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Lack of Evidence that Hematopoietic Stem Cells Depend on N-Cadherin-Mediated Adhesion to Osteoblasts for Their Maintenance

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SUMMARY

Recent studies have proposed that bone marrow hematopoietic stem cells (HSCs) are maintained via N-cadherin-mediated homophilic adhesion with osteoblasts. However, there is not yet any evidence that N-cadherin-expressing cells have HSC activity or that osteoblasts are required for HSC maintenance. We were unable to detect N-cadherin expression in highly purified HSCs by polymerase chain reaction, by using commercial anti-N-cadherin antibodies, or by β-galactosidase staining of N-cadherin gene trap mice. Only N-cadherin-negative bone marrow cells exhibited HSC activity in irradiated mice. Finally, *biglycan*-deficient mice had significant reductions in trabecular bone and osteoblasts but showed no defects in hematopoiesis, HSC frequency, or function. Thus, reductions in osteoblasts do not necessarily lead to reductions in HSCs. Most bone marrow HSCs in wild-type and *biglycan*-deficient mice localized to sinusoids, and few localized within five cell diameters of the endosteum. These results question whether significant numbers of HSCs depend on N-cadherin-mediated adhesion to osteoblasts.

INTRODUCTION

Hematopoietic stem cells (HSCs) persist throughout adult life in the bone marrow, where they continuously produce new blood cells. HSC maintenance is hypothesized to depend on the localization of HSCs to specialized microenvironments (niches) within the bone marrow (Adams and Scadden, 2006; Moore and Lemischka, 2006; Wilson and Trumpp, 2006). The identification of these niches has been a major goal of the field.

Osteoblasts are capable of influencing bone marrow HSC frequency. Osteoblasts secrete factors that regulate HSC maintenance and function (Taichman et al., 1996; Arai et al., 2004; Nilsson et al., 2005; Stier et al., 2005; Zhu et al., 2007), and genetic manipulations that increase osteoblast numbers in mice also increase the number of HSCs (Calvi et al., 2003; Zhang et al., 2003). Osteoclast function (Kollet et al., 2006), and the high levels of calcium thought to be generated from bone resorption (Adams et al., 2006), also regulates HSC migration and maintenance. These observations indicate that osteoblasts and the endosteum (the interface of bone and marrow where osteoblasts and osteoclasts localize) regulate HSC function and raise the question of whether HSCs localize to the endosteum itself or whether they are influenced at a distance (directly or indirectly) by extracellular factors that diffuse from the endosteum.

HSCs have been suggested to reside in direct contact with osteoblasts via homophilic adhesion between N-cadherin-expressing HSCs and N-cadherin-expressing osteoblasts (Zhang et al., 2003; Wilson et al., 2004). Ten percent of mouse c-kit$^+$lineage$^-$Sca-1$^+$ cells stained with a commercial anti-N-cadherin antibody, and BrdU label-retaining, N-cadherin$^+$ bone marrow cells were observed in contact with osteoblasts (Zhang et al., 2003). However, it has not yet been tested whether these N-cadherin$^+$ cells are HSCs or other cells that express similar markers. It also has not yet been tested whether N-cadherin is genetically required for HSC maintenance.

Two laboratories have published microarray analyses of highly purified HSCs that include data on *N-cadherin* (Ivanova et al., 2002; Kiel et al., 2005). Neither detected *N-cadherin* expression by HSCs, yet both detected *N-cadherin* expression by neural stem cells and embryonic stem cells using the same microarray platform (Ivanova et al., 2002; Molofsky et al., 2003). These studies raise the question of whether *N-cadherin* is expressed by HSCs.

Apart from the role of N-cadherin is the more general question of where HSCs reside in bone marrow. We systematically examined the localization of HSCs in the bone marrow using SLAM family receptors and found that most HSCs reside on the surface of sinusoidal blood

vessels (Kiel et al., 2005). SLAM family markers simplify and enhance the identification of HSCs as 45% of single $CD150^+CD48^-CD41^-$ bone marrow cells give long-term multilineage reconstitution in irradiated mice (Kiel et al., 2005). Almost all of these cells are c-kit^+, Sca-1^+, and $lineage^-$, so these markers can be used in conjunction with SLAM markers to purify the same population of HSCs, though they add little additional purity (Kiel et al., 2005). This "SLAM code" for HSCs has held up in every context of definitive hematopoiesis that we have examined (Kim et al., 2006; Yilmaz et al., 2006a). In our analysis, 60% of HSCs localized to sinusoids in the bone marrow, while 14% of HSCs localized to the endosteum (Kiel et al., 2005). Remaining HSCs were in other locations. This raised the possibility that most HSCs reside in sinusoidal vascular niches—a possibility that was further supported by the recent discovery that CXCL12-expressing reticular cells also reside adjacent to these sinusoids (Sugiyama et al., 2006).

It is unclear whether osteoblasts are physiologically required for HSC maintenance. To test this, it would be necessary to deplete osteoblasts in vivo to test whether they are not only sufficient to increase HSC numbers (Calvi et al., 2003; Zhang et al., 2003) but also necessary for HSC maintenance. This has been done by ablating osteoblasts from adult *Col1a1-TK* mice (Visnjic et al., 2004; Zhu et al., 2007). The authors did not observe an acute loss of HSCs. Rather, they observed an acute loss of differentiating cells, particularly B lineage progenitors. The frequency of c-$kit^+lineage^-Sca\text{-}1^+$ cells in bone marrow actually increased over time after osteoblast ablation (Visnjic et al., 2004; Zhu et al., 2007). HSCs depletion was not observed until weeks later, after bone marrow cellularity was severely reduced. This raises the question of whether HSCs are slowly lost as a direct consequence of osteoblast ablation or whether HSCs do not depend directly on osteoblasts but rather are ultimately depleted as a consequence of the loss of other cells. To resolve this question, the ideal approach would be to chronically deplete osteoblasts in vivo without inducing acute hematopoietic failure to test whether this depletes HSCs.

In this study, we have been unable to detect N-cadherin expression in highly purified HSCs by a variety of phenotypic and functional approaches. Thus, N-cadherin is unlikely to mediate homophilic adhesion between HSCs and osteoblasts. Reductions in trabecular bone and osteoblasts in *biglycan*-deficient mice had no effect on hematopoiesis, HSC frequency, or HSC function. We also confirmed that most HSCs localize to sinusoids within the bone marrow and that almost all HSCs are found within five cell diameters of a sinusoid. In contrast, only 8%–21% of HSCs were within five cell diameters of endosteum. These data suggest that most HSCs do not acutely depend on contact with osteoblasts for their maintenance, though it remains possible that a subset of HSCs is maintained at or near the endosteum by mechanisms other than N-cadherin-mediated adhesion. These results raise the question of whether the endosteum influences HSC localization and maintenance directly, by affecting a subset of HSCs that is present at the endosteum, or indirectly, by the secretion of diffusible factors that influence the ability of other cells to create niches for HSCs in other locations.

RESULTS

N-Cadherin Is Not Detectable in Highly Purified HSCs

We examined N-cadherin expression by a variety of approaches in highly purified HSCs. First, we tested whether HSCs expressed *N-cadherin* at the mRNA level by attempting to amplify *N-cadherin* from cDNA isolated from highly purified $CD150^+CD48^-CD41^-$c-$kit^+Sca\text{-}1^+lineage^-$ HSCs. $CD150^+CD48^-CD41^-$c-$kit^+Sca\text{-}1^+lineage^-$ cells represented 0.0076% ± 0.0021% of bone marrow cells in these experiments. This population contains all of the HSC activity from C57BL mouse bone marrow, and 47% of single cells within this population give long-term multilineage reconstitution in irradiated mice (Kiel et al., 2005). We were readily able to amplify *N-cadherin* from neonatal forebrain cells, which are known to express N-cadherin (Redies and Takeichi, 1993) (Figures 1A and 1B). However, we were never able to amplify *N-cadherin* from aliquots of $CD150^+CD48^-CD41^-$c-$kit^+Sca\text{-}1^+lineage^-$ HSCs (Figures 1A and 1B). We were also unable to amplify *N-cadherin* from aliquots of c-$kit^+Sca\text{-}1^+lineage^-Flk\text{-}2^-$ HSCs (Figures 1A and 1B), which represented 0.021% ± 0.001% of bone marrow cells and which are also highly enriched for HSCs (Christensen and Weissman, 2001; Kiel et al., 2005). In contrast, we were able to amplify similar amounts of *hypoxanthine phosphoribosyltransferase* (*HPRT*) from all of the samples (Figure 1B). Thus, we were unable to detect *N-cadherin* transcripts in highly purified HSCs.

To test N-cadherin expression at the protein level, we stained highly purified HSCs with two commercially available anti-N-cadherin antibodies and examined the staining by flow cytometry. These antibodies included the YS polyclonal anti-N-cadherin antibody that has been used previously to stain bone marrow (Zhang et al., 2003; Wilson et al., 2004), and the GC-4 monoclonal anti-N-cadherin antibody that has been used previously to characterize N-cadherin expression by epithelial progenitors (Hayashi et al., 2006). Both antibodies were able to stain neonatal forebrain cells by flow cytometry (Figure 1C). In contrast, neither antibody showed any staining above background on either $CD150^+CD48^-CD41^-$c-$kit^+Sca\text{-}1^+lineage^-$ HSCs or c-$kit^+Sca\text{-}1^+lineage^-Flk\text{-}2^-$ HSCs (Figure 1D). Thus, we were unable to detect N-cadherin expression on the surface of highly purified HSCs using commercially available antibodies.

If a subset of HSCs adheres to the endosteum, it is possible that these cells might be depleted within marrow samples that are obtained by flushing the marrow cavity with medium. To test whether this approach leaves behind significant numbers of HSCs, we used a bone burr (a drill, see the Experimental Procedures) to remove any cells remaining in the flushed marrow cavity. Representative images of sections through the marrow cavity from unmanipulated bone, flushed bone, and burred bone are shown

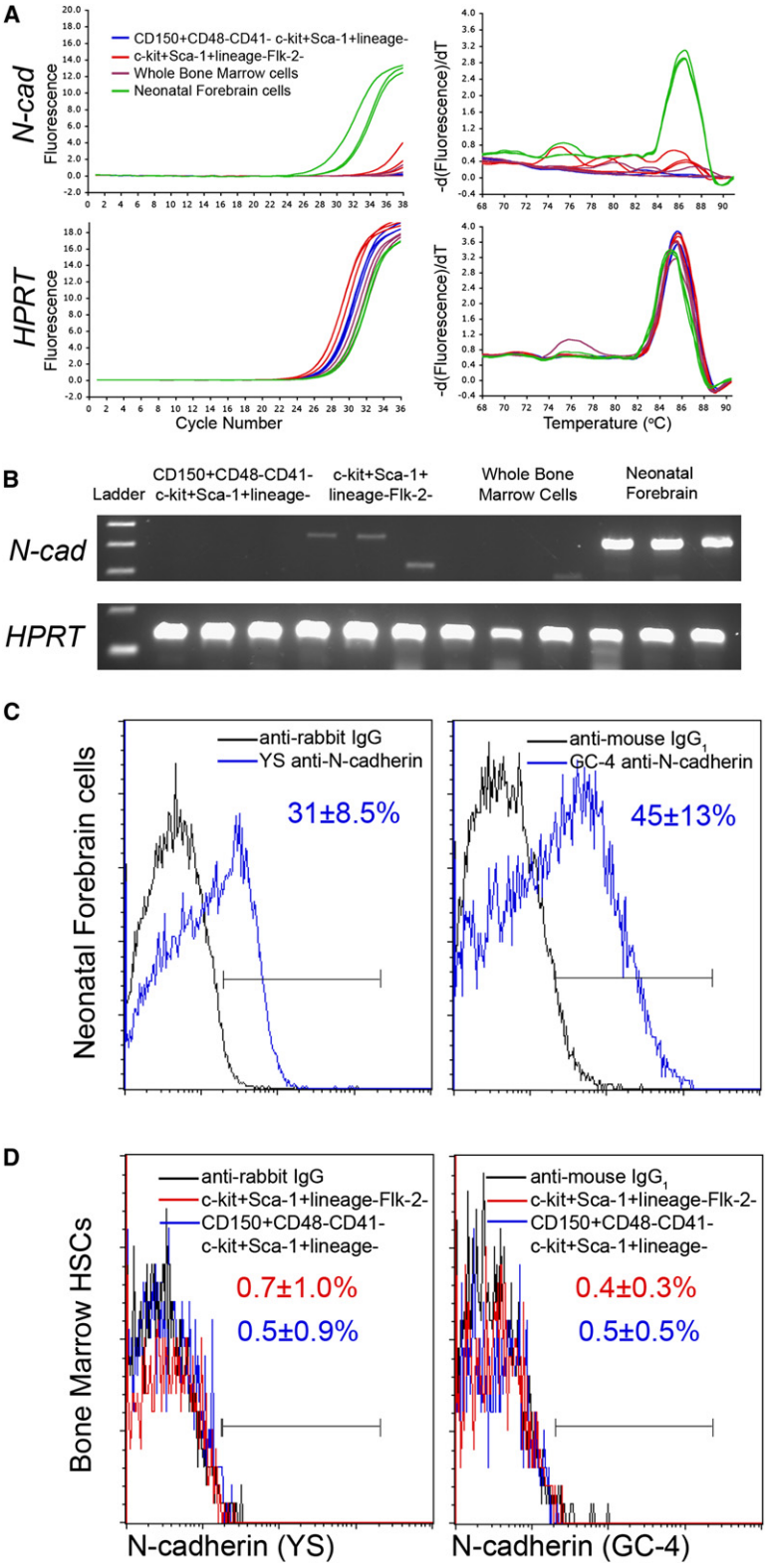


Figure 1. Failure to Detect *N-cadherin* mRNA or Protein Expression by Highly Purified HSCs

(A) *N-cadherin* could be readily amplified from three independent samples of neonatal forebrain cells, but not CD150$^+$CD48$^-$CD41$^-$c-kit$^+$Sca-1$^+$lineage$^-$ HSCs or c-kit$^+$Sca-1$^+$lineage$^-$Flk2$^-$ HSCs by quantitative PCR, despite amplifying similar amounts of *HPRT* from the same samples. Data show the amplification of transcript against cycle number (left panel) as well as the melting curve, demonstrating specific amplification of *HPRT* in all samples but *N-cadherin* only in forebrain samples. These data are representative of three independent experiments using four to five independently sorted HSC aliquots. Two hundred cell equivalents of cDNA were used per reaction.

(B) PCR products were separated on a 2% agarose gel that revealed a product of the expected size for *N-cadherin* only in forebrain samples. Sequencing revealed that this product was *N-cadherin* but that bands of other sizes in other lanes were nonspecific products.

(C) Polyclonal (YS) and monoclonal (GC-4) anti-N-cadherin antibodies were used to stain neonatal forebrain cells. Staining was examined by flow cytometry relative to control cells stained only with secondary antibodies.

(D) We could not detect N-cadherin staining above background with either the YS or GC-4 antibodies on CD150$^+$CD48$^-$CD41$^-$c-kit$^+$Sca-1$^+$lineage$^-$ HSCs or c-kit$^+$Sca-1$^+$lineage$^-$Flk2$^-$ HSCs. These data are representative of three to nine independent experiments.

in Figures 2A–2C. The vast majority of bone marrow cells were removed by simply flushing the bone marrow cavity: we did not observe residual bone marrow cells on most endosteal surfaces after flushing (Figure 2B). Nonetheless, there were some locations where residual bone marrow cells were present (Figure 2Bb). After scoring the endosteal surface with the bone burr, these residual cells were effectively removed (Figure 2C). We did not detect any

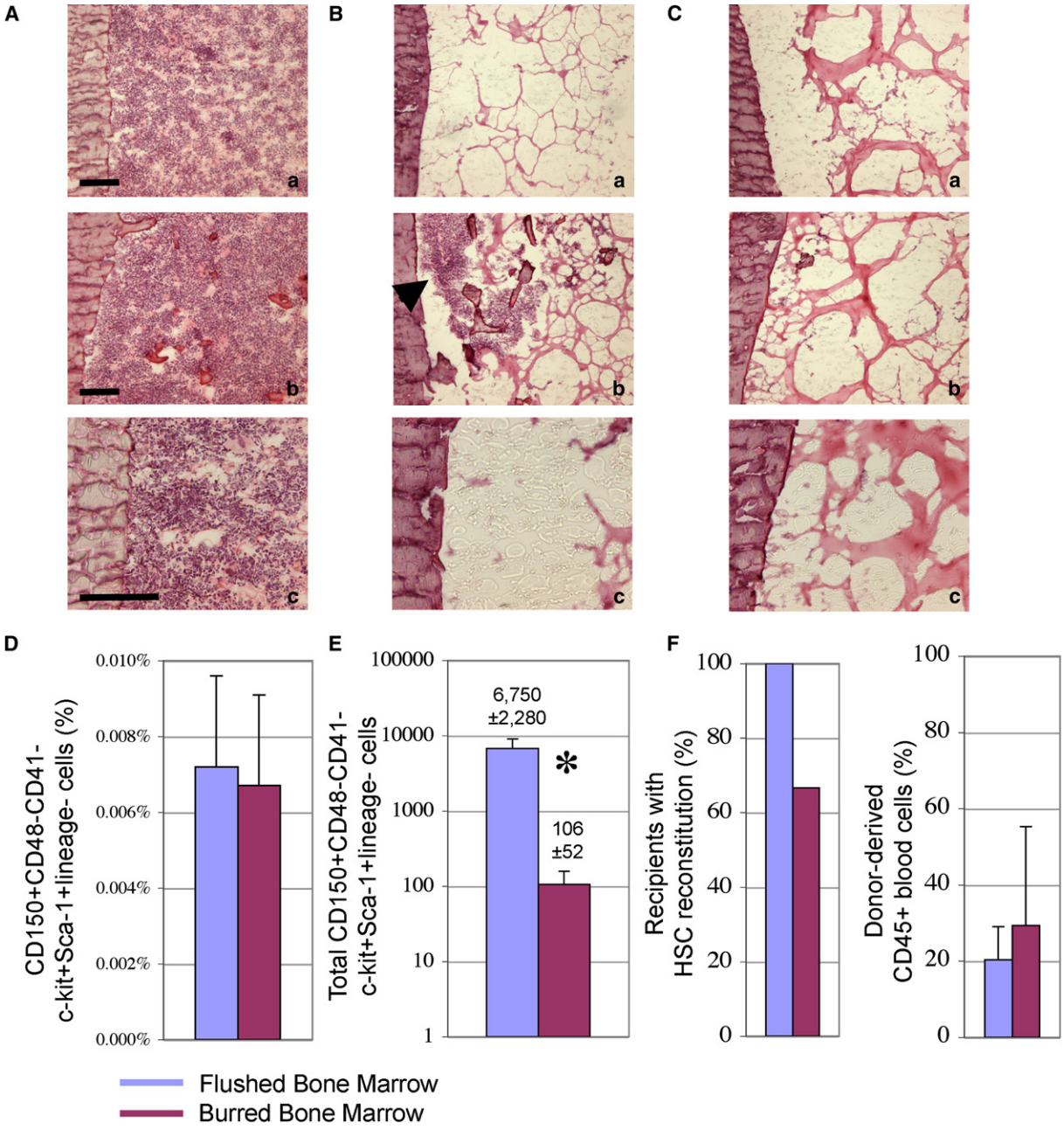


Figure 2. Flushing with Medium Washes the Vast Majority of Bone Marrow Cells and HSCs out of the Bone Marrow Cavity

(A–C) Hematoxylin and eosin stained sections through undecalcified, gelatin-embedded femurs (A) or femurs that were "flushed" with medium to remove marrow cells (B) or subsequently "burred" (drilling through the marrow cavity) to remove residual cells (C). Images show diaphysis sections (a) and epiphysis (b) sections as well as higher magnification images (c) (scale bar is always 150 μm). Only 1.6% of bone marrow cells was retained within the marrow cavity after flushing (arrowhead), and these residual cells were recovered by burring.

(D) The frequency of $CD150^{+}CD48^{-}CD41^{-}c\text{-}kit^{+}Sca\text{-}1^{+}lineage^{-}$ HSCs did not differ among bone marrow cells recovered by flushing versus burring ($p = 0.83$, $n = 3$).

(E) Taken together, this means that 98.5% of HSCs were recovered by flushing the bone marrow cavity.

(F) When 300,000 cells from either the flushed or burred fractions of bone marrow were injected into irradiated mice in competitive reconstitution assays, seven of seven recipients of flushed marrow cells and four of six recipients of burred marrow cells became long-term multilineage reconstituted by donor cells. There was no statistically significant difference in overall levels of donor cell reconstitution in these mice. Error bars represent SD.

difference in overall viability or in the frequency of $CD150^{+}CD48^{-}CD41^{-}c\text{-}kit^{+}Sca\text{-}1^{+}lineage^{-}$ HSCs in the burred fraction of bone marrow cells as compared to the flushed fraction of cells (Figure 2D). Moreover, the total number of bone marrow cells isolated in the burred fraction was only 1.6% of the bone marrow cells recovered

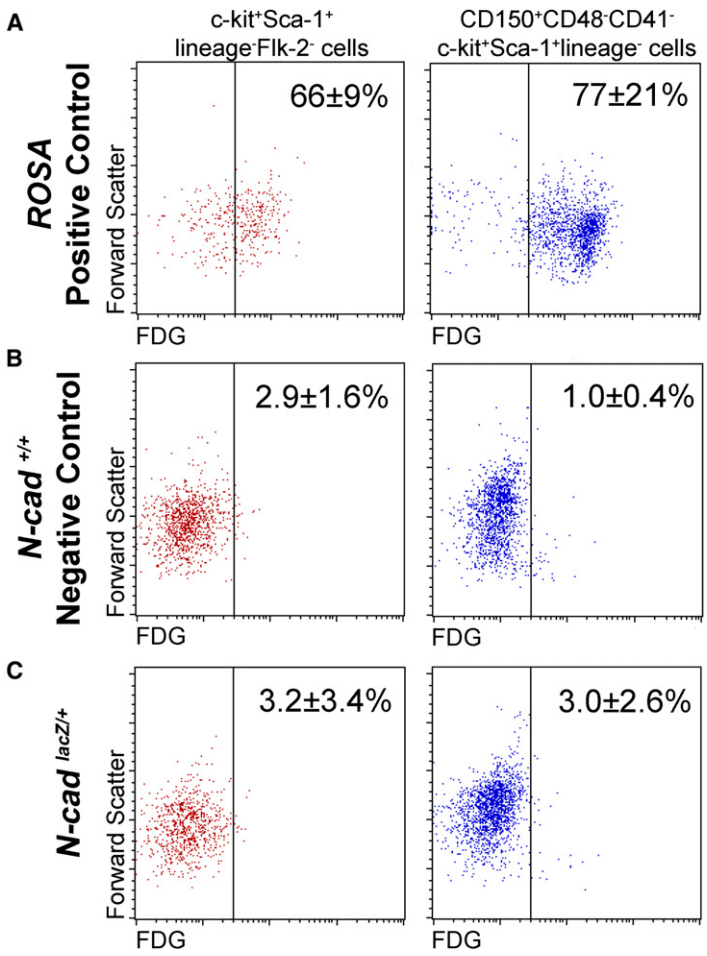


Figure 3. N-Cadherin Is Not Detectably Expressed by HSCs Isolated from *N-cadherin*$^{lacZ/+}$ Gene Trap Mice

c-kit$^+$Sca-1$^+$lineage$^-$Flk-2$^-$ HSCs or CD150$^+$CD48$^-$CD41$^-$c-kit$^+$Sca-1$^+$lineage$^-$ HSCs were isolated from Rosa mice (A), *N-cadherin*$^{lacZ/+}$ gene trap mice (C), or *N-cadherin*$^{+/+}$ (B) littermate controls and stained with FDG to measure β-galactosidase activity by flow cytometry. While most HSCs from Rosa mice exhibited FDG staining (A), no FDG staining was observed in HSCs from *N-cadherin*$^{lacZ/+}$ gene trap mice (C) above the background observed in negative control littermates (B) Flow cytometry plots depict typical results from a single experiment, while mean ± SD is presented for results from three independent experiments.

by flushing. Combining these statistics means that 98.5% of all HSCs in the bone marrow were removed by flushing: 6750 ± 2280 HSCs were isolated from the long bones (two femurs and two tibias) by flushing, and only an additional 106 ± 52 were recovered using the bone burr. This suggests that significant numbers of HSCs are not left behind when the marrow is carefully flushed out of the long bones.

To test whether the HSCs that were recovered using the bone burr were functionally distinct from the HSCs isolated by flushing, we performed competitive reconstitution assays on 300,000 flushed or burred bone marrow cells. Four of six recipients of burred bone marrow cells and seven of seven recipients of flushed bone marrow cells became long-term multilineage reconstituted by donor cells (Figure 2F). There was no statistically significant difference between the levels of donor cells that arose from flushed versus burred bone marrow cells (Figure 2F).

No Evidence for N-Cadherin Expression by HSCs in Gene Trap Mice

We sought to confirm the lack of N-cadherin expression by HSCs using an independent approach. *N-cadherin*^{lacZ} gene trap mice (Omnibank Sequence Tag OST 49160, generated by Lexicon Genetics) have been generated by retroviral insertion of lacZ into the first intron of *N-cadherin*, along with a strong splice acceptor sequence (Luo et al., 2005). lacZ is expressed in these mice under the control of the *N-cadherin* native promoter and other regulatory elements, allowing *N-cadherin* expression to be visualized based on β-galactosidase activity. β-galactosidase expression in these mice matches the known pattern of N-cadherin expression in the nervous system, heart, somites, and limbs (Luo et al., 2005). Myocardium sections from *N-cadherin*^{lacZ} gene trap mice, but not littermate controls, showed clear β-galactosidase activity throughout the myocardium (see Figure S1 in the Supplemental Data available with this article online), as reported previously (Luo et al., 2005). c-kit$^+$Sca-1$^+$lineage$^-$Flk-2$^-$ HSCs and CD150$^+$CD48$^-$CD41$^-$c-kit$^+$Sca-1$^+$lineage$^-$ HSCs from Rosa mice (which ubiquitously express β-galactosidase) showed β-galactosidase activity (Figure 3A), but not HSCs from *N-cadherin*^{lacZ} gene trap mice (Figure 3C) or littermate controls (Figure 3B). Thus, we were unable to detect N-cadherin expression by HSCs in *N-cadherin*^{lacZ} gene trap mice.

In case N-cadherin expression is induced by activation, we also examined N-cadherin expression by HSCs in culture and after mobilization by cyclophosphamide/G-CSF.

We cultured CD150$^+$CD48$^-$CD41$^-$c-kit$^+$Sca-1$^+$lineage$^-$ HSCs from Rosa mice and from *N-cadherin^{lacZ}* gene trap mice in liquid medium containing stem cell factor (SCF), thrombopoietin, insulin-like growth factor II, and acidic fibroblast growth factor (conditions that have been found to promote the maintenance of HSCs in culture) (Zhang and Lodish, 2005) for 4 days and then stained with X-Gal. Cells cultured from Rosa mice exhibited clear β-galactosidase activity, but not HSCs from *N-cadherin^{lacZ}* gene trap mice or littermate controls (Figure S2A). We were similarly unable to detect β-galactosidase expression by CD150$^+$CD48$^-$CD41$^-$c-kit$^+$Sca-1$^+$lineage$^-$ HSCs from the bone marrow or spleen of *N-cadherin^{lacZ}* gene trap mice or littermate controls that had been treated with cyclophosphamide/G-CSF, but we readily detected β-galactosidase activity in HSCs from Rosa mice (Figure S2B). Finally, we also failed to detect N-cadherin staining on CD150$^+$CD48$^-$CD41$^-$c-kit$^+$Sca-1$^+$lineage$^-$ HSCs from the spleen of cyclophosphamide/G-CSF-mobilized mice using either the YS or GC-4 antibodies. Thus, we were unable to detect N-cadherin expression by cultured HSCs or by mobilized HSCs.

N-Cadherin-Expressing Bone Marrow Cells Do Not Have HSC Activity

Using the YS anti-N-cadherin antibody and the GC-4 anti-N-cadherin antibody, we sorted bone marrow cells into N-cadherin$^+$ and N-cadherin$^-$ fractions (Figure 4A), then injected each fraction into irradiated mice in competitive reconstitution assays. The doses of cells were initially selected based on the fractions of N-cadherin$^+$ and N-cadherin$^-$ bone marrow cells that were contained in 200,000 whole bone marrow cells, as has been done in prior studies of marker expression by HSCs (Kiel et al., 2005). For example, in one experiment, N-cadherin$^+$ bone marrow cells that stained positively using the YS antibody represented 2% of bone marrow cells. As a result, we injected 4000 N-cadherin$^+$ bone marrow cells or 196,000 N-cadherin$^-$ bone marrow cells into irradiated mice. Most (13/17) of the mice injected with N-cadherin$^-$ bone marrow cells became long-term multilineage reconstituted by donor cells, whereas none (0/17) of the mice injected with N-cadherin$^+$ cells became long-term multilineage reconstituted by donor cells (Figures 4A–4C). We were thus unable to detect HSC activity among N-cadherin$^+$ cells.

While this suggested that most HSCs were N-cadherin$^-$, it left open the possibility that a minority of HSCs were N-cadherin$^+$ and that we did not inject enough cells to detect this subset of HSCs. To address this, we performed an independent experiment using the GC-4 antibody in which we injected the number of cells that would be present in 300,000 bone marrow cells. In this experiment, around 5% of bone marrow cells stained with GC-4, so we injected 15,000 N-cadherin$^+$ bone marrow cells or 285,000 N-cadherin$^-$ bone marrow cells into irradiated mice. To be certain that we did not miss any activity in the N-cadherin$^+$ fraction, we included a second group of mice that was injected with 45,000 N-cadherin$^+$ bone marrow cells. Mice injected with N-cadherin$^-$ bone marrow cells always (12/12) became long-term multilineage reconstituted by donor cells, whereas none (0/13) of the mice injected with either dose of N-cadherin$^+$ bone marrow cells became long-term multilineage reconstituted by donor cells (Figures 4A–4C). We thus failed to detect any HSC activity among N-cadherin$^+$ bone marrow cells.

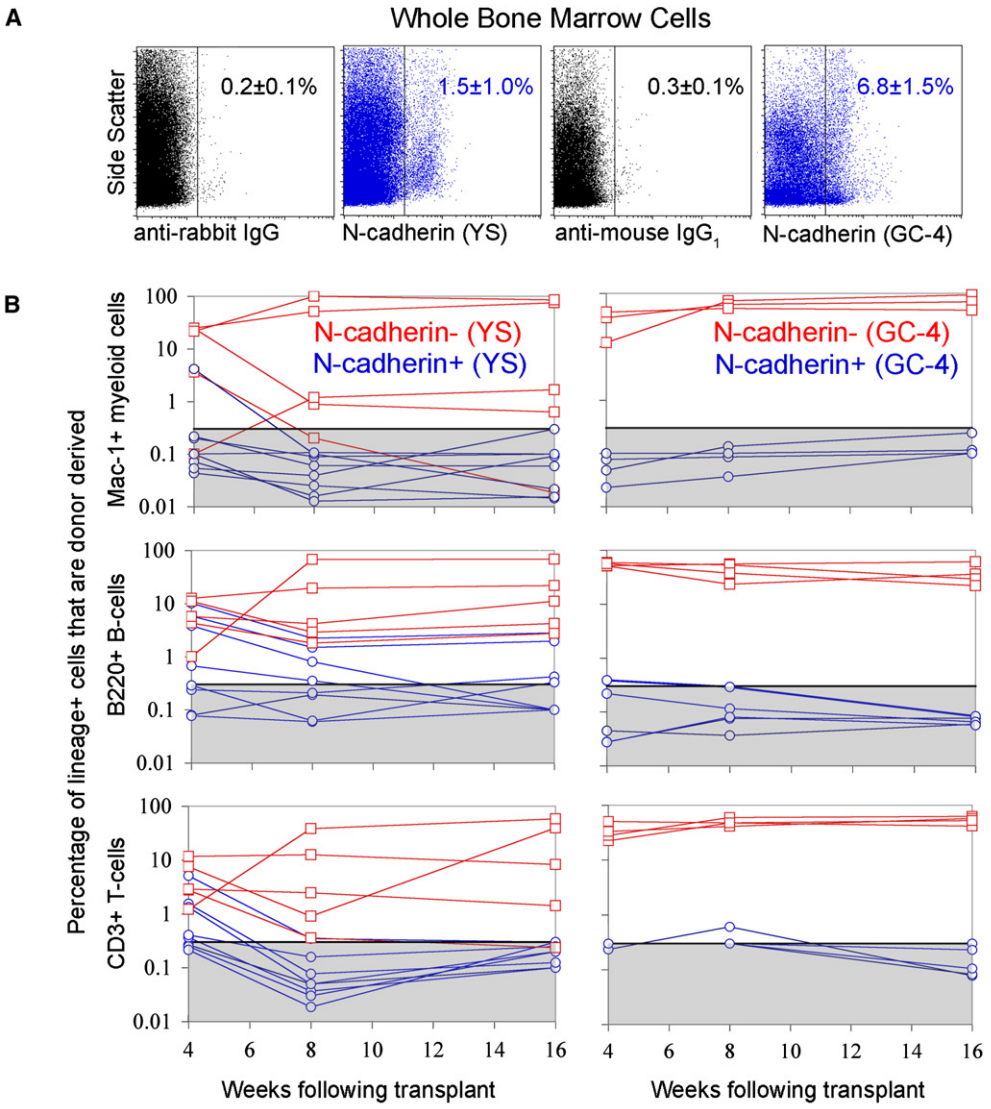
N-Cadherin-Expressing Bone Marrow Cells Have Little Progenitor Activity in Culture

To test whether N-cadherin$^+$ bone marrow cells have any progenitor activity, we plated N-cadherin$^+$ and N-cadherin$^-$ bone marrow cells in cytokine-supplemented methylcellulose medium and assayed the colony-forming ability of each fraction (Table 1). When highly purified HSCs (such as CD150$^+$CD48$^-$CD41$^-$c-kit$^+$Sca-1$^+$lineage$^-$ cells) are sorted into methylcellulose culture, more than 90% of the single cells form colonies, and the vast majority of the colonies are either CFU-GEMM (that contain granulocytes, erythrocytes, macrophages, and megakaryocytes) or CFU-GM (that contain granulocytes and macrophages) (Yilmaz et al., 2006b). Whether the YS or the GC-4 anti-N-cadherin antibody was used to fractionate bone marrow cells, virtually all colony-forming activity was in the N-cadherin$^-$ fraction of bone marrow cells, including all CFU-GEMM and more than 99% of all CFU-GM (Table 1). We also sorted N-cadherin$^+$ and N-cadherin$^-$ bone marrow cells into methylcellulose based on FDG staining of cells from *N-cadherin^{lacZ}* gene trap mice. Again, virtually all colony-forming activity was in the N-cadherin$^-$ fraction of bone marrow cells (Table 1). N-cadherin$^+$ bone marrow cells rarely formed colonies in culture, particularly the types of colonies that are formed by HSCs.

Osteoblast Depletion Does Not Necessarily Lead to HSC Depletion

The foregoing data suggest that N-cadherin is unlikely to act within HSCs to regulate their localization or function. However, this does not address the role of osteoblasts themselves in regulating bone marrow HSCs, as they could still influence HSCs by other mechanisms. To better understand the role that osteoblasts play in maintaining HSCs, we tested whether there was a quantitative relationship between osteoblast frequency and HSC frequency in the bone marrow. We examined *biglycan*-deficient mice to test whether depletion of osteoblasts and osteoblast progenitors would reduce the number of HSCs, as would be expected if osteoblasts are quantitatively required for HSC maintenance.

Biglycan is an extracellular matrix proteoglycan that is most prominently expressed by osteoblasts and chondrocytes (Xu et al., 1998) (Figure S3A). *biglycan*-deficient mice develop an osteoporosis-like phenotype, with less trabecular bone, fewer osteoblasts, and fewer osteoblast progenitors (Xu et al., 1998; Chen et al., 2002) (Figures S3B–S3D). This is evident in 3-month-old mice but worsens with age. We detected no statistically significant differences among *biglycan*-deficient mice and littermate



C

Antibody	Bone Marrow cells used for Transplantation	Mice that Engrafted	Mice with Long-Term Multilineage Reconstitution	Mice with Short-Term Multilineage Reconstitution
YS	196,000 N-cadherin-negative	17 of 17	13 of 17	4 of 17
YS	4,000 N-cadherin-positive	4 of 17	0 of 17	1 of 17
GC-4	285,000 N-cadherin-negative	12 of 12	12 of 12	0 of 12
GC-4	15,000 N-cadherin-positive	2 of 9	0 of 9	1 of 9
GC-4	45,000 N-cadherin-positive	1 of 4	0 of 4	1 of 4

Figure 4. All Detectable HSC Activity Is Contained within the N-Cadherin$^-$ Fraction of Bone Marrow Cells

(A) Of bone marrow cells, 1.5%–6.8% stained positively for N-cadherin, depending on the antibody used (mean ± SD from three to eight independent experiments). (B) Bone marrow cells were fractionated into N-cadherin$^-$ and N-cadherin$^+$ cells using either YS or GC-4 antibodies and injected into lethally irradiated mice. Cell doses were based on the fraction of N-cadherin$^-$ versus N-cadherin$^+$ cells that were contained within 200,000 (YS) or 300,000 (GC-4) bone marrow cells as indicated in the table (C). To ensure that we did not miss infrequent N-cadherin$^+$ HSCs, we also tripled the

Table 1. The N-Cadherin$^+$ Fraction of Bone Marrow Cells Has Little Capacity to Form Colonies in Culture and Lacks the Ability to Form Primitive CFU-GEMM Colonies

	Cells Added to Culture	Percentage of Cells that Formed Colonies	Percentage of All Colonies that Derived from This Fraction	Percentage of All CFU-GEMM that Were in This Fraction	Percentage of All CFU-GM that Were in This Fraction
YS antibody	N-cadherin$^+$	0.01% ± 0.02%	0.03% ± 0.08%	0%	0.05% ± 0.11%
	N-cadherin$^-$	0.41% ± 0.08%	99.97% ± 0.08%	100%	99.95% ± 0.11%
GC-4 antibody	N-cadherin$^+$	0.2% ± 0.2%	0.7% ± 0.4%	0%	0.8% ± 0.6%
	N-cadherin$^-$	0.5% ± 0.03%	99.3% ± 0.4%	100%	99.2% ± 0.6%
N-cadherin$^{+/+}$	FDG$^+$	0.53% ± 0.19%	1.4% ± 1.7%	0%	1.3% ± 1.5%
	FDG$^-$	0.51% ± 0.02%	98.6% ± 1.7%	100%	98.7% ± 1.5%
N-cadherin^{lacZ}	FDG$^+$	0.56% ± 0.31%	2.4% ± 0.89%	0%	1.9% ± 0.7%
	FDG$^-$	0.48% ± 0.04%	97.6% ± 0.89%	100%	98.1% ± 0.7%

N-cadherin$^+$ or N-cadherin$^-$ bone marrow cells were sorted based on staining with the YS polyclonal antibody or the GC-4 monoclonal antibody, or based on FDG staining for β-galactosidase activity in *N-cadherin*$^{+/+}$ wild-type (control) mice or *N-cadherin*^{lacZ} gene trap mice. Cells were plated in methylcellulose medium supplemented with SCF, IL-3, IL-6, and erythropoietin. After 10–14 days, the number and type of colonies was counted. The data represent four to six independent experiments in which an average of 16,000–17,500 N-cadherin$^-$ bone marrow cells or 3000 to 5400 N-cadherin$^+$ bone marrow cells were plated per experiment (based on antibody staining) and two independent experiments in which 18,000–27,000 FDG$^-$ bone marrow cells or 900 FDG$^+$ bone marrow cells were plated per experiment. There was no statistically significant difference in the colonies formed by the FDG$^+$ fraction of cells from control wild-type mice as compared to *N-cadherin*^{lacZ} gene trap mice, suggesting that the FDG staining in rare colony-forming progenitors may be attributable to background. The three columns on the right side of the table show the percentage of all CFU-C, CFU-GEMM, and CFU-GM from bone marrow that fall into the N-cadherin$^+$ or N-cadherin$^-$ fractions of cells.

controls of any age in the frequency of myeloid, erythroid, B, or T lineage cells in the bone marrow (Figure S4A) or spleen (Figure S4B). We also did not detect any differences in bone marrow cellularity (Figure 5A) or the number (Figure 5B) or type (Figure 5C) of colony-forming progenitors in the bone marrow. The depletion of osteoblasts in *biglycan*-deficient mice did not lead to discernable hematopoietic defects.

We also directly assayed HSC frequency and function in these mice. We did not detect any differences among *biglycan*-deficient mice and littermate controls at 3, 9–15, or 24 months of age in the frequency (Figure 5D) or absolute number (Figure 5E) of CD150$^+$CD48$^-$CD41$^-$c-kit$^+$Sca-1$^+$ HSCs in the bone marrow. To test HSC function, we transplanted 300,000 whole bone marrow cells from 3-month-old *biglycan*-deficient mice or littermate controls into 3- to 6-month-old *biglycan*-deficient or control recipients, along with 300,000 wild-type recipient bone marrow cells for radioprotection. Donor mice in these experiments were CD45.2$^+$, while recipient mice were CD45.1$^+$, irrespective of genotype. The *biglycan*-deficient bone marrow cells gave similar levels of long-term multilineage reconstitution as wild-type donor cells, whether they were transplanted into wild-type or *biglycan*-deficient recipients (Figure 5F). These data indicate that *biglycan* deficiency had no effect on the level of reconstituting activity in competitive reconstitution assays, irrespective of whether the donors, the recipients, or both were *biglycan* deficient. Thus, we detected no effect of *biglycan* deficiency on HSC frequency or function.

HSCs in Wild-Type and *biglycan*-Deficient Bone Marrow Localize Primarily to Sinusoids

Using methods described previously (Kiel et al., 2005), we cut sections through the bone marrow of wild-type and *biglycan*-deficient mice and stained with SLAM family markers to localize HSCs. We observed no statistically significant differences among wild-type and *biglycan*-deficient mice in terms of the proportions of all bone marrow cells that were adjacent to or within five cell diameters of sinusoids or endosteum (Figure 6B). While not statistically significant, *biglycan*-deficient mice tended to have a lower proportion of bone marrow cells adjacent to or within five cell diameters of endosteum (Figure 6B), consistent with the reduced trabecular bone in these mice (Figures S3B and S3C).

dose of N-cadherin$^+$ bone marrow cells that was transplanted in one experiment (C). Recipient mice were monitored for engraftment by donor myeloid, B, and T cells for 16 weeks after transplantation. Recipients of N-cadherin$^-$ bone marrow cells almost always (YS) or always (GC-4) exhibited high levels of long-term multilineage reconstitution by donor cells, while recipients of N-cadherin$^+$ donor cells never exhibited long-term multilineage reconstitution by donor cells. In (B), each line represents a single mouse, and the black line at 0.3% represents the level of background below which donor cell engraftment could not be detected (shaded area). Data are from three to four independent experiments.

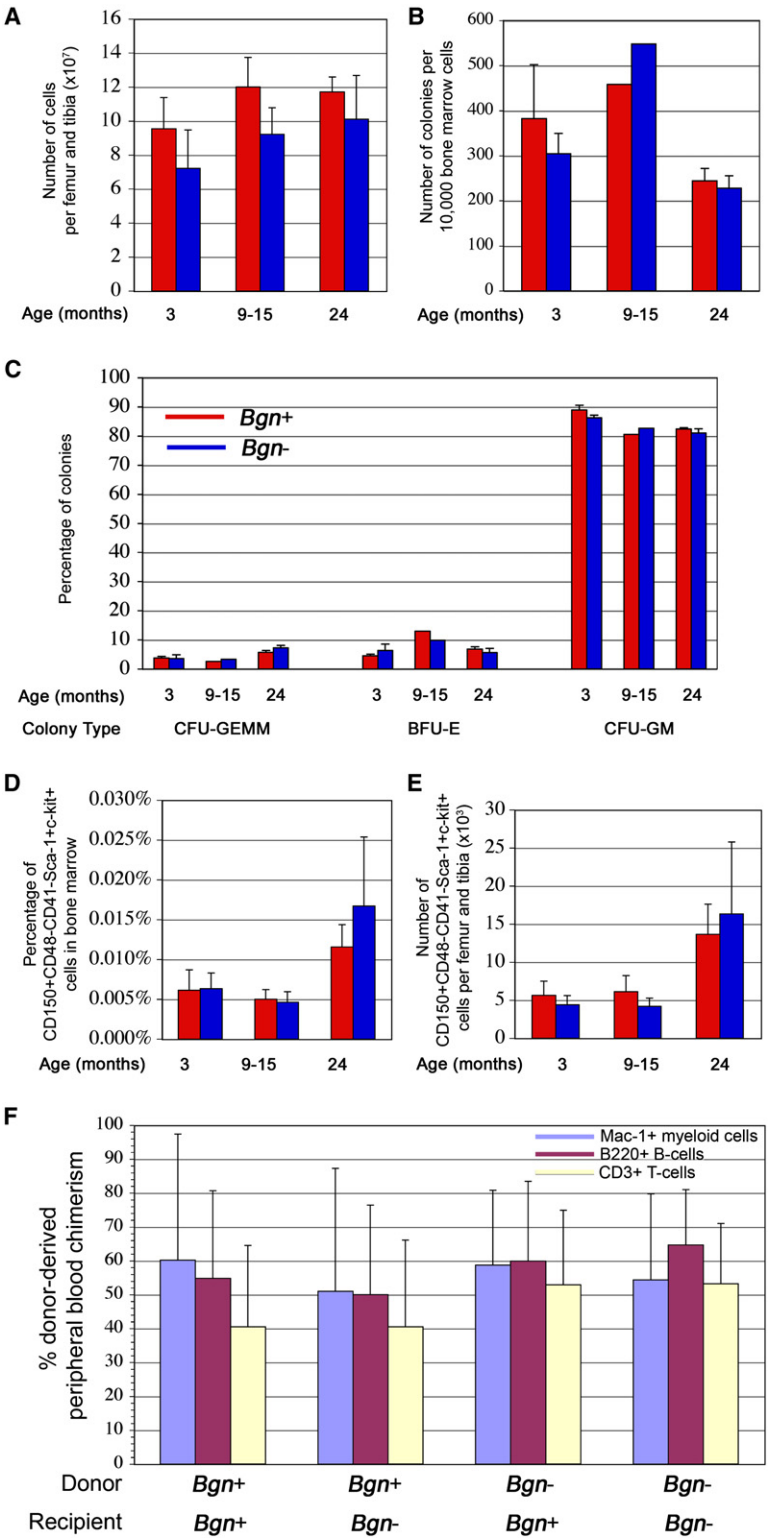
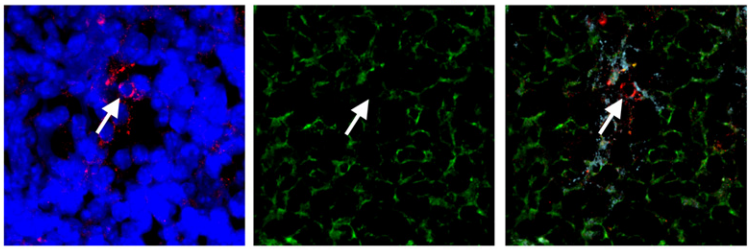


Figure 5. Normal Hematopoiesis and HSC Function despite Osteoblast Depletion in the Absence of *biglycan*

(A) Slightly fewer bone marrow cells were recovered per femur and tibia of *biglycan*-deficient (blue bars) mice as compared to littermate controls (red bars) at 3, 9–15, or 24 months of age, though in no case was the difference statistically significant ($p = 0.09$–0.31; data from five independent experiments with two to five mice per genotype per age). Numbers (B) and types (C) of colonies that formed in culture from *biglycan*-deficient (blue bars) mice and littermate controls (red bars) were similar at all ages. Frequency (D) and absolute number (E) of $CD150^+CD48^-CD41^-c\text{-}kit^+Sca\text{-}1^+$ HSCs in bone marrow from control littermates (Bgn^+, red bars) and *biglycan*-deficient (Bgn^-, blue bars) mice at 3, 9–15, or 24 months of age ($p = 0.46$–0.89). Data represent mean ± SD from two to five animals of each genotype in one to three independent experiments per age group. (F) HSC engraftment and reconstitution in competitive repopulation assays was not affected by *biglycan* deficiency, irrespective of whether donors, recipients, or both were *biglycan* deficient. Bone marrow cells (300,000) from Bgn^+ or Bgn^- donors (along with 300,000 recipient bone marrow cells) were transplanted into Bgn^+ or Bgn^- recipients. No statistically significant differences were detected in the level of donor chimerism in any lineage in any treatment. The data represent the results obtained 16 weeks after transplantation from one of two independent experiments. A total of 12–17 recipients were analyzed for each treatment with two to three independent donors per genotype. All error bars represent SD.

Most bone marrow HSCs appeared to localize to sinusoids. $CD150^+CD48^-CD41^-lineage^-$ cells are very highly enriched for HSCs (Kiel et al., 2005), and represented 0.0042%–0.0049% of cells in bone marrow sections in these experiments (Figure 6C). In both wild-type and *biglycan*-deficient mice, 58% of $CD150^+CD48^-CD41^-lineage^-$ cells that we identified in sections were adjacent to sinusoids, and almost all of these cells (92%–95%) were

A



B

All bone marrow cells	*Bgn+*	*Bgn-*
Cells adjacent to sinusoidal endothelium	11±4%	13±5%
Cells within 5 cell diameters of sinusoidal endothelium	52±17%	52±16%
Cells adjacent to endosteum	1.2±0.5%	0.9±0.3%
Cells within 5 cell diameters of endosteum	5.9±2.3%	3.8±1.6%

C

CD150+CD48-CD41-lineage- (SLAM) cells	*Bgn+*	*Bgn-*
Images examined	176	174
SLAM cells identified/nucleated cells analyzed	24/488,400 (0.0049%)	19/451,500 (0.0042%)
SLAM cells adjacent to sinusoidal endothelium	14/24 (58%)	11/19 (58%)
SLAM cells within 5 cell diameters of sinusoidal endothelium	22/24 (92%)	18/19 (95%)
SLAM cells adjacent to endosteum	0/24 (0%)	4/19 (21%)
SLAM cells within 5 cell diameters of endosteum	2/24 (8%)	4/19 (21%)

Figure 6. $CD150^+CD48^-CD41^-lineage^-$ HSCs Mainly Localize to Sinusoids in Wild-Type and *biglycan*-Deficient Mice

(A) Sections through the bone marrow of 9- to 15-month-old wild-type and *biglycan*-deficient mice were analyzed by immunofluorescence for the localization of $CD150^+CD48^-CD41^-$ $lineage^-$ HSCs. Slides were also stained with an antibody against the panendothelial antigen MECA-32 and the nuclear dye DAPI. The localization of all bone marrow cells (B) and $CD150^+CD48^-CD41^-lineage^-$ HSCs (C) was assessed for proximity to MECA-32^+ sinusoidal endothelium or to endosteum. Arrow points to a $CD150^+CD48^-CD41^-lineage^-$ cell adjacent to a MECA-32^+ sinusoid (A). Data were obtained from two to three mice per genotype and represent mean ± SD.

within five cell diameters of a sinusoid. This suggests that HSCs are approximately 5-fold more likely than other bone marrow cells to be adjacent to sinusoids, consistent with our earlier observations (Kiel et al., 2005). Moreover, the fact that nearly all HSCs are close to sinusoids suggests that nearly all HSCs are likely to be influenced by factors secreted by perivascular cells (Sugiyama et al., 2006).

A smaller and potentially more variable proportion of HSCs localized to the endosteum. In wild-type mice, we did not detect any $CD150^+CD48^-CD41^-lineage^-$ cells at the endosteum, while in *biglycan*-deficient mice, 4 of 19 (21%) $CD150^+CD48^-CD41^-lineage^-$ cells localized to the endosteum. In our prior study (Kiel et al., 2005), we observed 14% of wild-type $CD150^+CD48^-CD41^-lineage^-$ cells localized to the endosteum. Thus, these results are consistent with our earlier study in suggesting that a minority of bone marrow HSCs are present at the endosteum at any point in time. We also did not detect many HSCs that were within five cell diameters of the endosteum: only 8%–21% of HSCs were visibly within five cell diameters of the endosteum in these experiments. Nonetheless, this suggests that HSCs are more likely than other bone marrow cells to be close to the endosteum, even in mice in which osteoblasts and trabecular bone have been depleted. These results are consistent with the possibility that the endosteum may represent a niche for a subset of HSCs despite the depletion of osteoblasts; however, most HSCs either do not require contact with cells at the endosteum or require only intermittent contact.

DISCUSSION

A recent model of the HSC niche holds that HSCs adhere to the surface of osteoblasts by N-cadherin-mediated homophilic adhesion and that HSCs acutely depend upon this interaction for their maintenance. But while this model has received considerable attention, no study has yet shown that N-cadherin-expressing bone marrow cells have HSC activity or that N-cadherin is required by HSCs for their maintenance.

When we examined N-cadherin expression in very highly purified HSCs, we were unable to detect N-cadherin expression by PCR (Figures 1A and 1B), by staining with the YS anti-N-cadherin antibody (Figure 1D), by staining with the GC-4 anti-N-cadherin antibody (Figure 1D), or by analyzing β-galactosidase expression in *N-cadherin*^{lacZ} gene trap mice (Figure 3C). This failure to detect N-cadherin expression did not depend on the use of SLAM family markers, as we were also unable to

detect N-cadherin expression in c-kit$^+$Sca-1$^+$lineage$^-$ Flk-2$^-$ cells. These results are consistent with earlier results from us and others that failed to detect N-cadherin expression by HSCs by microarray analysis (Ivanova et al., 2002; Kiel et al., 2005).

Consistent with this, all of the HSC activity in long-term reconstitution assays came from the N-cadherin$^-$ fraction of bone marrow cells, irrespective of whether we used the YS or the GC-4 anti-N-cadherin antibodies (Figure 4). This did not simply reflect an inability of N-cadherin$^+$ bone marrow cells to engraft in vivo, as N-cadherin$^+$ bone marrow cells were rarely able to form primitive colonies in culture (Table 1). We were thus unable to detect primitive progenitor activity from N-cadherin$^+$ bone marrow cells in vitro or in vivo. The only exception is that 3 of 30 irradiated mice that were injected with N-cadherin$^+$ bone marrow cells became transiently multilineage reconstituted by low levels of donor cells (Figures 4B and 4C). This raises the possibility that a small subset of transiently reconstituting multipotent progenitors might express N-cadherin, though it is also possible that N-cadherin$^-$ cells contaminated the N-cadherin$^+$ fraction.

Although these data indicate that HSCs are unlikely to be autonomously regulated by N-cadherin, it remains possible that osteoblasts play an important role in the maintenance of bone marrow HSCs through other mechanisms. But if HSCs do not adhere to the surface of osteoblasts using N-cadherin, how do osteoblasts influence HSC frequency?

A first step is to determine whether there is a quantitative relationship between HSCs and osteoblasts in bone marrow. Although *biglycan* deficiency leads to progressive osteoblast depletion with age (Xu et al., 1998), we did not detect any effect of *biglycan* deficiency on hematopoiesis or on HSC frequency or function irrespective of age (Figure 5). These data indicate there is not a quantitative relationship between HSCs and osteoblasts in the bone marrow: while modest increases in osteoblast numbers modestly increase HSC numbers (Calvi et al., 2003; Zhang et al., 2003), modest decreases in osteoblast numbers do not necessarily decrease HSC numbers. Osteoblasts are therefore not limiting for the creation of adult HSC niches. Nonetheless, osteoblasts were never completely eliminated from *biglycan*-deficient mice (Figure S3), so this study does not suggest that these cells are not involved in HSC maintenance.

We also confirmed our observation that approximately 60% of bone marrow CD150$^+$CD48$^-$CD41$^-$lineage$^-$ cells localize to sinusoids at any one time (Figure 6). Moreover, we extended this observation by finding that almost all bone marrow CD150$^+$CD48$^-$CD41$^-$lineage$^-$ cells localize within five cell diameters of sinusoids (Figure 6). This suggests that almost all bone marrow HSCs are present in or near perivascular environments. When combined with the observation that perivascular reticular cells are a major source of CXCL12, a factor that is required for HSC maintenance (Sugiyama et al., 2006); that endothelial cells can promote the maintenance of HSCs in culture (Ohneda et al., 1998; Li et al., 2004); and that endothelial cell function is required for adult bone marrow hematopoiesis in vivo (Yao et al., 2005), these data suggest that at least some bone marrow HSCs are maintained in perivascular niches.

A variety of other factors that regulate HSCs and hematopoiesis are also expressed around the vasculature in bone marrow (Kopp et al., 2005). It is also possible that mechanisms that have been suggested to regulate endosteal cells, such as sympathetic nervous system activity (Katayama et al., 2006), also regulate perivascular cells. Nonetheless, additional studies will be required to test whether perivascular cells act directly on HSCs to promote their maintenance. It is also important to bear in mind that some HSCs observed near sinusoids may be migrating through these environments (Wright et al., 2001) rather than being maintained there.

It was recently observed that most bone marrow HSCs reside within relatively hypoxic environments (Parmar et al., 2007). Although it has not yet been determined which regions of the bone marrow are most hypoxic, it is conceivable that sinusoids are among the most hypoxic microenvironments. Sinusoids not only carry venous circulation, but it is slow venous circulation that is not designed to transport oxygen so much as to allow cells to enter and exit circulation. In contrast, a rich supply of arterial blood flows by and through the endosteum to nourish bone. This issue will require additional studies.

While our data emphasize the potential role played by perivascular cells in the regulation of HSCs, they do not rule out a role for osteoblasts. We have observed a minority of CD150$^+$CD48$^-$CD41$^-$lineage$^-$ cells that localize to the endosteum in this (Figure 6) and prior studies (Kiel et al., 2005). However, these cells are considerably more likely than other bone marrow cells to localize to the endosteum (Figure 6B versus Figure 6C). This is consistent with the suggestion that a subset of HSCs localizes to the endosteum (Nilsson et al., 2001; Zhang et al., 2003; Arai et al., 2004; Kiel et al., 2005; Sugiyama et al., 2006; Suzuki et al., 2006) and that osteoblasts and osteoclasts may regulate the maintenance and migration of at least some HSCs (Calvi et al., 2003; Zhang et al., 2003; Arai et al., 2004; Adams et al., 2006; Kollet et al., 2006). Nonetheless, our data suggest that osteoblasts are unlikely to regulate HSCs by N-cadherin-mediated homophilic adhesion. As with perivascular cells, in order to formally test whether there is an endosteal niche for HSCs, it will be necessary to test whether endosteal cells act directly on HSCs to promote their maintenance through mechanisms that are required in vivo.

Our demonstration that N-cadherin is not likely to regulate the adhesion of HSCs to osteoblasts, and the prior demonstration that Notch1/Jagged1 interactions are not required for HSC maintenance (Mancini et al., 2005), means that all of the mechanisms by which endosteal cells have been proposed to regulate HSCs involve secreted factors. This raises a number of questions. First, do these factors act directly on HSCs or do they act indirectly through third-party cells? Endosteal cells may regulate HSC maintenance primarily by recruiting other bone

marrow cells, like vascular cells (Tombran-Tink and Barnstable, 2004), raising the possibility that osteoblasts could promote HSC maintenance by recruiting vascular niches. Second, if factors secreted from the endosteum act directly on HSCs, do they act only on the subset of HSCs that localizes to the endosteum or do they diffuse to other microenvironments? Third, are the HSCs that localize near sinusoids different from the HSCs that localize near the endosteum? So far there is no evidence for this, as nearly all $CD150^+CD48^-CD41^-lineage^-$ cells are quiescent (Kiel et al., 2005) and there is no evidence for a deeply quiescent subset of HSCs that goes months without dividing (Cheshier et al., 1999). Nonetheless, it remains possible that HSCs in different locations differ in other ways. Finally, it would be interesting to be able to test how often HSCs migrate between sinusoidal and endosteal environments.

Much remains to be learned regarding the identities of the cells that contribute to the maintenance of HSCs and the mechanisms by which they contribute. These mechanisms will likely be complex, involving multiple cell types and gene products, some of which act directly on HSCs and others that act indirectly through third-party cells. While the direct genetic experiments are conducted to elucidate these mechanisms, it will be important for working models of HSC niches to acknowledge this potential complexity.

EXPERIMENTAL PROCEDURES

Mice

C57BL/Ka-Thy-1.1 (Ly5.2) and C57BL/Ka-Thy-1.2 (Ly5.1) mice were housed in the Unit for Laboratory Animal Medicine at the University of Michigan. Adult mice were sacrificed between 8 and 10 weeks of age unless otherwise stated. Neonatal mice were sacrificed 1–4 days after birth. Experiments to characterize hematopoiesis in *biglycan*-deficient mice (an X-linked gene) were performed only on males.

Flow Cytometry and the Isolation of HSCs

Bone marrow cells were flushed from the long bones (tibias and femurs) with Hank's buffered salt solution without calcium or magnesium, supplemented with 2% heat-inactivated calf serum (GIBCO, Grand Island, NY; HBSS+). Cells were triturated and filtered through nylon screen (45 μm, Sefar America, Kansas City, MO) to obtain a single-cell suspension. For isolation of $c\text{-}kit^+Sca\text{-}1^+lineage^-$ cells, whole bone marrow cells were incubated with PE-conjugated monoclonal antibodies to lineage markers including B220 (6B2), CD3 (KT31.1), CD4 (GK1.5), CD8 (53-6.7), Gr-1 (8C5), Mac-1 (M1/70), Ter119, and IgM in addition to FITC-conjugated anti-Sca-1 (Ly6A/E; E13-6.7) and biotin-conjugated anti-c-kit (2B8). c-kit staining was visualized using streptavidin APC-Cy7. Where indicated, an antibody against Flk-2 (A2F10.1) was used to isolate $Flk\text{-}2^-$ progenitors. For isolation of $CD150^+CD48^-CD41^-c\text{-}kit^+Sca\text{-}1^+lineage^-$ HSCs, bone marrow cells were incubated with PE-conjugated anti-CD150 (TC15-12F12.2; BioLegend, San Diego, CA), FITC-conjugated anti-CD48 (HM48-1; BioLegend), FITC-conjugated anti-CD41 (MWReg30; BD PharMingen, San Diego, CA), biotin-conjugated anti-Sca-1 (E13-6.7), and PE-Cy5-conjugated anti-c-kit (2B8) antibody, in addition to antibodies against the following FITC-conjugated lineage markers: Ter119, B220 (6B2), Gr-1 (8C5), and CD2 (RM2-5). HSCs were sometimes pre-enriched by selecting $Sca\text{-}1^+$ or $c\text{-}kit^+$ cells using paramagnetic microbeads and autoMACS (Miltenyi Biotec, Auburn, CA). Nonviable cells were excluded from sorts and analyses using the viability dye DAPI (1 μg/ml).

For isolation of bone marrow cells using the bone burr, bones were flushed with HBSS+; then the lumen of bones was briefly drilled with a high-speed microdrill using a 0.5 mm diameter steel bone burr (Fine Science Tools, Foster City, CA). The bones were then eluted a second time with HBSS+, triturated, and passed through a 27G needle several times prior to filtering through nylon mesh as above.

Methods for staining bone marrow sections for the localization of HSCs are as described previously (Kiel et al., 2005). See the Supplemental Data for details. Methods for FDG staining of bone marrow cells, quantitative PCR, and details regarding long-term competitive reconstitution assays of irradiated mice are also present in the Supplemental Data.

N-Cadherin and Biglycan Antibody Staining

Cells were stained with antibodies against the extracellular domain of N-cadherin (YS, IBL, Japan; GC-4, SIGMA, St. Louis, MO) at 1:50–1:100 for 25 min on ice. Cells were then stained with APC or Cy5-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA). Biglycan staining in sections was performed using a polyclonal antiserum provided by Dr. Larry Fisher (Bianco et al., 1990).

Methylcellulose Cultures

Sorted bone marrow cells were plated in wells of 96-well plates (Corning, Corning, NY) containing 100 μl 1.0% methylcellulose (Stem Cell Technologies, Vancouver, BC) as previously described. The methylcellulose was supplemented with 1% penicillin/streptomycin (GIBCO), 50 ng/ml SCF, 10 ng/ml interleukin-3 (IL-3), 10 ng/ml interleukin-6 (IL-6), and 3 U/ml erythropoietin (Epo). Colonies were maintained at 37°C in humidified incubators at 6% O_2. Colony formation was scored after 10–14 days of culture.

Supplemental Data

Supplemental Data include four figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/1/2/204/DC1/.

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