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ABSTRACT

It is now evident that hemopoietic stem cells (HSC) are located in close proximity to bone lining cells within the endosteum. Accordingly, it is unlikely that the traditional method for harvesting bone marrow (BM) from mice by simply flushing long bones would result in optimal recovery of HSC. With this in mind, we have developed improved methodologies based on sequential grinding and enzymatic digestion of murine bone tissue to harvest higher numbers of BM cells and HSC from the endosteal and central marrow regions. This methodology resulted in up to a sixfold greater recovery of primitive hemopoietic cells (lineage⁻Sca⁺Kit⁺ [LSK] cells) and HSC as shown by transplant studies. HSC from different anatomical regions of the marrow exhibited important functional differences. Compared with their central marrow counterparts, HSC isolated from the endosteal region (a) had 1.8-fold greater proliferative potential, (b) exhibited almost twofold greater ability to home to the BM following tail vein injection and to lodge in the endosteal region, and (c) demonstrated significantly greater long-term hemopoietic reconstitution potential as shown using limiting dilution competitive transplant assays. STEM CELLS 2007; 25:1062–1069

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The concept of a hemopoietic stem cell (HSC) niche was first proposed by Schofield [1], who suggested that HSC reside within a distinct three-dimensional structure. He proposed that these fixed tissue spaces or niches were responsible for regulating stem cell fate and defining the size of the HSC pool. Many studies, including our own, strongly suggest that the HSC niche is located within the endosteal region of the bone marrow (BM) at the interface between the bone and BM [2–11]. Recognizing the importance of the endosteum as the site for the HSC niche, we have developed a methodology based on grinding bones and enzymatic digestion to yield a significant number of HSC from the endosteal region that are not collected by traditional flushing methods of BM harvesting. This method, involving the precise combination of mechanical disaggregation with enzymatic digestion at physiological temperature, allows the separate isolation and comparison of HSC from the endosteal and central BM regions. Although other investigators have used aspects of our approach to prospectively isolate HSC [12–14], they have not analyzed the impact of modified BM cell harvesting methodologies on the recovery and enrichment of HSC. Our data demonstrate that deviations from our precise method significantly affect stem cell yield, the repertoire of cell adhesion markers expressed on their surface, and the measurement of their functional potential. We show that HSC isolated from the endosteal region exhibit significantly increased in vitro (proliferation) and in vivo (homing, lodgement, and reconstituting) hemopoietic potential compared with their central marrow counterparts.

MATERIALS AND METHODS

Mice

Congenic C57Bl/6 (Ly5.2) and PTRPA (Ly5.1) mice were purchased from Animal Resources Center (Perth, WA, Australia, http://www.arc.wa.gov.au) and housed clean conventionally for at least a week prior to experimental use. All mice received mouse chow (Barastok, St. Arnaud, VIC, Australia) and acidified water ad libitum.

Irradiation

The ability of cells to reconstitute hemopoiesis was analyzed in mice receiving a near-lethal dose of irradiation (9.5 Gy) in two equal fractions separated by a 4-hour interval, delivered from two opposing ¹³⁷Cs sources (Gammacell 40; Atomic Energy of Canada, Ottawa, ON, Canada, http://www.aecl.ca) at a dose rate of 1.4 Gy/minute.

Isolation of Hemopoietic Cells from Different Marrow Regions

Mice were killed by cervical dislocation. BM was routinely collected from femurs, tibias, and iliac crests using the following methods.

Central Marrow Isolation. For the isolation of central marrow, bones were flushed using a 23-gauge (femurs and tibias) or 26-

gauge needle (iliac crests) and 1 ml of phosphate-buffered saline (PBS)-2% heat-inactivated (HI) fetal calf serum (FCS) (HyClone, Logan, UT, http://www.hyclone.com) (femurs and tibias vs. iliac crests respectively) and the bones discarded. The cells were washed by centrifuging at 400g for 5 minutes at 4°C, resuspended in PBS-2% HI FCS, and then refiltered through a 40-μm filter (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) and diluted to 10^7 cells per milliliter.

Endosteal Marrow Isolation. For the isolation of endosteal marrow, bones were flushed as described above for the isolation of the central marrow fraction. The flushed cells were discarded, and the marrow-depleted bones were ground in a mortar and pestle in PBS 2% HI FCS. The bone fragments were washed twice and filtered through a 40-μm filter, collecting a total of 200 ml for 10 mice. Bone fragments were incubated in 5 ml of 3 mg/ml Collagenase I (Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com) and 4 mg/ml Dispase II (Worthington, Lakewood, NJ, http://www.worthington-biochem.com), available in a stem cell isolation kit (Chemicon, Temecula, CA, http://www.chemicon.com; catalog no. SCR051-S) in PBS (310 mosm) in a shaking incubator (37°C; 250 rpm for 5 minutes). The bone fragments were then washed with PBS 2% HI FCS by vigorous shaking and filtering through a 40-μm filter, collecting a total of 100 ml. All collected cells were washed by centrifuging at 400g for 5 minutes at 4°C, resuspended in PBS 2% HI FCS, filtered through a 40-μm filter, pooled, and diluted to 10^7 cells per milliliter.

Hemopoietic Cell Enrichment Strategies

Low-density BM mononuclear cells (<1.077 g/cm³) were isolated by discontinuous density gradient centrifugation using Nycoprep for animals (Accurate Chemical and Scientific Corporation, Westbury, NY, http://www.accuratechemical.com). Isolated cells were washed prior to isolation of an enriched population of hemopoietic lineage antigen-negative (Lin⁻) cells. Briefly, low-density cells were labeled with a cocktail of unconjugated primary rat anti-mouse antibodies: B220 (CD45R; B cells), Mac-1 (CD11b; macrophages), Gr-1 (Ly-6G; neutrophils), and Ter119 (erythroid cells). Lin⁺ cells were then removed by immunomagnetic selection using sheep anti-rat IgG Dynal beads (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Initially, cells and beads were incubated with constant mixing at a ratio of 0.5 beads per cell in 10^8 cells per milliliter for 5 minutes at 4°C, prior to positive cells being removed by magnetic separation. This process was repeated to yield an enriched population of hemopoietic precursors.
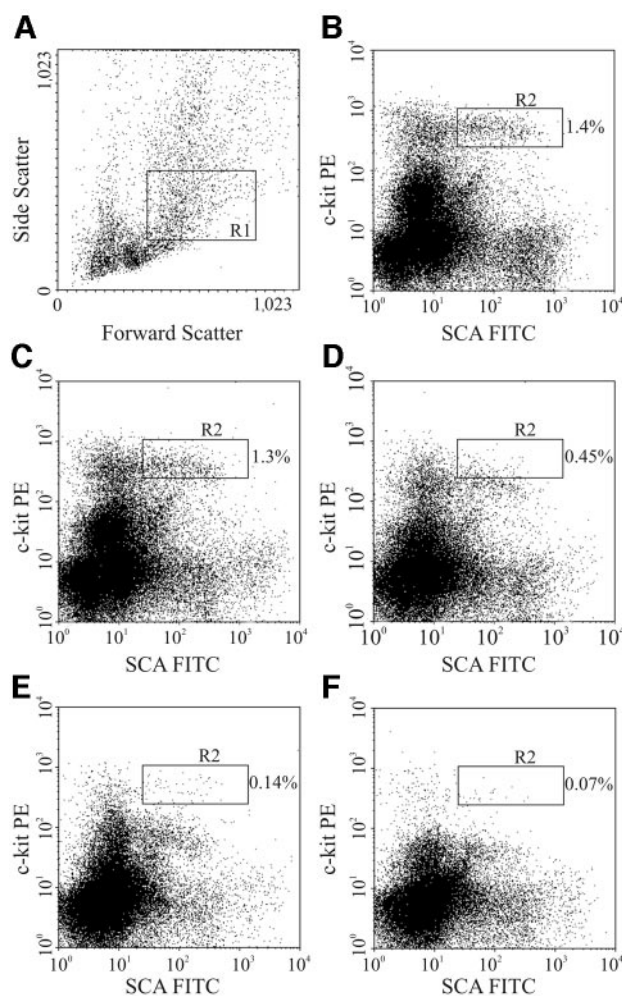
Stem Cell Antigen 1 (Sca-1) and c-kit Labeling

Lin⁻ cells were washed and resuspended in a cocktail of Sca-1-fluorescein isothiocyanate (FITC) (BD Pharmingen, San Diego, http://www.bdbiosciences.com/pharmingen; 1 μg per 5 × 10^6 cells), c-kit-phycoerythrin (PE) (BD Pharmingen; 1 μg per 5 × 10^6 cells), and streptavidin-Red 670 (Gibco, Grand Island, NY, http://www.invitrogen.com; final concentration, 1:160) for the detection of residual Lin⁺ cells, on ice in the dark for 20 minutes. Cells were washed and resuspended at 5 × 10^6 cells per milliliter prior to fluorescence-activated cell sorting (FACS).

Flow Cytometry

Labeled cells were sorted on a FACStar^PLUS cell sorter equipped with a 5-W argon ion laser (Coherent Innova 90, Palo Alto, CA, http://www.coherent.com) emitting 488 nm light at 200 mW. Light-scatter signals were collected through a 488 ± 10-nm band-pass filter and a 1-decade logarithmic neutral density filter in the forward light scatter path. FITC-emitted green fluorescence pulses were collected through a 530 ± 15-nm band-pass filter. Orange fluorescence pulses emitted following excitation of PE were reflected through a 440 dichroic short-pass mirror and collected through a 575 ± 26-nm band-pass dichroic filter. Pulses emitted following the excitation of Red 670 were collected through a long-pass RG655 filter. Lineage⁻Sca⁺Kit⁺ (LSK) cells were sorted at approximately 10,000 input cells per second and collected in serum-coated tubes on ice (Fig. 1). Overlap of the FITC, PE, and

Figure 1. Flow cytometric analysis of the effects of collagenase/dispase enzymatic digestion of the bone fragments on c-kit expression. For the identification of lineage⁻Sca⁺Kit⁺ (LSK) cells, a blast window (R1) was created on a forward versus side scatter profile from Lin⁻ magnetic cell sorting-depleted marrow **(A)**. Back-gating of Sca⁺Kit⁺ cells demonstrated that at least 95% of LSK cells were located within R1. Sca⁺Kit⁺ cells were identified (R2) and comprised 1.4% of R1 after 5 minutes initial collagenase/dispase enzymatic digestion of the bone fragments **(B)**. The proportion of LSK cells with the same levels of c-kit expression rapidly and significantly decreased to 1.3%, 0.45%, 0.14% and 0.07% of R1 after 15, 30, 60, and 120 minutes (**[C–F]**, respectively). Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; SCA, stem cell antigen.

Red 670 emission spectra was compensated for electronically. Sorted cells were counted and reanalyzed for purity.

Analysis of Homing Receptors

At least 1 × 10^5 LSK cells from the endosteal and central BM regions were labeled with antibodies against CXCR₄, CD49d, and PSGL-1 (BD Pharmingen), followed by a PE secondary antibody. Cells were washed and resuspended at 5 × 10^6 cells per milliliter prior to FACS analysis.

Low- and High-Proliferative Potential Colony-Forming Cell Assays

Low- and high-proliferative potential colony-forming cells (LPP-CFC and HPP-CFC, respectively) were assayed by plating 500 LSK cells per 35-mm Petri dish in a double-layer nutrient agar culture system as previously described [15], except that stem cell factor

(SCF) was added to colony-stimulating factor 1, interleukin (IL)-1, and IL-3 to analyze HPP-CFC.

LSK Cell Culture

Sorted LSK cells were cultured under serum-free conditions in 100 μl per well of CellGro (Cellgenix, Freiburg, Germany, http://www.cellgenix.com) supplemented with rat SCF (75 ng/ml), human FLT3 ligand (50 ng/ml), IL-6 (10 ng/ml), and IL-11 (100 ng/ml) in a 96-well flat-bottom plate at a density of 100 cells per well. All cells were cultured at 37°C in 5% O_2, 10% CO_2, and 85% N_2. Cells were grown for 6 days prior to counting.

Competitive Limiting Dilution Assay

Sorted LSK cells were transplanted in a competitive limiting dilution assay into irradiated recipients. Specifically, limiting numbers of endosteal LSK cells (between 1,000 and 30) isolated from male PTPRCA donors were competed with 300 central LSK cells isolated from male C57Bl/6 donors and transplanted in 0.2 ml of PBS by injection into the lateral tail vein of female C57Bl/6 recipients.

Long-Term Transplant Analysis

Bone marrow was isolated from individual transplant recipients 12 weeks post-transplant using the method described above for isolating the endosteal fraction without initially flushing. Whole BM was labeled with a rat anti-mouse Ly5.1-PE (PTPRCA) antibody and a rat anti-mouse Ly5.2-FITC (C57Bl/6) antibody to determine the proportion of PTPRCA LSK cells. All Ly5.2-FITC$^+$ cells were sorted, and the proportion of male C57Bl/6 LSK cell donor contribution was determined using real-time polymerase chain reaction (PCR) for Y chromosome. The proportion of donor cells was then mathematically calculated.

DNA Isolation and Real-Time PCR Analysis

Cells were dry-pelleted and resuspended in 600 μl of 50 mM NaOH. Cells were heated for 10 minutes at 90°C and vortexed, and 100 μl of 1M Tris, pH 8, was added. Cells were centrifuged at 13,000 rpm for 10 minutes, and the supernatant was collected. PCR using gene-specific primers and an internal oligonucleotide probe was used to quantitate the relative DNA levels of Y chromosome in a similar manner to that previously described by Peters et al. [16]. Duplicate technical replicates were done for each sample, and all samples were assayed at a similar cDNA template concentration. For the Y chromosome internal probe, the reporter dye 6-carboxylfluorescein and quencher 6-carboxytetramethyl rhodamine was labeled at 5′ and 3′, respectively. For 18S internal probes, the reporter 6-carboxylfluorescein was substituted with VIC (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). The PCR was done in a final volume of 20 μl and consisted of 10 μl of Taqman reaction mix, 300 nM Y chromosome forward and reverse primer, 100 nM probe, 100 nM each 18S primer, and 100 nM 18S probe. PCR amplification was done by denaturation for 10 minutes at 95°C followed by annealing for 2 minutes at 50°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Thermocycling and fluorescence measurements were done in an ABI Prism 7500 sequence detection system (Applied Biosystems). Relative quantitation was done by normalizing threshold cycle (Ct) values of each sample gene with Ct values of 18S. qCt corresponds to the difference between the Ct of the Y chromosome gene of interest and the Ct of 18S. Data were then compared with a standard curve created using known proportions of male DNA (average r^2 = .99), and the percentage of male cellular contribution was determined.

5- (and 6)-Carboxyfluorescein Diacetate Succinimidyl Ester Labeling

Cells to be tested for homing ability or spatial distribution analysis were labeled with 5- (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFDA, SE) (Molecular Probes, Eugene, OR) as previously described [7] and then transplanted in 0.2 ml of PBS by injection into the lateral tail vein.

Homing and Spatial Distribution Assay

Sorted CFSE$^+$ LSK cells were transplanted in 0.2 ml of PBS by injection into the lateral tail vein of 2–4 female C57Bl/6 recipients per group. Between 27,000 and 28,000 and 70,000 and 105,000 CFSE$^+$ cells were transplanted into each nonablated recipient for homing and spatial distribution analysis, respectively, together with 200,000 unlabeled whole bone marrow cells.

Analysis of Cell Homing and Spatial Distribution

The homing ability and spatial distribution of CFSE-positive central or endosteal LSK cells was analyzed 15 hours post-transplant into nonablated recipients. For the analysis of homing, endosteal and central marrow samples were prepared from individual mice as described above. Marrow cells were labeled with a rat anti-mouse CD45-PE-conjugated antibody (BD Pharmingen) for 15 minutes on ice. Cells were washed, and the analysis of the proportion of CFSE$^+$ donor cells in each fraction was performed using CD45-PE as the denominator for the total number of cells analyzed by flow cytometry. Preparation of samples for spatial distribution was as previously described [7]. Briefly, the location of CFSE-labeled cells (positive cells) from at least six longitudinal sections per transplant recipient was recorded. Central longitudinal sections were analyzed, as opposed to transverse sections, as each individual section encompasses more of the entire femur. To ensure that individual cells were only analyzed once, every other 3.5-μm section was analyzed. The location of positive cells was designated either endosteal (previously arbitrarily defined as within 12 cells of the endosteum) or central (more than 12 cells from either endosteum).

Statistical Analysis

Differences between means were evaluated using Student's t-test. Contribution of each donor cell population to hemopoietic recovery during the short- and long-term competitive limiting dilution assays was evaluated by comparing the contribution of the LSK cells isolated from the endosteum to that mathematically expected if there was equal contribution. Based on the data not being normally distributed or having equal variance, analysis was done using a Spearman rank order correlation test, where a p value $\leq$ .05 shows a significant correlation and p >.05 shows no significant relationship. This test allows for the correlation of each data point in the context of the entire data set (n = 56 at 6 weeks and n = 42 at 12 weeks) and is appropriate when comparing a large number of data points in different groups with an expected outcome.

RESULTS

Endosteal and Central Bone Marrow Have Equivalent Proportions of LSK Cells

We analyzed the proportion and total number of LSK cells isolated from the central and endosteal regions from total femurs, tibias, and iliac crests using the isolation methods described (Table 1). The incidence of LSK cells was equivalent in both the central and endosteal bone marrow fractions. However, a significant number of LSK cells, which remain within the endosteal region (33%) following the isolation of BM cells from the central marrow core, would be lost using the traditional flushing methodology.

In developing this methodology, we observed that the time of incubation of marrow cells with collagenase/dispase is a critical determinant to subsequent immunolabeling and isolation of HSC based on the LSK phenotype. As shown in Figure 1, when cells are incubated in this enzyme combination, there is a time-dependent loss of c-kit immunoreactivity, with incubation periods longer than 5 minutes resulting in a progressive loss of c-kit. Consequently, cells could no longer be isolated based on the expression of the c-kit receptor. Furthermore, longer periods of enzymatic digestion also resulted in a loss of expression in Sca-1.

Table 1. The proportion and total number of LSK cells isolated from the central and endosteal region of bone marrow

	Total nucleated cells ($\times\ 10^6$)	% LSK in total BM[a]	Total LSK isolated[b]
Central BM	88.4 ± 2.7	0.17 ± 0.016	$74,247 \pm 3,941$
Endosteal BM	46.6 ± 0.9	0.18 ± 0.02	$37,210 \pm 4,150$

Data represent the mean $\pm$ SEM of nine different LSK isolations from two femurs, two tibias, and two iliac crests.
[a]The incidence of LSK cells calculated from total BM.
[b]Actual number of LSK cells obtained after high-speed fluorescence-activated cell sorting of the entire marrow harvest. It must be remembered that the absolute number of stem cells isolated using any method will be significantly influenced by the pre-enrichment strategies used and their associated nonspecific cell losses.
Abbreviations: BM, bone marrow; LSK, lineage$^-$Sca$^+$Kit$^+$.

LSK Cells Within the Endosteal Region Have an Increased Proliferative Potential

To determine whether LSK cells isolated from the endosteal region had increased proliferative potential, cells were cultured in serum-free conditions for 6 days. LSK cells isolated from the endosteal region had a significantly higher proliferative potential (1.8-fold; $p = .02$) under these conditions compared with those from the central marrow core (Fig. 2). The increased cell production in this in vitro assay may be attributed to either an increased proportion of primitive cells with enhanced proliferative potential within the endosteal LSK fraction or, alternatively, to the isolation of a greater number of committed progenitors within the LSK fraction that proliferate rapidly during the 6 days of culture. In vitro clonogenic assays were therefore performed to address this issue.
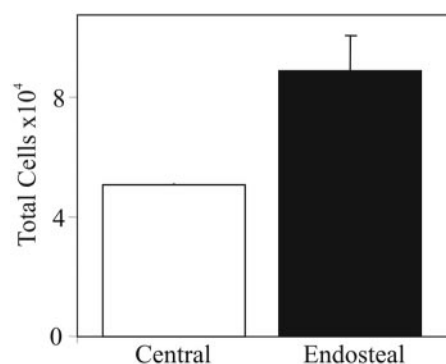
LSK Cells with High Proliferative Colony-Forming Potential Are Preferentially Enriched Within the Endosteal Region

The clonogenic characteristics of LSK cells isolated from the endosteal and central marrow regions were examined using the double-layer agar colony-forming assay as described by Bartelmez et al. [15]. The incidence of HPP-CFC in the LSK cell fraction isolated from the endosteal region was significantly higher (1.4-fold; $p < .01$) than that of the LSK fraction isolated from the central marrow region (Fig. 3C). Conversely, endosteal marrow LSK cells had a significantly reduced proportion (1.4-fold; $p < .01$) of more committed LPP-CFC (Fig. 3B). Significantly, even though the endosteal region only makes up approximately 14% of the marrow area (excluding the central vein) [7], there was a significant increase in the total number of HPP-CFC in this region (1.9-fold; $p < .01$) (Fig. 3D).

To determine whether endosteal LSK cells were characterized by an enhanced in vivo as well as in vitro hemopoietic potential compared with those isolated from the central BM region, we compared their relative homing potential and spatial distribution post-transplant, and their relative long-term hemopoietic reconstituting ability.

LSK Cells Isolated from the Endosteal Region Home More Efficiently to the Bone Marrow and Preferentially Reseed in the Endosteal Region

Following transplantation in a 15-hour homing assay in a nonablated mouse model, LSK cells isolated from the endosteal region had a twofold increased homing efficiency to the BM compared with LSK cells isolated from the central marrow core ($p < .05$) (Fig. 4A). More than 14% of endosteal LSK cells homed to the BM of nonablated recipients, compared with the 7% of LSK cells isolated from the central marrow core. This latter value of 7% is comparable to the seeding efficiency routinely quoted for HSC to the BM post-transplantation [17–
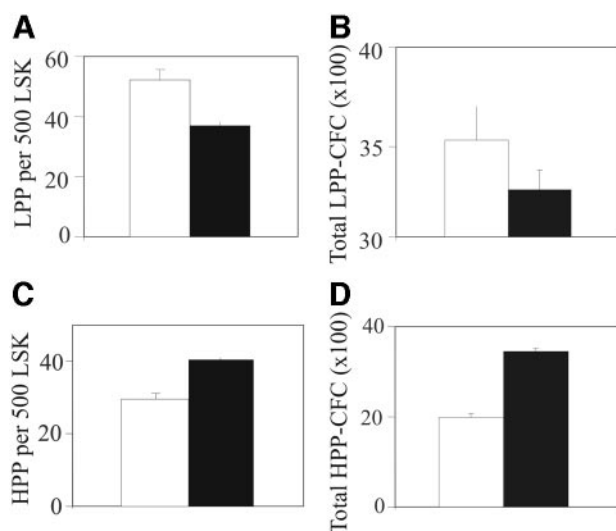
Figure 2. Lineage$^-$Sca$^+$Kit$^+$ (LSK) cells within the endosteal region have significantly increased proliferative potential in vitro. Sorted LSK cells (100 per well) isolated from the endosteal region (black bar) and the central marrow core (white bar) were grown in serum-free media in a potent combination of four early-acting growth factors (SCF, FLT3 ligand, interleukin [IL]-6, and IL-11). After 6 days, there was a significant ($p = .02$, Student's t test) increase in the number of cells generated from endosteal LSK cells compared with those isolated from the central marrow core. Data are from a representative experiment ($n = 4$) showing the mean $\pm$ SEM from quadruplicate wells.

23]. However, analysis of CXCR$_4$, CD29d, and PSGL-1, cell surface receptors with well-defined roles in the homing of hemopoietic cells to the BM, revealed no differences in the proportion of cells expressing these receptors or the levels of expression between central or endosteal LSK cells (data not shown).
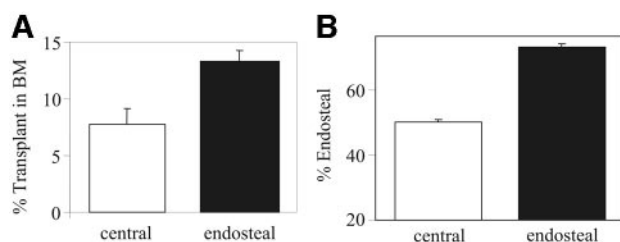
Significantly, following transplantation, both endosteal and centrally located LSK cells preferentially lodged within the region of the BM from which they were originally isolated. Specifically, 73% $\pm$ 1.2% of LSK cells isolated from the endosteal region relodged within this region 15 hours post-transplant, with a significantly higher (1.5-fold; $p < .05$) efficiency than those isolated from the central marrow core (50% $\pm$ 1%) (Fig. 4B).

LSK Cells Isolated from the Endosteal Region Have Enhanced Hemopoietic Potential In Vivo

Based on these functional differences, we conducted competitive limiting dilution transplant experiments to compare the short- and long-term hemopoietic potential of LSK cells isolated from these distinct anatomical regions. Increasing numbers of LSK cells isolated from the endosteal marrow region were competed against a constant number of LSK cells isolated from the central marrow core. Analysis of donor cells in the peripheral blood 6 weeks post-transplant revealed no differences in short-term hemopoietic repopulating ability between LSK cells isolated from the endosteal region and those isolated from the central marrow core (Fig. 5A). The proportion of donor cells correlated with the mathematically expected outcomes for an equal contribution from endosteal and central LSK cells (corre-
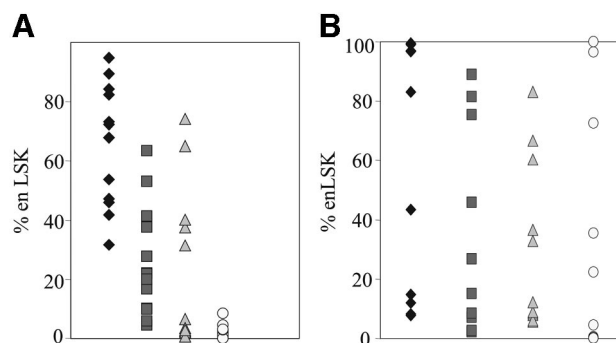
Figure 3. LSK cells with high-proliferative colony-forming potential are significantly enriched within the endosteal region. The frequency and total content of LPP-CFC (**A, B**) and HPP-CFC (**C, D**) within LSK cells isolated from the central (white bar), and endosteal regions (black bar). Compared with the central marrow core, there was a significant decrease in the proportion of LPP-CFC within LSK cells isolated from the endosteal region ($p < .05$) (**A**). In contrast, there was a significant increase in both the incidence and total content of HPP-CFC within LSK cells isolated from the endosteal region compared with LSK cells isolated from the central marrow core ($p < .05$) (**C, D**). Data show a representative experiment ($n = 6$) with the mean ± SEM from triplicate dishes of LSK cells isolated from two femurs, two tibias, and two iliac crests from three animals. Abbreviations: CFC, colony-forming cells; HPP, high proliferative potential; LPP, low proliferative potential; LSK, lineage⁻Sca⁺Kit⁺.

Figure 4. Lineage⁻Sca⁺Kit⁺ (LSK) cells within the endosteal region have significantly increased homing efficiency to the BM in vivo. LSK cells isolated from the endosteal region and transplanted into nonablated recipients had a significantly higher homing efficiency to the BM after 15 hours compared with LSK cells isolated from the central marrow core ($p < .05$) (**A**). In addition, LSK cells isolated from the endosteal region and transplanted into nonablated recipients had a significantly greater affinity for the endosteal region of the BM after 15 hours compared with LSK cells isolated from the central marrow core ($p < .05$) (**B**). Data are the mean ± SEM from both endosteal and central marrow LSK cells isolated from two separate harvests of 15 animals and each of the central and endosteal fractions transplanted into 2–4 individual recipients. Abbreviation: BM, bone marrow.

lation coefficient = 0.82; $p < .001$. However, analysis of the BM 12 weeks post-transplant revealed a significantly greater contribution (correlation coefficient = 0.28; $p > .05$) by endosteal donor cells compared with central LSK cells to donor hemopoiesis than that mathematically expected (Fig. 5B). This was particularly evident in recipients transplanted with limited numbers of endosteal LSK cells (30 cells per mouse), where the proportion of donor hemopoiesis in 50% of the recipients was far greater than the predicted 9%. Notably, two of the recipients transplanted with 30 LSK cells exhibited 100% donor hemopoiesis.

Figure 5. Lineage⁻Sca⁺Kit⁺ (LSK) cells within the endosteal region have an equivalent or increased transplant potential in vivo in a competitive limiting dilution assay. Limiting numbers of LSK cells (diamond, 1,000; square, 300; triangle, 100; circle, 30) isolated from the endosteal region were competitively transplanted with 300 LSK cells isolated from the central marrow core into irradiated recipients. Donor contribution to WBC were analyzed in the peripheral blood 6 weeks post-transplant (**A**) and in the BM 12 weeks post-transplant (**B**). Data are the values for individual recipients ($n = 10–15$ per group) from three biological transplant repetitions. The expected mathematical values if the competing cells had equivalent hemopoietic reconstituting abilities would be 77%, 50% 25%, and 9%, respectively. In both instances, the data were compared with the mathematically expected outcome using a Spearman rank order correlation. Abbreviation: enLSK, endosteal lineage⁻Sca⁺Kit⁺.

DISCUSSION

For more than half a century, studies, including our own, have strongly suggested that the HSC niche is located within the endosteal region of the BM or the interface between the bone and BM [2–11]. At this location, HSC reside in very close proximity to bone and participate in adhesive interactions with both cellular and extracellular components of the hemopoietic microenvironment. It is therefore surprising that these adhesive interactions and the endosteal location of the HSC niche have not been considered when developing methods for murine BM harvesting and HSC purification. In contrast to these considerations, the advances in HSC isolation have focused on the use of additional or combinations of markers to further subset an already enriched population of BM HSC. Although this approach has been successful in identifying HSC with differing transplant potential, the quality and total number of HSC that can be isolated will ultimately be governed by the contents of the BM sample initially harvested. In addition, the absolute number of stem cells isolated from the initial BM sample will then be significantly influenced by the pre-enrichment strategies used.

We have described methods, based on grinding bones and enzymatic digestion to recover maximal numbers of marrow cells and HSC, that enable the purification of HSC from the endosteal and central marrow regions and, for the first time, a direct comparison of their hemopoietic potential. Using these methods, we have shown that HSC with a well-defined phenotype (LSK), isolated from the endosteal marrow region, have significantly enhanced hemopoietic potential compared with their central marrow counterparts. Endosteal LSK cells have a greater proliferation potential when cultured in stromal-free conditions in serum-deprived media supplemented with a potent combination of early acting synergistic growth factors and contain a significantly greater number of HPP-CFC.

Furthermore, endosteal LSK cells had greater in vivo hemopoietic potential. Following transplantation into nonablated re-

cipients, a twofold increase in the homing efficiency of endosteal LSK cells was observed, combined with a greater preference to relodge within the endosteal region of the BM. In addition, a limiting dilution competitive transplant model demonstrated equivalent short-term hemopoietic reconstituting potential but increased long-term reconstituting potential of endosteal LSK cells compared with those isolated from the central BM core. Such a functional difference in HSC within a highly enriched BM subfraction isolated using the same phenotype has not previously been described, but it could in part be attributed to their enhanced ability to home and lodge within the endosteal region. In addition, the endosteal LSK fraction might be enriched for primitive long-term repopulating cells compared with the LSK fraction isolated from central marrow. Accordingly, it is tempting to speculate that these more primitive endosteal cells have cell-autonomous properties that enable them to outcompete central LSK cells. Our initial studies did not demonstrate any differences in expression of cell-adhesion molecules, known to be involved in regulating homing of HSC to the BM, including $CXCR_4$, CD49d, and PSGL-1.

The reasons endosteal LSK cells have enhanced homing and in vivo hemopoietic potential remains unclear but clearly warrants further investigation. It is possible that treatment of bone fragments with collagenase and dispase could affect cell surface receptors involved in the short-term homing of HSC (LSK cells) to the bone marrow, or their survival and proliferation in vivo. Moreover, treatment of bone fragments with these enzymes might also release bone proteins or factors that influence HSC functions and contribute to the observed difference in hemopoietic potential of endosteally or centrally isolated LSK cells. To investigate this possibility, we conducted a series of experiments in which central LSK cells were incubated with collagenase and dispase or with supernatant from enzyme digested bone fragments and then assessed the immunoreactivity of antibodies to cell surface receptors, including Sca-1, c-kit, PSGL-1, CD49d, $CXCR_4$, CD34, and flt3. In each case, the immunoreactivity of antibodies to each of these cell surface receptors was unchanged. Accordingly, we suggest that the increased functional characteristics of LSK cells isolated from the endosteal region is not attributable to the effect of enzymes on these specific receptors. Nevertheless, it is possible that previously unrecognized cell surface receptors involved in regulating stem cell homing, lodgement, and survival might be cleaved, revealed, or upregulated by this method of isolation and thus contribute to the enhanced hemopoietic activity of endosteal LSK cells. Despite this caveat, our data demonstrate that HSC isolated from the endosteal region exhibit greater hemopoietic potential than those harvested from the central marrow region, an observation that has direct implications for BM transplantation.

Our data also demonstrate that the BM harvesting methodology directly affects the number and quality of the HSC subsequently isolated. This has important implications for the field. The vast majority of studies that address the immunophenotypic functional properties of HSC have been performed on BM obtained using the simple, traditional flushing technique [13, 24–30]. A critical consideration in determining the initial BM sample harvested using this method is the needle size, although the gauge is rarely mention in publications [31–37]. Even more of a concern is that a description of how the BM was harvested is often not included [38–40]. Although the method of marrow harvest has been modified in some studies to include the grinding of bones to isolate marrow cells [41–45], these studies have never assessed whether the method of marrow harvest influences the yield or hemopoietic potential of isolated HSC.

The impact of the method of BM harvesting may be far-reaching. For example, studies analyzing the cell cycle rate of HSC isolated by flushing the central core [46, 47] may have been analyzing a more mature HSC with less hemopoietic potential but a faster cell cycle rate than its endosteal counterpart. Mathematical models based on these findings [48] would also be affected. In addition, differences in marrow harvesting methods may explain some of the large disparity in the reported homing efficiencies of single HSC. In a recent study, Matsuzaki et al. [49] reported an "unexpectedly" efficient HSC homing capacity of 96% compared with other studies, where engraftment levels never exceeded 30%. Significantly, although not identified, the study by Matsuzaki et al. [49] is the only one where marrow was isolated by grinding bones, whereas in all other studies, either the marrow was isolated by flushing [50, 51] or the methodology for marrow isolation was not described [52, 53].

It is surprising that studies aimed at identifying components of the HSC niche and their regulatory roles in maintaining the HSC pool have also neglected to consider that the method of BM harvesting results in the isolation of HSC from different regions of the BM with different properties [54]. By exploiting the ability of different isolation methods, we could further delineate the roles of specific molecules in HSC regulation.

One previous study specifically analyzed the impact of the initial cell harvesting procedure on the quantity and quality of hemopoietic progenitors isolated from the BM [55]. Rosendaal and Adam [55] showed that following vigorous flushing, a significant number of marrow cells remained, but some of these could be subsequently released by grinding. Although the hemopoietic potential of these populations was only assayed using surrogate in vitro assays, their data support the notion that more primitive cells are located in proximity to the bone-BM interface. Importantly, the authors identify a population of hemopoietic cells that remain on the bone fragments postgrinding, which they postulate are the "hemopoietic reserve that by being located close to stromal regulatory cells cycle very rarely." We suggest that the endosteal BM fraction isolated by our grinding and enzymatic digestion methodology includes the "hemopoietic reserve" described by Rosendaal and Adam [55]. An additional study investigating the distribution of progenitor cells within the marrow of different murine bones using in vitro surrogate assays also harvested marrow cells by grinding bones in a mortar and pestle [14]. However, this study did not involve the enzymatic digestion of bones to release hemopoietic cells known to remain attached to bone fragments, nor did it separate and directly compare the hemopoietic potential of HSC isolated from the endosteal and central bone marrow regions.

In a study by Funk et al. [13], associations of HSC and stromal cells isolated in marrow cell aggregates were analyzed after digestion with 2 mg/ml collagenase for 1.5–2 hours at 37°C. The authors analyzed the phenotype and hemopoietic potential of cells using a variety of in vitro and in vivo assays, but the potential effect of the enzymatic digestion on cell surface antigens and processes, including homing, lodgement, proliferation, and differentiation, were not considered [13]. More recently, in a study by Arai et al. [12], bone-associated marrow cells were isolated using 2 mg/ml collagenase and 2.4 U of dispase for 2 hours at 37°C. However, the potential effects of enzymatic digestion on cell surface antigen expression were not analyzed or discussed.

Research into normal and perturbed hemopoiesis is underpinned by the isolation, characterization, and utilization of murine HSC and has resulted in more than 11,500 publications since 1970. Methods for reproducible harvesting of BM and optimal isolation of defined populations of hemopoietic stem and progenitor cells are critical for this research. As HSC are a rare subpopulation of cells within the BM, any improvements to methods for isolating greater numbers of cells that represent a

more homogeneous HSC population with increased in vitro and in vivo potential are of significant value to experimental hematologists. The method we describe for harvesting of BM and the isolation of HSC results in the recovery and isolation of HSC from the endosteal region that display greater hemopoietic potential than phenotypically identical (LSK) cells isolated from the central marrow area. We therefore suggest that this method be adopted as the method of choice for isolation of HSC for the analysis of molecules important in the HSC niche, transcriptional and proteomic profiling, and functional studies, including transplantation, gene marking, and ex vivo expansion. The broad use of this method for harvesting BM and subsequent isolation of HSC from mouse BM will make results from different laboratories more comparable.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Hemopoietic Stem Cells with Higher Hemopoietic Potential Reside at the Bone Marrow Endosteum

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