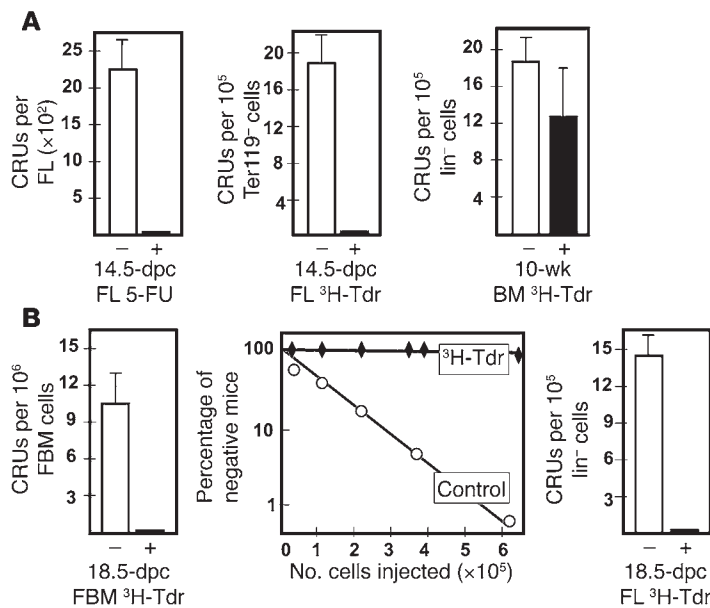
BM and reinitiate hematopoiesis. Quiescent adult mouse HSCs can execute this process at near unit efficiency in suitably myelo-suppressed hosts, as shown by their detection at purities of 20% and higher following intravenous injection (15, 17, 18). However, notable changes in HSC engrafting potential have also been found to accompany the progression of HSCs through the cell cycle both in vitro and in vivo (3, 19–21). Specifically, HSC activity was not detectable in suspensions of adult or neonatal hematopoietic cells in the S/G₂/M phases, even when substantial HSC activity was found in the corresponding G₁ cells. The transient nature of the silencing of HSC homing activity during the progression of these cells through S/G₂/M is inferred from the fact that the populations studied did not contain G₀ HSCs or were expanding their HSC content, although the reacquisition of repopulating activity by incapacitated S/G₂/M HSCs was not formally documented. The molecular mechanisms that control the BM-homing activity of HSCs are not fully elucidated, although a number of cell-surface ligand-receptor interactions with known involvement in cell adhesion and chemotaxis have been implicated. These include CXC chemokine ligand 12 (CXCL12; also referred to as stromal cell-derived factor 1 [SDF-1]) and CXC chemokine receptor 4 (CXCR4), Steel factor (SF) and c-kit, CD44 and hyaluronic acid or osteopontin, VLA4 and VCAM1, and P2Y-like receptor and an unknown ligand (22–25). The expression and activity of some of these appear to be variably affected by cytokine exposure (26, 27); however, their specific involvement in the engraftment defect of HSCs in S/G₂/M has remained unclear.

In the mouse embryo, pluripotent hematopoietic cells with long-term repopulating ability first appear in the aorta-gonad-mesonephros region on the ninth day of gestation (28). These cells then migrate to the fetal liver (FL) and later to the BM, their numbers continually expanding until young adulthood is reached (29). Most of the HSCs in the 14.5-dpc FL have phenotypic characteris-

Nonstandard abbreviations used: $\alpha 4$ int, $\alpha 4$ -integrin; CRU, competitive repopulating unit; CXCL12, CXC chemokine ligand 12; FACS, fluorescence-activated cell sorting; FL, fetal liver; 5-FU, 5-fluorouracil; HA, hyaluronic acid; HF/2, Hank's balanced salt solution containing 2% FCS; Hst, Hoechst 33342; ³H-Tdr, ³H-thymidine; lin, lineage marker; OPN, osteopontin; PI, propidium iodide; Py, pyronin Y; Rho, rhodamine-123; SF, Steel factor; SP, side population.

Conflict of interest: The authors have declared that no conflict of interest exists.

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**Figure 1**

All fetal HSCs are sensitive to cell cycle-specific drugs. Cells from different mouse embryonic tissues were analyzed for CRU content either 16 hours after injection of the pregnant mother with 100 mg/kg 5-FU (+) or PBS (-) or after in vitro incubation of the cells for 16 hours with (+) or without (-) high-specific activity $^3\text{H-Tdr}$. (A) Shown are the effects of 5-FU injection on 14.5-dpc FL CRUs (left panel; data pooled from 3 independent experiments) as well as the effects of $^3\text{H-Tdr}$ on 14.5-dpc FL CRUs (middle panel) and the lack of effect of $^3\text{H-Tdr}$ on CRUs from adult (10-week-old) mice assessed in parallel (right panel; data pooled from 6 independent experiments). (B) The effects of $^3\text{H-Tdr}$ on 18.5-dpc fetal BM (FBM) and FL CRUs (left and right panels; data pooled from 4 independent experiments). The middle panel shows the complete data set from the limiting dilution analysis of the 18.5-dpc fetal BM cells. $**P < 0.001$.

tics of activated adult HSCs (CD38⁺, Mac1⁺, CD34⁺, Rho⁺, non-SP) (13, 14), as might be expected for an expanding HSC population. The proportion of HSCs in the 14.5-dpc FL that are proliferating has been previously estimated from phenotyping studies to be approximately 35% (3), although a more direct measurement of this fraction has not been reported.

As a first step toward elucidating the mechanisms that regulate changes in HSC turnover and engraftment properties during ontogeny, we designed experiments to assess their proliferative status in mice at different stages of development. Our results showed that the entire HSC population remained in cycle until the third week after birth regardless of the tissue in which the HSCs were located. Then within 1 week, the majority of the HSCs switched abruptly from an actively dividing to a quiescent state. Until this switch occurred, those HSCs that were in S/G₂/M showed the same engraftment defect previously demonstrated for adult HSCs that have been stimulated to divide. Interestingly, prior to the establishment of a quiescent HSC population, the HSCs in S/G₂/M were found to express higher levels of CXCL12 than those in G₁, and their defective engraftment activity could be completely reversed, either by holding them ex vivo for a few hours until they reentered G₁ or by pretreating the host with a specific antagonist of CXCL12.

Results

All HSCs in the 14.5-dpc FL are rapidly proliferating. To measure the proportion of HSCs that are in cycle in the 14.5-dpc FL, we used 3 complementary strategies. In the first, we injected pregnant mice at 13.5 dpc with 100 mg/kg of 5-fluorouracil (5-FU) and then removed the fetuses 16 hours later, prepared cell suspensions from the FLs, and measured the number of HSCs present using a limiting dilution transplantation assay for long-term (16-week) competitive repopulating units (CRUs) (1). In these experiments, we detected very few CRUs in the FLs of the 5-FU-treated embryos (Figure 1A). A comparison of the CRU yield from the 5-FU-treated FLs with the control FLs from pregnant mice injected 13.5 dpc with PBS indicated that the 5-FU treatment had reduced the expected CRU population in vivo by more than 1,000-fold.

We then assessed the cycling status of HSCs in the 14.5-dpc FL by measuring the proportion of CRUs that survived a 16-hour exposure to high-specific activity $^3\text{H-thymidine}$ ($^3\text{H-Tdr}$) (30). Sixteen hours was anticipated to be sufficient to allow all cycling HSCs to enter S-phase, which we later confirmed (see below), with minimal exit of any quiescent cells from G₀ (31), as demonstrated for adult BM HSCs, most of which were in G₀ (Figure 1A). For these experiments, the Ter119⁺ (erythroid) cells were first removed from the FL cells to give a 10-fold enrichment in HSC content, and cells were then incubated in a serum-free medium supplemented with 50 ng/ml SF only. This growth factor condition was chosen based on other data demonstrating that freshly isolated 14.5-dpc FL CRUs are maintained at the same numbers for 16 hours under these conditions (M.B. Bowie and C.J. Eaves, unpublished observations). The results of the $^3\text{H-Tdr}$ suicide experiments showed that this treatment reduced the number of CRUs in the suspensions of 14.5-dpc FL cells more than 100-fold ($P < 0.001$; Figure 1A), whereas the same treatment had no significant effect on the recovery of CRUs in similarly treated lineage marker-negative (lin⁻) BM cells from young adult (10-week-old) mice compared with either control cells incubated without $^3\text{H-Tdr}$ ($P = 0.17$) or starting values (data not shown).

We then assessed the distribution of CRUs between the G₀ and G₁/S/G₂/M fractions of Ter119⁺ 14.5-dpc FL cells. These subsets were isolated by fluorescence-activated cell sorting (FACS) on the basis of their staining with Hst and Pyronin Y (Py) (32). A representative FACS profile of the Hst- and Py-stained cells is shown in Figure 2A. The combined results of in vivo assays of 14.5-dpc sorted cells from 4 independent experiments are shown in Figure 3 and indicate that all transplantable CRU activity was confined to the G₁/S/G₂/M fraction. Based on the total number of G₀ cells assayed, the proportion of quiescent HSCs was estimated to be less than 0.02%.

HSCs undergo a complete and abrupt change in cycling activity between 3 and 4 weeks after birth. Since HSCs are known to be present in the BM of fetal mice at later times of gestation, it was of interest to investigate whether HSCs first become quiescent in the fetus at that site. To address this question, we used the 16-hour $^3\text{H-Tdr}$ suicide assay to determine the cycling status of the CRUs present

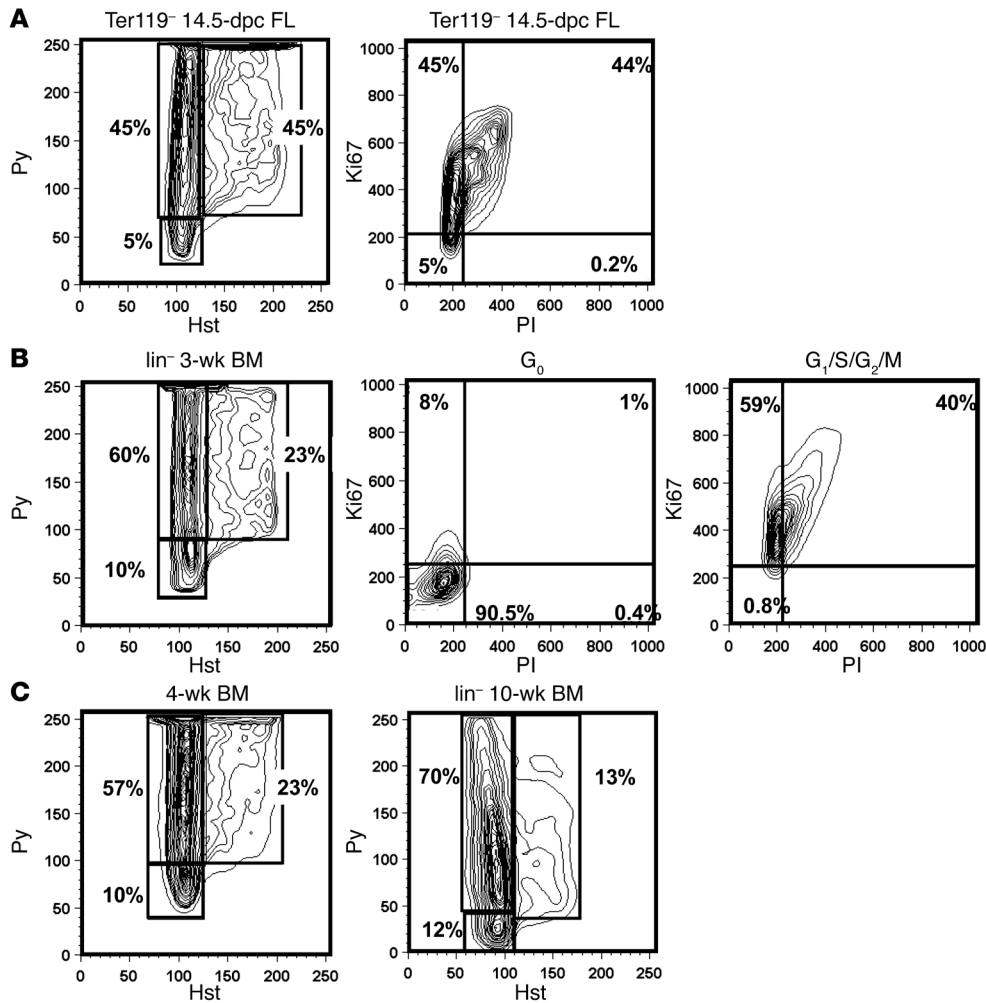


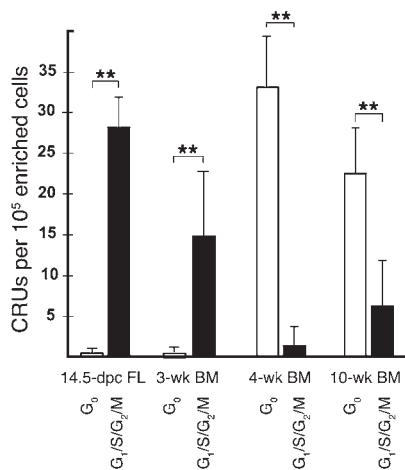
Figure 2
 FACS profiles of the distribution of G₀, G₁, and S/G₂/M cells in different lin⁻ populations. **(A)** Representative FACS contour plot for 14.5-dpc Ter119⁻ FL cells after staining with Hst and Py and for Ki67. **(B)** Representative FACS contour plot for lin⁻ 3-wk BM cells after staining with Hst and Py; sorted G₀ cells after staining for Ki67 (>90% of the G₀ cells showed no Ki67 expression); and sorted G₁/S/G₂/M cells after staining for Ki67 (>99% of the G₁/S/G₂/M cells expressed Ki67). **(C)** Representative FACS contour plots for lin⁻ 4- and 10-wk BM cells after staining with Hst and Py. Percentages indicate the proportion of total cells found within the indicated gate.

in the BM of mice at 18.5 dpc. For comparison, we also evaluated the cycling status of CRUs in the 18.5-dpc FL. The frequency of CRUs in these 2 tissues was 1 per 10⁵ and 1 per 7 × 10⁴ total nucleated cells (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI28310DS1); i.e., approximately 5- and 3.5-fold lower, respectively, than in adult BM (1 per 2 × 10⁴ total nucleated cells; ref. 33) and 6- and 4-fold lower, respectively, than in the 14.5-dpc FL (1 per 1.7 × 10⁴ total nucleated cells; ref. 33). After overnight exposure to high-specific activity ³H-Tdr, no CRUs were detected in the suspensions of either the 18.5-dpc fetal BM cells or the 18.5-dpc FL cells, in contrast to the control cells incubated in the same medium without ³H-Tdr (Figure 1B). Thus all HSCs in the fetus, regardless of their location, appear to be rapidly proliferating.

To further investigate the pace and timing of the transition of HSCs into a largely quiescent population, we analyzed the cycling status of CRUs in lin⁻ BM cell suspensions from 3- and 4-week-old (weanling) mice (lin⁻ 3- and 4-wk BM, respectively). In initial experiments, the CRU frequencies in the lin⁻ 3- and 4-wk BM cells were found to be the same (1 per 6.5 × 10³ and 1 per 6.3 × 10³ lin⁻ cells, respectively; Supplemental Table 2), approximately 2-fold lower than in the lin⁻ 10-wk BM cells (young adult mice; 1 per 2.9 × 10³ lin⁻ cells). We then fractionated 3- and 4-wk BM cells by FACS into their component G₀ and G₁/S/G₂/M subsets based on the gates

shown in the left panels of Figure 2, B and C, and the sorted G₀ and G₁/S/G₂/M cells were assayed separately for CRU activity. Reanalysis of the sorted G₀ and G₁/S/G₂/M fractions after staining for Ki67 confirmed that the cells expressing this proliferation marker were confined to those we had designated as G₁/S/G₂/M (Figure 2B). Remarkably, the results of the in vivo assays showed that all of the CRUs detected in the 3-wk BM were also confined to the G₁/S/G₂/M fraction, whereas more than 98% of the CRUs in the 4-wk BM were found in the G₀ fraction (Figure 3). Thus, there was a rapid down-regulation of CRU proliferative activity in the BM of mice between 3 and 4 weeks of age with little change in CRU number.

HSCs in S/G₂/M show a specific and reversible engraftment defect regardless of their developmental origin or route of injection into assay recipients. Given the previously reported engraftment defect of adult HSCs stimulated to enter S/G₂/M (19), it was of interest to determine whether the number of proliferating HSCs present early in development might be routinely underestimated due an inability to detect those in S/G₂/M. To investigate this possibility, the G₁/S/G₂/M population of Ter119⁻ 14.5-dpc FL cells was subdivided into its component G₁ and S/G₂/M fractions, and each of these 2 subsets was assayed separately for CRUs. In this case, the gate settings chosen to separate the G₁ (2n DNA) and S-phase cells (>2n DNA) were validated by the profiles obtained when the sorted cells were stained with propidium iodide (PI) and reanalyzed by FACS (Figure 4A).



All CRU activity detectable in the G₁/S/G₂/M fraction of Ter119⁻ 14.5-dpc FL cells was confined to the G₁ subset (Figure 4B and Figure 5A). Similar experiments performed with lin⁺ 3-wk BM cells showed that the CRUs in the G₁/S/G₂/M population from this source were likewise confined to the G₁ fraction (Figure 5A and data not shown). It is of interest to note that the S/G₂/M defect was specific to repopulating cells able to produce progeny in all lineages for at least 16 weeks. In contrast, cells with short-term repopulating activity (8 weeks) were readily detected in the S/G₂/M fraction as well as in the G₁ fraction, thus confirming the restriction of this cell cycle-dependent engraftment defect to cells with sustained multilineage repopulating activity (34, 35).

To determine whether the apparent engraftment defect of proliferating CRUs was reversible, we first assayed the CRU content of aliquots of the same isolated G₁ and S/G₂/M cells after they had been incubated for 6 hours at 37°C in serum-free medium containing 50 ng/ml SF. During this time, many of the G₁ cells progressed into S/G₂/M and many of the S/G₂/M cells moved into G₁, as seen by their altered PI (Figure 4A) and Hst (data not shown) staining profiles. In vivo assays showed that CRU activity reappeared when the S/G₂/M cells were cultured for 6 hours, whereas the CRU activity originally present in the G₁ cells was partially lost (Figure 4B).

Figure 4

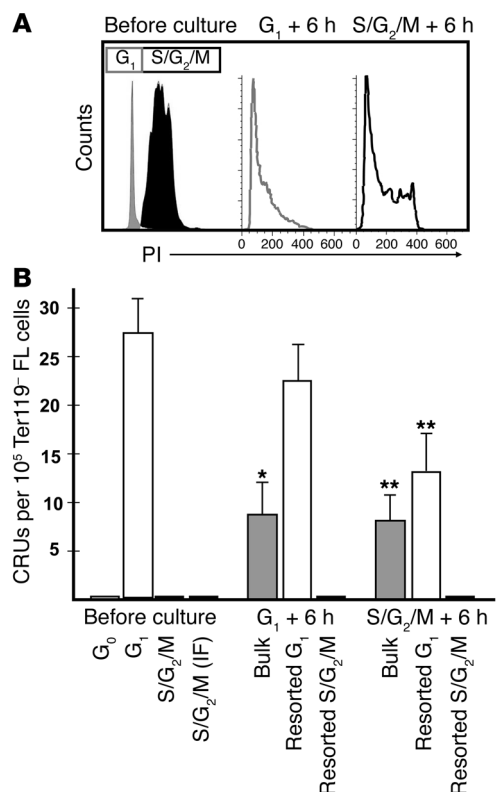
Hst/Py-sorted HSCs display an absolute but transient S/G₂/M engraftment defect. (A) Ter119⁻ 14.5-dpc FL cells in G₁/S/G₂/M were fractionated into their component G₁ and S/G₂/M subsets, leaving a slight separation between them. Aliquots of the sorted subsets were then stained with PI as well as with Hst/Py (data not shown). The sorted cells were cultured for 6 hours and then stained again with PI. We found that during this 6-hour culture period, approximately one-third of the cells originally in G₁ had progressed into S/G₂/M, and a similar proportion of the cells originally in S/G₂/M had progressed into G₁. (B) CRUs per 10⁵ initial Ter119⁻ FL cells for G₁ and S/G₂/M fractions before and after 6 hours in culture. There was a 3.5-fold loss of CRUs when G₁ cells were cultured for 6 hours, but no loss when the cultured cells were re-sorted for G₁ cells (*P* = 0.36). Conversely, we detected a greater than 65-fold increase in the number of CRUs detected when CRUs in S/G₂/M were cultured and a greater than 128-fold increase when the cultured cells were sorted for G₁ cells. IF, intrafemoral. Values are mean ± SEM of results from at least 3 experiments. **P* < 0.01, ***P* < 0.001 versus respective cell types before culture.

Figure 3

The cycling activity of CRUs is downregulated between 3 and 4 weeks of age. Shown are the number of CRUs per 10⁵ initial total viable cells. FL cells were depleted of Ter119⁺ cells; for the 3- and 4-wk BM cells, all lin⁺ cells except Mac1⁺ cells were removed; and for the 10-wk BM cells, all lin⁺ cells including Mac1⁺ cells were removed. Values are mean ± SEM from data pooled from at least 3 experiments per tissue. ***P* < 0.001.

We next asked whether the inability of intravenously transplanted CRUs in S/G₂/M to engraft recipient mice might be overcome by injecting the cells directly into the femoral BM space. However, we did not detect any CRUs after intrafemoral injection in this subset of Ter119⁻ 14.5-dpc FL cells (Figure 4B), even though the CRU numbers measured in the corresponding G₁ 14.5-dpc FL cells after intrafemoral injection were the same as after intravenous transplantation (1 per 3.6 × 10³ and 1 per 3.8 × 10³ cells, respectively).

The S/G₂/M engraftment defect of HSCs is overcome by pretreatment of the host with a CXCL12 antagonist. Previous reports have shown that CXCL12 can promote both the mobilization (36) and the homing (37–39) of HSCs. However, the mobilization of primitive hematopoietic cells can also be stimulated by blocking CXCL12/CXCR4 signaling, as achieved by in vivo administration of AMD3100, a CXCL12 antagonist (40). In addition, it has recently been shown that in vivo administration of AMD3100 can increase the competitive engrafting ability of transplanted BM cells in unirradiated hosts (41). These findings suggested that targeting the CXCL12/CXCR4 pathway might also affect the variable engraftment properties of cycling HSCs by influencing either the HSCs themselves or the transplanted host. To



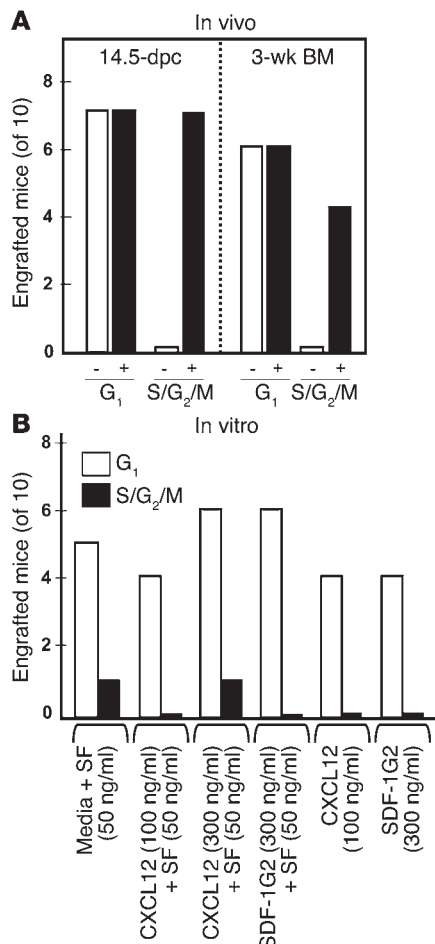


Figure 5

The engraftment defect of HSCs in S/G₂/M is corrected by treatment of the host, but not the cells, with SDF-1G2. **(A)** Effect of injecting prospective recipients 2 hours after irradiation and 2 hours prior to transplant with 10 ng/ml SDF-1G2 (+) or PBS (-). Starting equivalents of 4,000 G₁ cells per recipient mouse or 12,000 S/G₂/M cells per recipient mouse were similarly tested. Both 14.5-dpc FL and 3-wk BM HSCs in S/G₂/M engrafted only when transplanted into SDF-1G2-treated recipients, whereas treated recipients were no more likely to be engrafted long-term by HSCs in G₁ than were untreated recipients. Results are combined from 3 independent experiments. **(B)** Effect of in vitro treatment of sorted Ter119⁻ FL cells in G₁ or S/G₂/M for 30 minutes at 37°C in serum-free medium plus the indicated additives on CRU detection. In vitro treatment had no significant effect on the number of mice that subsequently showed multilineage repopulation from starting cells in either G₁ or S/G₂/M. Results are combined from 3 independent experiments.

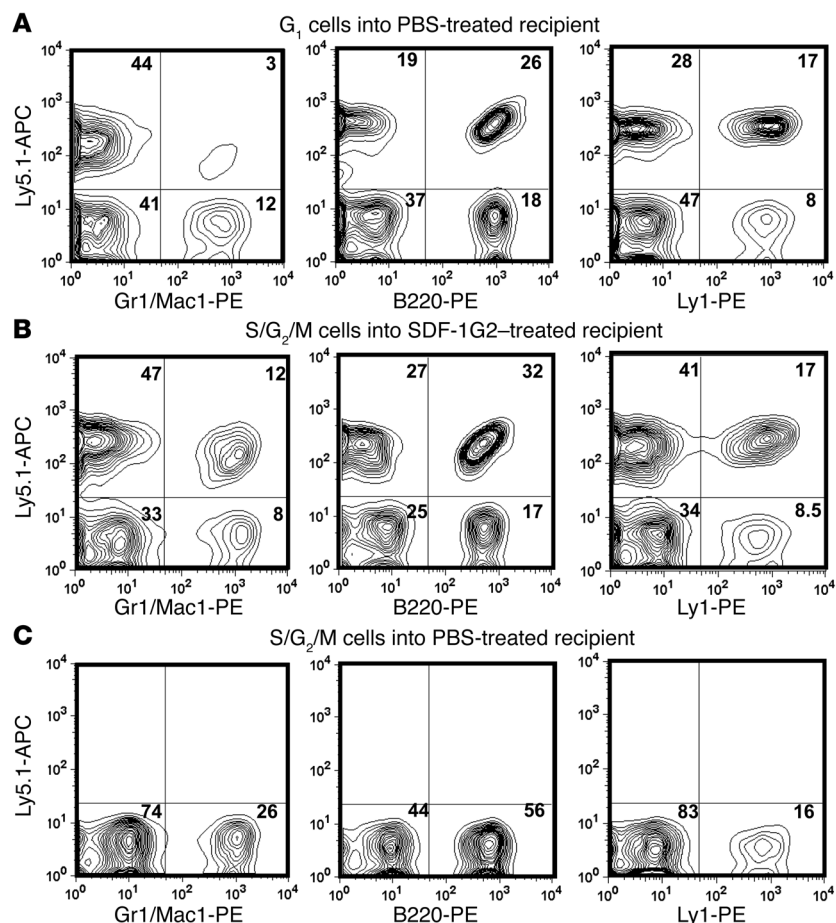
no difference in the engrafting activity of the transplanted G₁ or S/G₂/M cells was seen compared with untreated controls over a wide range of SDF-1G2 and CXCL12 concentrations tested, either with or without added SF (Figure 5B).

HSCs in S/G₂/M express higher levels of CXCL12 transcripts than do HSCs in G₁. To begin to understand the mechanism behind the observed HSC S/G₂/M engraftment defect and how it might be overcome by SDF-1G2 pretreatment of the host, we isolated highly purified populations of HSCs from 14.5-dpc FLs and from 3-wk BM (lin⁻Sca-1⁺CD43⁺Mac1⁺ cells representing approximately 20% pure HSCs; M.B. Bowie and C.J. Eaves, unpublished observations) and sorted these into their corresponding G₀/G₁ and S/G₂/M fractions by Hst staining. Aliquots of approximately 200–800 cells were collected from each fraction in 3 independent sorting experiments, and transcript levels for *Gapdh*, *c-Kit*, *c-mpl*, *CD44*, *α4-integrin (α4int)*, *VCAM1*, *CXCR4*, and *CXCL12* were measured by quantitative real-time analysis of the cDNAs prepared from the isolated RNA extracts as described in Methods. Transcripts for all these genes were consistently detected in both the G₀/G₁ and S/G₂/M fractions of the highly purified suspensions of HSCs from 14.5-dpc FL and 3-wk BM, including *CXCL12*, which had not previously been shown to be expressed by HSCs (Figure 7). Interestingly, *CXCL12* was also the only one of the genes assessed that was found to be expressed at significantly different levels in G₀/G₁ and S/G₂/M HSCs (9-fold higher in S/G₂/M HSCs; *P* < 0.05).

Discussion

This study presents 2 clinically relevant features of HSC regulation. The first is the unanticipated sudden and complete change in HSC proliferative activity that occurred in juvenile mice between 3 and 4 weeks of age. Both the abruptness and the reproducibility of this change suggest an underlying mechanism that is tightly controlled and broadly active. It is notable that this change was not linked to the migration of HSCs during late embryogenesis from the microenvironment of the FL to that of the BM, but rather was strictly associated with the developmental status of the donor. Thus, although differences between BM and FL niches and stromal cells have been sought and described (45–48), these differences do not appear to directly determine the cycling activity of the HSCs they are thought to regulate. The present data are more consistent with a model in which the mechanism of HSC cycling control in vivo is indirectly controlled by external cues, perhaps via changing stimulation of the stromal cells that then alter the signals they deliver, as suggested by studies of the long-term BM culture system

investigate these possibilities, we first asked whether pretreating either the cycling HSCs to be transplanted or their hosts with a specific antagonist of CXCL12 might alter the level of repopulation obtained 16 weeks later. The CXCL12 antagonist used in these experiments was SDF-1G2 (also called P2G because it is identical to stromal cell-derived factor 1 except that the proline at position 2 has been converted to glycine; ref. 42). SDF-1G2 is thus structurally quite different from AMD3100 but similar in its ability to block CXCL12 from binding to CXCR4 without activating CXCR4 (42, 43). SDF-1G2 and AMD3100 also share an ability to elicit effects on primitive hematopoietic cells both in vitro and in vivo (44). Mice were injected with 10 μg of SDF-1G2 or PBS 2 hours prior to the transplantation of FACS-sorted G₁ or S/G₂/M cells and analyzed for the presence of donor-derived blood cells 16 weeks later, and the results for 14.5-dpc FL and 3-wk BM cells were similar (Figure 5A). Treatment of recipients with SDF-1G2 had no effect on the repopulating activity of CRUs in G₁. In contrast, SDF-1G2 pretreatment of recipients of S/G₂/M cells enabled long-term multilineage repopulation to be readily detected (in 7 and 4 mice of 10 transplanted with 14.5-dpc FL and 3-wk BM S/G₂/M cells, respectively, versus 0 of 10 in PBS-injected controls for each). Moreover, the SDF-1G2-pretreated hosts showed levels of repopulation by both sources of S/G₂/M cells that were indistinguishable from those seen in mice transplanted with G₁ cells (Figure 6). On the other hand, when SDF-1G2 was applied directly to the cells to be transplanted for 30 minutes before injection,

**Figure 6**

Donor-derived repopulation of SDF-1G₂-treated mice. Shown are representative FACS profiles of donor-specific cells detected after dual staining for the donor-type Ly5 allotype and various lineage-specific markers. (A) Example of a positively engrafted PBS-treated recipient of FL cells in G_1 . (B) Example of a positively engrafted SDF-1G₂-treated recipient of FL cells in S/G₂/M. (C) Example of a PBS-treated recipient of FL cells in S/G₂/M that did not show donor-derived hematopoiesis. Numbers within graphs indicate the proportion of total cells found within the indicated gene.

(44, 49) and of elements of the BM microenvironment in vivo (25). However, internally programmed changes in HSC responsiveness to external factors could also contribute to a developmentally controlled alteration in HSC cycling activity.

In humans, an abrupt change in HSC proliferative activity at an analogous point in development (2–4 years) has been inferred from measurements of the rate of decline in telomere length of circulating leukocytes (50). This suggests that the mechanisms involved in regulating HSC proliferative activity during ontogeny may be preserved across these species and that the mouse will be a relevant model for their future elucidation. It is interesting to note that, in the mouse, a number of other changes in hematopoietic cell properties or output parameters have already been found to change during ontogeny in concert with this transition of the HSC compartment from a predominantly cycling to a predominantly quiescent population. These changes include the initial acquisition of an SP and Rho^{dull} phenotype by HSCs (15) and the completion of appearance and rapid cycling of adult-type (Ly49⁺) natural killer cells and peripheral T cells (51, 52).

Many other differences in the properties of fetal and adult HSCs and the programs they dictate have also been described (13, 53, 54). It will clearly be of interest to determine the extent to which these may be programmatically linked to the mechanisms that precipitate the change in HSC cycling that occurred in mice between 3 and 4 weeks of age. Several genes have been implicated in the differential control of HSC behavior at different stages of development. These include genes encoding various transcription factors, i.e., *Runx1* (55), *Notch* (56), *Scl* (57), *bmi1* (58, 59), and *Gfi-1* (60, 61), as well as the growth factor receptors *c-Kit* (62, 63) and *Tie2* (64). Further delineation of the molecular basis of the unique programs operative in fetal HSCs and how these regulate fetal HSC cycling are of major interest, as this information could lead to new strategies for expanding HSCs and offer potential insights into mechanisms of leukemogenesis.

The second significant set of findings from our studies indicates the universality and pronounced extent of the engraftment defect found to characterize cycling HSCs in the S/G₂/M phases of the cell cycle, the specificity of this effect for hematopoietic cells with prolonged versus short-term repopulating activity, and the reversibility of this defect seen either following their progression into G_1 or when the host was pretreated with a specific antagonist of CXCL12. Interestingly, the corrective effect of in vivo-administered SDF-1G₂ could not be replicated by treatment of the cells with this agent prior to injection. The in vivo effect of SDF-1G₂ could also not be mimicked by intrafemoral injection of the test cells. The inability of intrafemoral injection to overcome the defective engraftment of HSCs in S/G₂/M suggests that this defect is likely mediated by events that affect the transplanted HSCs after they have entered the BM environment.

Quantitative analysis of the level of expression of 7 genes in the G_1 and S/G₂/M subsets of purified cycling HSCs from both FL and 3-wk BM sources confirmed the expected expression of *c-Kit*, *c-mpl*, *CD44*, *$\alpha 4int$* , *VCAM1*, and *CXCR4* and further revealed that these cells also all contained *CXCL12* transcripts. Moreover, although the transcript levels were not different between the G_1 and S/G₂/M fractions for *c-Kit*, *c-mpl*, *CD44*, *$\alpha 4int$* , *VCAM1*, or *CXCR4*, a 9-fold increase in *CXCL12* expression was noted in the S/G₂/M HSCs. Previous work has suggested that the ability of transplanted HSCs to reach a niche within the BM that can support their self maintenance may depend on the strength of the CXCL12 gradient they encounter within the BM space, causing them to migrate toward the osteoblasts that line the bone (22). According to such a model, the ability of HSCs to express CXCL12 themselves, in the absence of changes in their expression of CXCR4, might be anticipated to regulate their ability to respond to other more distal sources of CXCL12. Upregulated expression of CXCL12 during the progression of HSCs through S/G₂/M, as demonstrated here, might then be sufficient to interfere with an appropriate intra-BM migratory

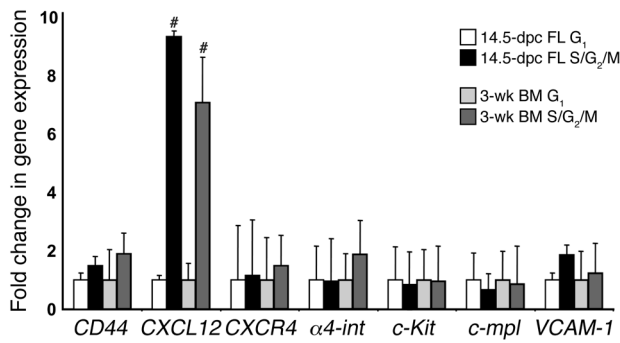


Figure 7
Gene expression analysis of the G₁ and S/G₂/M subsets of highly purified lin⁻Sca1⁺CD43⁺Mac1⁺ HSCs from 14.5-dpc FL and 3-wk BM. Gene expression in G₁ was set as 1, and the fold change in transcript levels in the corresponding S/G₂/M fraction is shown. Results are mean ± SEM of data from 2–3 biological replicates measured in triplicate. #P < 0.05 versus respective G₁ samples.

response, resulting in the rapid differentiation, death, or irreversible sequestration of these cells in a site where they could not be stimulated to divide. Timed blockade of CXCR4 on cells within the BM by injected SDF-1G2 might then increase the level of intra-BM CXCL12 to a point that transiently restores an effective chemoattractant gradient for the otherwise-insensitive HSCs in S/G₂/M. Such a possibility has, in fact, recently been modeled in the zebrafish, where overexpression of CXCL12 in the germ cells was found to prevent the normal migration of these cells toward endogenous CXCL12 signals (65).

In the hematopoietic system, it is interesting to note that long-term repopulating CXCL12^{-/-} HSCs engrafted irradiated hosts, whereas only short-term repopulation was obtained from CXCR4^{-/-} cells (66, 67). In addition, as would be predicted from the explanation we have advanced, forced overexpression of CXCR4 in retrovirally transduced (i.e., proliferating) human HSCs was able to enhance the in vivo engrafting activity of these cells (68), and, conversely, treatment with antibodies to CXCR4 had the opposite effect (69). However, CXCL12 levels in the BM are also subject to regulation, as occurs, for example, following the administration of G-CSF (70).

Recently, Chen et al. (41) found that administration of AMD3100 pretransplant can produce a modest improvement in the engraftment of quiescent adult BM HSCs transplanted into nonirradiated hosts. The mechanism proposed was that the injected AMD3100 initiated the mobilization of endogenous HSCs within the BM, thereby improving the ability of the incoming transplanted HSCs to compete for niche occupancy. The studies of Chen et al. thus differed in several respects from those described here, where the effects on engraftment were assessed in sublethally irradiated hosts and found to be exclusive for transplanted HSCs in S/G₂/M at the time of injection. Therefore, it seems unlikely that the mechanisms responsible for the enhanced engraftment seen in both experimental models are identical, in spite of the fact that they are both initiated by treatment of the host with a CXCL12 antagonist.

The fact that proliferating human HSCs show the same engraftment defect when they transit S/G₂/M is noteworthy (20) and underscores the clinical implications of these findings. For example, our results predict that intrafemoral injection of transplants is unlikely to be a useful strategy for improving the therapeutic effectiveness of HSCs induced to expand in vitro. To date, interference

of CXCL12 action by specific CXCR4 inhibitors has been used primarily for enhancing the yield of HSCs from donors for transplantation into myeloablated patients (71, 72). Another application of such inhibitors suggested by the findings reported here could be to treat recipients of cycling cell transplants. Thus significant benefit might also be derived by pretreatment of the host, particularly when transplants of genetically modified or cultured cells are to be administered, since half of the HSCs in an asynchronously dividing population would be expected to be in S/G₂/M.

Methods

Mice. Ly5-congenic strains of C57BL/6 mice were used as donors and recipients. All recipients were also homozygous for the *W⁴¹* allele. Mice were bred and maintained in microisolators with sterile food, water, and bedding at the BC Cancer Research Centre. All experimental protocols were approved by the University of British Columbia Animal Care Committee.

Cells. Single-cell suspensions were prepared in Hank's balanced salt solution containing 2% FCS (HF/2; StemCell Technologies). Enriched populations of HSCs were obtained by immunomagnetic removal of Ter119⁺ or lin⁺ cells from FL and BM cell suspensions, respectively (using EasySep; StemCell Technologies). Antibodies used for isolation of lin⁻ cells between 4 and 10 weeks of age were anti-B220, Ter119, anti-Gr1, anti-Ly1, and anti-Mac1 (StemCell Technologies). To isolate lin⁻ cells from 3-week-old mice, the Mac1 antibody was omitted because Mac1 is known to be expressed on fetal and cycling HSCs (13, 14, 73).

Tritiated ³H-Tdr suicide assay. Cells were suspended at 10⁶/ml in Iscove's medium containing 5 × 10⁻⁵ mol/l 2-mercaptoethanol, a serum substitute (BIT 9500; StemCell Technologies), and 50 ng/ml murine SF (StemCell Technologies). Equal volumes were then incubated at 37°C in 5% CO₂ in air for 16 hours in 35-mm petri dishes in the presence or absence of 20 μCi/ml ³H-Tdr (25 Ci/mmol; Amersham Biosciences). The cells were then harvested and washed twice with Iscove's medium containing 2% FCS, and limiting dilution CRU assays were performed.

FACS isolation and analysis of cells in different cell cycle phases. Cells were suspended in HF/2 containing 1 μg/ml Hst (Invitrogen) and either 10 μmol/l fumitremorgin C (a gift from S. Bates, NIH, Bethesda, Maryland, USA) or 50 μmol/l reserpine (Sigma-Aldrich) and then incubated at 37°C for 45 minutes. Py (Sigma-Aldrich) was added at 1 μg/ml, and the cells were incubated for another 45 minutes at 37°C. Cells were washed twice in HF/2 with 1 μg/ml PI (Sigma-Aldrich) in the second wash and were finally resuspended in HF/2 with PI and kept on ice in the dark until being sorted (PI-cells only) on a 3-laser FACS Vantage (BD). For analysis of DNA content, cells were either restained with Hst only using the same protocol or stained with PI at least 1 hour after storage at 4°C of cells that had been washed twice in ice-cold PBS with 0.1% glucose and fixed in 1 ml ice-cold 70% ethanol. To stain with PI, cells were washed twice with 2% PBS and resuspended in PBS with 0.1% glucose, 5 μg/ml PI, and 200 μg/ml RNase A. Cells were then incubated for at least 1 hour at 4°C and analyzed directly on a FACSCalibur (BD). To stain sorted cells for Ki67, the cells were washed, resuspended in 50 μl ice-cold 80% ethanol, and incubated at -20°C for at least 2 hours. The fixed cells were washed twice in 300 μl PBS with 1% FCS and 0.09% NaN₃ (pH 7.2). FITC-conjugated anti-human Ki67 antibody (BD) was then added, and the cells were incubated for 30 minutes at room temperature in the dark. Cells were then analyzed by FACS, with cells stained with a FITC-conjugated mouse IgG₁ (BD) serving as a control.

In vitro treatment of S/G₂/M HSCs. Sorted cells were incubated at 37°C in 5% CO₂ in air in the wells of a round-bottom 96-well plate in serum-free media (as in the ³H-Tdr suicide assays) with 1 of the following 6 additions: 100 ng/ml CXCL12 (a gift from I. Clark-Lewis, University of British Columbia, Vancouver, British Columbia, Canada) or 300 ng/ml SDF-1G2 (also a gift from I.



Clark-Lewis, now available at cost as a requested peptide from the Biomedical Research Centre, University of British Columbia) but no SF; 50 ng/ml SF alone; 50 ng/ml SF plus 100 ng/ml CXCL12 or 300 ng/ml CXCL12; or 300 ng/ml SDF-1G2. Cells were then harvested from the wells, and equal aliquots were injected into recipient mice such that each mouse received the equivalent of either 4×10^3 starting G₁ cells or 1.2×10^4 starting S/G₂/M cells.

CRU assay. Recipient (W^{41}/W^{41}) mice were sublethally irradiated with either 400 cGy ¹³⁷Cs γ -rays or 360 cGy 250 kVp X-rays and then injected intravenously with the test cells, except when injected intrafemorally as indicated. Intrafemoral injections were performed as described previously (74). CRUs were identified by their ability to have generated $\geq 1\%$ donor Ly5-type blood cells including Ly1⁺ (T cell), B220⁺ (B cell), and Gr1/Mac1⁺ (granulocyte/monocyte) subsets that could be detected 16 weeks or later after transplantation (75). CRU frequencies were calculated using the L-calc program (StemCell Technologies) from the proportions of mice given various doses of test cells that were negative for this endpoint. Recipients treated with SDF-1G2 were injected intravenously with 10 μ g per mouse of SDF-1G2 dissolved in PBS 2 hours after being irradiated and were then transplanted another 2 hours later. This schedule was used in an attempt to minimize direct interaction of the injected HSCs with SDF-1G2 in the circulation (based on the likely rapid clearance of SDF-1G2) and to maximize any potential effect on the host by keeping the interval between SDF-1G2 injection and the transplant as short as possible. Controls were injected with PBS instead of the SDF-1G2.

Real-time PCR. Cells were sorted into 1 ml HF/10, and RNA was isolated using the PicoPure RNA Isolation Kit (Arcturus) as recommended by the supplier including a 15-minute DNase1 treatment (QIAGEN) on the column at room temperature. RNA was eluted into an 11- μ l volume and stored at -80°C . A cDNA preparation was then generated using the SuperScript III First-Strand Synthesis System for RT-PCR (18080093; Invitrogen) as recommended by the manufacturer, with the reaction scaled up to use 25 μ l. Quantitative real-time PCR was performed using the following primer pairs (5' to 3'): α 4int (NM_010576.2) forward, AGGACACACCAGGCATTTCAT; reverse, CCTCAGTGTTCGTTTGGTG; CD44 (NM_009851.1) forward, CTTTATCCGGAGCACCTTGCCACC; reverse, GTCACAGTGGCGGAAGTCC; *c-Kit* (NM_021099.2) forward, ACAAGAG-

GAGATCCGCAAGA; reverse, GAAGCTCAGCAAATCATCCAG; *c-mpl* (NM_010823.1) forward, AGTGGCAGCACCAGTCATCT; reverse, GAGATGGCTCCAGCACCTT; *CXCR4* (NM_009911.2) forward, CGGAGTCAGAATCCTCCAGT; reverse, CTGGTCAGTCTTATATCTGGAAAA; *Gapdh* (NM_008084) forward, AACTTTGGCATTGTGGAAGG; reverse, ATGCAGGGATGATGTTCTGG; *CXCL12* (NM_001012477) forward, GAGCCAACGTCAAGCATCTG; reverse, CGGGTCAATGCACACTTGTG; *VCAM1* (NM_011693.2) forward, TGATTGGGAGAGACAAAGCA; reverse, AACACCGAATCCCCAACTT. The relative expression changes were determined as previously described (76), and the housekeeping *Gapdh* gene transcript was used to normalize the results.

Statistics. Comparisons were made using the Wald test. *P* values of less than 0.05 were considered significant.

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Address correspondence to: Connie J. Eaves, Terry Fox Laboratory, 675 West 10th Avenue, Vancouver, British Columbia V5Z 1L3, Canada. Phone: (604) 675-8122; Fax: (604) 877-0712; E-mail: ceaves@bccrc.ca.

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