IDENTIFICATION, LOCALIZATION AND CHARACTERIZATION OF HEMATOPOIETIC STEM CELLS AND THEIR NICHE

by

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I wish to thank Toshihide Iwashita for contributing significantly to the completion of the microarray studies and the in vivo localization of hematopoietic stem cells as described in Chapter 2 of this dissertation. His presence in this and in other projects carried out in the lab was characterized by both excellence and selflessness.

I wish to thank Omer Yilmaz with whom I worked closely during my medical school coursework. He and I together discovered the differential expression of SLAM family members on hematopoietic progenitors and conducted much of the experiments in Chapter 2 in tandem.

I wish to thank Shenghui He for his support and friendship during the last several years of my coursework. His intelligent and enthusiastic input was essential to the promulgation and accurate interpretation of most experiments described in Chapter 4 of this dissertation.

PREFACE

The work described in this dissertation comprises much of the work that I conducted in the Morrison lab beginning in September, 2000.

Part of the contents of Chapters 1 (Introduction) and 5 (Conclusion) were originally published in *Nature Reviews Immunology 8: 290-301*.

Chapter 2 describes the identification of SLAM marker differential expression and localization of hematopoietic stem cells in tissue sections originally published in *Cell 2005 121:1109-21*. I initiated this work by sorting out purified populations of hematopoietic stem cells (HSCs) from fetal, young adult and old adult mouse hematopoietic tissue for gene expression profiling by microarray. Toshihide Iwashita conducted meticulous control experiments for amplifying gene product from small sample sizes and performed the molecular experiments required for preparation of the samples for microarray hybridization. I confirmed the differential expression of a list of candidate genes by quantitative PCR and identified the SLAM-family member CD150 as being differentially expressed on HSCs. Omer Yilmaz used one of the microarray datasets to identify the SLAM-family member CD48 as a marker of more lineage-committed progenitors. He and I then worked together to further characterize the differential expression of these two markers in addition to the other SLAM-family member CD244. Toshihide Iwashita and I worked together to perfect the immunohistochemical localization of HSCs using CD150 and CD48 in tissue sections.

Additionally, Omer Yilmaz and I worked together to define the surface marker expression profile of mulitpotent progenitors. Subsequent to this work, I further characterized the differential expression of the SLAM-family members CD2, CD84 and CD229 on early hematopoietic progenitors (manuscript in preparation, data not included in this dissertation).

Chapters 3 and 4 demonstrate that N-cadherin is not required for the cell-autonomous regulation of HSC function in the endosteal niche and that the reduction in the number of osteoblasts in biglycan-deficient mice does not lead to reductions in HSC number. Chapter was published in *Cell Stem Cell 2007 1: 204-17*. Chapter 4 has not yet been published (manuscript in preparation). I alone conducted all of the experiments described in this chapter. Glenn Radice provided N-cadherin genetrap reporter mice.

Chapter 5 demonstrates that HSCs cannot be identified according to bromodeoxyuridine (BrdU)-label retention and that HSCs do not segregate their chromosomes according to the immortal strand model. This work was published in *Nature 2007 449:238-42*. I initiated this work by demonstrating that BrdU-label retention did not significantly enrich for phenotypically defined HSCs in multiple contexts. I discussed biological details of HSCs and chromosome segregation with Sara Gentry, Rina Ashkenazi and Trachette Jackson who developed a mathematical model of BrdU-retention in HSCs and assisted in other aspects pertaining to statistical interpreation of BrdU-retention and immortal strand segregation data. I developed the chloro-deoxyuridine (CldU)/iodo-deoxyuridine (IdU) assay with Monica Teta and Jake Kushner who provided details about reagents. During the latter stages of the project Shenghui He and I had many productive conversations that lead to a more precise understanding of the

data and its interpretation. Additionally, he assisted in the collection of the in vitro BrdU-segregation experiments by painstakingly isolating and immunostaining the progeny of many individually cultured HSCs.

Additional work detailing the use of SLAM-family members to improve the purity of HSCs from old, mobilized and previously reconstituted mice was published in *Blood 2006 107:924-30.* This work will not be explicitly discussed in this dissertation. All of the experiments contained in this publication were performed jointly by Omer Yilmaz and me.

In work that will not be discussed in this dissertation, I performed additional experiments to address the differential expression of SLAM-family members on human hematopoietic stem and progenitor cells. Michael Savona and Michael Perron are completing this work.

Finally, prior to the completion of this dissertation, I performed experiments to characterize the HSC and hematopoietic defects in mice deficient for expression of the hematopoietic transcription factors GATA-1 and NF-E2. Melih Acar is continuing this work.

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ABSTRACT

To improve our ability to identify HSCs and their localization in vivo we compared the gene expression profiles of highly purified hematopoietic stem cells (HSCs) and non-self-renewing multipotent progenitors (MPPs). Cell surface receptors of the SLAM family, including CD150 and CD48, were differentially expressed among functionally distinct progenitors such that HSCs were highly purified as CD150+CD48- cells whereas MPPs were CD150-CD48-. The ability to purify HSCs based on a simple combination of SLAM receptors allowed us to identify HSCs in tissue sections. Most HSCs were associated with sinusoidal endothelium in the spleen and bone marrow. This raised the possibility that HSCs reside in perivascular niches.

Our data suggesting that HSCs reside perivascularly was in contrast to studies which proposed that HSCs identified by the thymidine analogue bromo-deoxyuridine (BrdU)-label retention are associated with osteoblasts via N-cadherin-mediated homophilic adhesion. We therefore tested the hypotheses that N-cadherin was important for HSC maintenance and that HSCs could be identified by BrdU-label retention. We did not detect N-cadherin expression in HSCs by polymerase chain reaction, using anti-N-cadherin antibodies, or by ß-galactosidase staining of *N-cadherin* gene-trap mice. Moreover, N-cadherin deficiency did not affect bone marrow cellularity, the numbers of colony-forming progenitors, the frequency of HSCs, the ability of HSCs to sustain hematopoiesis over time, or their ability to reconstitute irradiated mice. These results

indicate that HSCs do not depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. We tested whether HSCs might retain BrdU, either because they segregate chromosomes asymmetrically or because they divide slowly, by administering BrdU to newborn, cyclophosphamide/G-CSF mobilized, and normal adult mice for 4 to 10 days, followed by 70-days without BrdU. In each case, less than 6% of HSCs retained BrdU and less than 0.5% of BrdU-retaining hematopoietic cells were HSCs, revealing poor specificity and poor sensitivity as an HSC marker. Sequential administration of chloro-deoxyuridine (CldU) and iodo-deoxyuridine (IdU) suggested that all HSCs segregate their chromosomes randomly. Division of individual HSCs in culture revealed no asymmetric segregation of label. HSCs therefore cannot be identified based on BrdU label-retention and BrdU-label retaining cells are highly unlikely to be HSCs.

CHAPTER 1

**IDENTIFICATION, LOCALIZATION AND CHARACTERIZATION OF
HEMATOPOIETIC STEM CELLS AND THEIR IN VIVO NICHE[1]**

INTRODUCTION

Hematopoietic stem cells (HSCs) are the self-renewing, multipotent progenitors

that give rise to all types of mature blood cells.   HSCs persist throughout life and

dynamically regulate their numbers after injury by undergoing self-renewing divisions

that depend upon both cell-intrinsic (Molofsky et al., 2004) and cell-extrinsic (Adams and

Scadden, 2006) mechanisms. With respect to the cell-extrinsic mechanisms, HSCs are

thought to reside within specialized microenvironments within hematopoietic tissues

created by supporting cells that express membrane-bound and secreted factors that

promote HSC maintenance (survival and self-renewal), and that regulate HSC migration,

quiescence, and differentiation. The bone marrow is the major site of adult

hematopoiesis, but HSCs can undergo hematopoiesis in the adult spleen and liver during

periods of hematopoietic stress (Figure 1.1). Specialized HSC niches have long been

hypothesized within these hematopoietic tissues (Schofield, 1978), and recent advances

have begun to characterize the mechanisms of cell-extrinsic regulation of HSCs (Table

1.1).

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title "Uncertainty in the niches that maintain haematopoietic stem cells" with authors
listed as MJ Kiel and SJ Morrison.

A major question in the field of HSC biology has been to identify the cells that are responsible for this cell-extrinsic regulation of HSC maintenance and that comprise the HSC niche. Until recently, this question was too difficult to address adequately due to the technical difficulty associated with isolation of HSCs. As a result, much work in this regard relied on the use of markers of HSC identity that were either untested or that resulted in low HSC purity. This dissertation describes the use of SLAM-family members for the improved and simplified isolation of HSCs, the subsequent localization of HSCs in tissue sections in a perivascular niche in the bone marrow and spleen, and the examination of other previously utilized strategies to identify putative HSCs in vivo adjacent to osteoblasts, including N-cadherin expression and bromo-deoxyuridine (BrdU)-label retention.

In the past, many different techniques have been employed to isolate cells with the ability to reconstitute the hematopoietic compartment of lethally irradiated recipient mice in all lineages for long periods of time (HSCs). These techniques have included density separation, differential dye efflux ability and analysis of expression of surface antigens by antibody staining. Use of antibodies recognizing surface markers expressed only on mature hematopoietic cells provided an effective initial depletion strategy to enrich for HSC activity. The use of additional antibodies recognizing HSCs and other progenitors, such as Sca-1 and c-kit, or progenitors and not HSCs, such as Flk-2, further improved HSC isolation strategies. Other more specific positive markers of HSCs were also identified, including CD201 (Balazs et al., 2006) and Endoglin (Chen et al., 2003; Chen et al., 2002). Nevertheless, the purification of HSCs still required the use of a complex combination of antibodies against these markers and considerable technical

expertise to recognize the different immunophenotypic populations.   Importantly, in order to localize HSC niches in tissue sections, a simplified strategy was required that would be amenable to immunohistochemical analysis without compromising the purity of the target population.

Some of the early attempts to identify the HSC niche localized putative HSCs near osteoblasts in the bone marrow. A number of these studies reported that enriched populations of HSCs preferentially home to areas of bone marrow that are close to the endosteum after being injected into irradiated or non-irradiated mice. It is difficult to interpret the results of some of these experiments because the transplanted cells were enriched using markers that give low HSC purity, and it remains uncertain whether the cells that localized close to the endosteum were HSCs or were other cells. In other cases, relatively highly enriched HSCs were infused, and approximately 60% of the cells found in bone-marrow sections fifteen hours after infusion were within twelve cell diameters of the endosteum (Nilsson et al., 2001a). This supports the idea that HSCs are more likely than other bone marrow cells to localize near the endosteal surface. Nonetheless, a variety of distinct microenvironments are located within 12 cell diameters of the bone. This is particularly true in trabecular bone, where a high proportion of HSCs appear to localize (Calvi et al., 2003; Kiel et al., 2005; Zhang et al., 2003) and where many cells are close to a bone surface. These results raise the question of whether the endosteal surface is itself a niche, or whether endosteal cells instead contribute to the formation of niches that are close to, but not at, the endosteal surface.

One widely discussed current model proposes that HSCs adhere to the surface of osteoblasts using N-cadherin-mediated homotypic adhesion and that HSCs are acutely

dependent upon this interaction for their maintenance. This inference was based on the observation of N-cadherin+ cells at the endosteum that express some HSC markers (Wilson et al., 2004; Zhang et al., 2003). However, these studies did not test whether the N-cadherin expressing cells had HSC activity or whether N-cadherin was necessary for HSC maintenance. As described in Chapter 3 of this dissertation, we have been unable to detect N-cadherin expression by cells with HSC activity (Kiel et al., 2007) and have been unable to detect any defect in hematopoiesis or HSC function due to loss of N-cadherin in hematopoietic cells.

Similarly, BrdU-label retention assays were postulated to identify HSCs because of their relative quiescence as compared to other bone marrow cells. These assays are carried out by administering a short pulse of BrdU to label dividing cells, then removing BrdU for a longer chase period to allow cells that are actively dividing to dilute out the label through subsequent divisions in the absence of BrdU. The fraction of cells that remains BrdU+ for long chase periods is believed to be enriched for HSCs.

Using these strategies, previous attempts to localize putative HSCs in tissue sections identified a subset of cells in the bone marrow that was distinguished by both N-cadherin expression and long-term BrdU-label retention adjacent to osteoblasts at the endosteum. Despite the fact that neither the specificity nor the sensitivity of these techniques was addressed, these N-cadherin+ BrdU-label retaining cells were postulated to be HSCs that reside in direct contact with osteoblasts.

For these reasons, many HSCs are believed to reside at, or near, the endosteum in the bone marrow. Endosteal cells are known to secrete factors that promote HSC maintenance (Adams and Scadden, 2006; Li and Xie, 2005; Suda et al., 2005). Therefore,

the endosteum has an important role in promoting HSC maintenance and regulating HSC function. However, other bone marrow cells probably also play important roles in the creation of HSC niches. For instance, many HSCs are now known to reside adjacent to sinusoidal blood vessels in the bone marrow (Kiel et al., 2007; Kiel et al., 2005), and perivascular cells also secrete factors that regulate HSC maintenance (Sacchetti et al., 2007; Sugiyama et al., 2006).

Herein I present evidence suggesting the existence of perivascular niches and challenge some of the evidence suggesting the existence of osteoblastic niches. Later I will consider the evidence for the involvement of diverse cell types, hematopoietic microenvironments, and growth factors in the regulation of HSC maintenance, and finally will consider the diverse models of HSC niches that are consistent with currently available evidence.

ENDOSTEAL CELLS CONTRIBUTE TO THE MAINTENANCE OF HSCs

Much of the work pertaining to the characterization of environmental mechanisms that regulate HSC maintenance has been focused on the role of the bone-marrow endosteum. The endosteum is the inner surface of the bone at the interface of bone and bone marrow (Figure 1.1A). The endosteal surface is covered by a protective layer of bone-lining cells that can differentiate into bone-forming osteoblasts, but is also marked by the presence of bone-resorbing osteoclasts. Osteoblasts and osteoclasts are present in dynamic equilibrium under steady-state conditions but can increase or decrease in frequency depending on whether new bone is forming or being remodelled (Franz-Odendaal et al., 2006; Seeman and Delmas, 2006). The endosteal surface interfaces with

haematopoietic progenitors in the marrow as well as with a rich supply of arterial blood that passes through the endosteum to nourish the bone (De Bruyn et al., 1970). It is likely that the interaction of bone cells, haematopoietic cells, and vascular cells at the endosteum regulates both haematopoiesis and bone formation.

The endosteum has long been viewed as having a role in the regulation of primitive haematopoietic progenitors. Beginning in the 1970s, it was reported that progenitors that can form multilineage colonies in the spleens of irradiated mice (colony-forming unit-spleen; CFU-S), which were initially regarded as HSCs but which were later found to be mainly transiently reconstituting multipotent progenitors (Morrison and Weissman, 1994; Spangrude et al., 1995; Wolf et al., 1993), are more enriched near the endosteum than within the central bone marrow (Lord et al., 1975). At least some of these CFU-S were thought to reside at the endosteal surface (Gong, 1978), though other studies questioned this conclusion and observed CFU-S throughout the bone marrow (Maloney et al., 1978). Complicating matters further, the CFU-S near the endosteum were more rapidly dividing than CFU-S in the central bone marrow (Lord et al., 1975), suggesting that the cells near the endosteum might not be quiescent HSCs. Although the implications of these observations for HSC localization are uncertain, these studies made the general point that primitive haematopoietic progenitors tend to be enriched near the endosteum.

Important recent studies have shown that factors that regulate HSC maintenance are secreted by cells at the endosteum. Genetic manipulations that increase osteoblast numbers and trabecular bone in mice also increase the number of HSCs in bone marrow (Calvi et al., 2003; Zhang et al., 2003).   Osteoblasts express factors that appear to regulate the maintenance or the numbers of HSCs in the bone marrow, including positive

regulators such as angiopoietin (Arai et al., 2004) and negative regulators such as

osteopontin (Nilsson et al., 2005; Stier et al., 2005). Coupled with evidence that at least

some HSCs reside at or near the endosteum (Arai et al., 2004; Kiel et al., 2007; Kiel et

al., 2005; Nilsson et al., 2001b; Suzuki et al., 2006; Zhang et al., 2003), these data

suggest the existence of an endosteal niche for HSCs. Very specific models of this

endosteal niche have been widely discussed in the literature, though currently available

data remain compatible with a variety of ways in which endosteal cells could promote

HSC maintenance (Figure 1.2).

In addition to N-cadherin, osteoblasts have also been proposed to promote HSC

maintenance by expressing Jagged, thereby activating Notch expressed by HSCs

adhering to osteoblasts (Calvi et al., 2003). Notch activation by Jagged does promote

HSC maintenance in culture (Calvi et al., 2003). However, conditional deletion of both

Jagged1 and Notch1 from bone marrow cells did not affect HSC maintenance *in vivo*

(Mancini et al., 2005). It remains possible that HSCs require Notch signaling under

certain circumstances but Notch activation does not appear to be a crucial general

component of the niche. Additionally, we have determined that HSCs do not depend on

N-cadherin expression for their maintenance based on conditional deletion of N-cadherin

in HSCs. Nevertheless, if N-cadherin and Notch ligands are not required for HSC

maintenance, it is possible that endosteal cells promote HSC maintenance by secreting

diffusible factors that act indirectly or at a distance (Figure 1.2). Additional work will be

required to determine whether a subset of HSCs adheres to the surface of osteoblasts by

other mechanisms or whether endosteal cells mainly secrete factors that act on HSCs

nearby, but not at, the endosteum.

Osteoblasts are capable of supporting the maintenance of primitive haematopoietic progenitors in culture (Taichman and Emerson, 1994), but it remains unclear whether osteoblasts are acutely required for HSC maintenance *in vivo*. This has been tested by ablating osteoblasts from adult *Col1a1-TK* mice (Visnjic et al., 2004; Zhu et al., 2007). These authors did not observe an acute loss of HSCs. Rather they observed an acute loss of differentiating cells, particularly B-lineage progenitors, followed by a delayed depletion of c-kit+lineage-Sca-1+ cells, which are enriched for HSCs. In fact, the frequency of c-kit+lineage-Sca-1+ cells in bone marrow actually increased over time after osteoblast ablation (although absolute numbers of such cells declined), whereas B-lineage progenitors and other differentiating cells were severely depleted in frequency and absolute number within days of osteoblast ablation (Visnjic et al., 2004; Zhu et al., 2007). This suggests that some types of restricted progenitors and differentiating cells depend more acutely on osteoblasts than HSCs do. This raises the question of whether HSCs were slowly lost as a direct consequence of osteoblast ablation or whether HSCs were ultimately depleted as a secondary consequence of the loss of other bone-marrow cells.

Apart from the potential role of non-endosteal bone-marrow cells in the regulation of HSCs, even the role of the endosteum itself is likely to be complex (Figure 1.3). Osteoclast activity (Kollet et al., 2006) and the calcium ions that are generated by bone resorption (Adams et al., 2006) have also been implicated in HSC maintenance and localization. Osteoclasts secrete proteases such as matrix metalloproteinase-9 (MMP9) and cathepsin K as well as growth factors such as CXCL12 (Kollet et al., 2006) that regulate HSC maintenance and localization within the bone marrow (Ara et al., 2003;

Peled et al., 1999; Sugiyama et al., 2006; Zou et al., 1998). The proteases are required for bone remodeling as well as to release membrane-bound growth factors, such as steel factor, that also regulate HSC frequency and localization in the bone marrow (Heissig et al., 2002; Kollet et al., 2006). One interpretation of the late disappearance of HSCs from the endosteum after osteoblast ablation (Visnjic et al., 2004; Zhu et al., 2007) is that the ongoing ability of osteoclasts to resorb bone, to secrete growth factors, and to enzymatically enhance the release of growth factors synthesized by other cells, may have sustained HSCs for a considerable period of time in the absence of osteoblasts, though this remains to be tested.

THE EVOLUTIONARILY CONSERVED ROLE OF VASCULAR CELLS IN HSC MAINTENANCE

The vasculature has a crucial role in the formation and expansion of HSCs during embryonic development, and probably in the regulation of adult HSCs. Indeed, there are vertebrate species in which haematopoiesis never occurs in association with bone. In zebrafish, for example, haematopoietic progenitors arise in close association with the dorsal aorta and ultimately establish haematopoiesis in the kidney and thymus (Murayama et al., 2006). Evolutionarily, bone marrow was first used as a site of haematopoiesis in certain amphibian species (Zon, 1995).

Even in mammals, the ability of HSCs to self-renew and differentiate during fetal development, prior to the creation of bone marrow cavities, suggests that HSCs arise and are maintained in vascular niches prior to birth. There is a close developmental relationship between the haematopoietic and endothelial lineages (Huber et al., 2004; Kennedy et al., 1997), and HSCs appear to arise from a perivascular progenitor during

embryonic development (Cumano et al., 1996; de Bruijn et al., 2002; Medvinsky and Dzierzak, 1996; Mikkola and Orkin, 2006; North et al., 2002; Sanchez et al., 1996). In addition to these data demonstrating the presence of perivascular HSCs in the yolk sac, aorta-gonad-mesonephros region, and vitelline arteries, HSCs also reside and undergo haematopoiesis in association with blood vessels in the placenta (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). Since there is no bone or endosteum in any of these tissues, the data suggest that HSCs probably reside primarily in perivascular niches during embryonic and fetal development. Nonetheless, these niches remain to be characterized and could depend on critical contributions by non-vascular cells. Furthermore, these niches only transiently contain HSCs raising the possibility that the existence of supportive niches in these tissues is only transient or that HSCs are induced to migrate from these tissues by signals from subsequent sites of hematopoiesis such as the liver and bone marrow.

THE CONTRIBUTION OF THE VASCULATURE TO THE MAINTENANCE OF ADULT HSCS

Vascular cells likely contribute to the creation of HSC niches in extramedullary tissues. HSCs are present throughout adult life in extramedullary tissues, such as the liver and spleen (Taniguchi et al., 1996), and extramedullary haematopoiesis can flourish in these tissues for long periods of time despite the absence of bone or endosteum (Johnson et al., 1992; Yang et al., 1995). This implies that there are cells other than osteoblasts that can create environments that sustain adult HSCs. As described in Chapter 2, most HSCs mobilized to the adult spleen localized adjacent to sinusoids (Kiel et al., 2005), suggesting that HSCs in extramedullary tissues may reside within perivascular niches.

Sinusoids are specialized blood vessels that are present only in haematopoietic tissues and that carry venous circulation. The walls of sinusoids are fenestrated endothelium through which haematopoietic cells can enter and exit the circulation. The case for extramedullary vascular niches remains circumstantial as we are not aware of any data that directly demonstrate that perivascular cells in extramedullary tissues can promote the maintenance of HSCs.

As described in Chapters 2 and 3, many HSCs in adult bone marrow can also be found around sinusoids. When the localization of HSCs in bone marrow was examined using SLAM-family markers about 60% of bone-marrow HSCs were adjacent to sinusoids and up to 20% of HSCs localized to the endosteum (Kiel et al., 2007; Kiel et al., 2005). The remaining HSCs were scattered throughout the bone marrow, and were not located adjacent to sinusoids or endosteum. HSCs were thus significantly more likely than other bone marrow cells to localize to sinusoids or the endosteal surface, consistent with the idea that there might be niches in these locations (Kiel et al., 2007). Virtually all HSCs were within 5 cell diameters of a sinusoid in the bone marrow (Kiel et al., 2007), raising the possibility that even the HSCs that are near the endosteum may be influenced by perivascular cells.

The observation that substantial numbers of HSCs localize to sinusoids in the bone marrow makes sense, given that HSCs can be mobilized within minutes of administering interleukin (IL)-8 to mice (Laterveer et al., 1995). Furthermore, HSCs deficient for *Rac1* and *Rac2* (genes that encode proteins that control cell migration) are highly defective in their ability to migrate and yet conditional deletion of *Rac1* and *Rac2* from adult haematopoietic cells (Gu et al., 2003) or administration of a RAC inhibitor

leads to the mobilization of HSCs into circulation within hours (Cancelas et al., 2005).

This rapid mobilization of HSCs that lack the ability to migrate normally supports the

idea that significant numbers of HSCs are present on sinusoids, poised to enter the

circulation. All of these data raise the question of whether endothelial cells or other

perivascular cells actually create a perivascular niche that helps to maintain HSCs (Kiel

et al., 2005; Kopp et al., 2005) (Figure 1.4A,C) or whether HSCs transiently migrate

through perivascular sites on their way in and out of circulation (Wright et al., 2001)

(Figure 1.4B).

Similar to osteoblasts, endothelial cells can promote the maintenance of HSCs in

culture (Li et al., 2004; Ohneda et al., 1998) and normal endothelial cell function is

required for haematopoiesis *in vivo* (Yao et al., 2005). When the gene encoding the

common cytokine receptor subunit, *GP130*, was conditionally deleted from endothelial

cells, the postnatal bone marrow became hypocellular, mice became anemic by

adulthood, and died by 1 year of age (Yao et al., 2005). In this study, a floxed allele of

GP130 was conditionally deleted using *Tie2-Cre*, a transgene in which Cre-recombinase

is expressed in HSCs and in endothelial cells (Koni et al., 2001), making it possible to

conditionally delete floxed genes from these cell types. The authors reported that

cellularity around sinusoids in the bone marrow markedly declined, whereas cellularity at

the endosteum remained normal. The defects were not attributable to a loss of *GP130* in

HSCs as *GP130*-deficient bone-marrow cells were able to reconstitute irradiated wild-

type recipients, but wild-type bone marrow cells remained unable to reconstitute *GP130*-

deficient mice. These data provide strong genetic evidence of the importance of

endothelial cells to haematopoiesis *in vivo*, though it remains unclear from these data

whether endothelial cells are required for the maintenance of HSCs or just for their differentiation, and whether the endothelial cells act directly or indirectly on HSCs.

Similar to the endosteum, the perivascular environment also contains multiple cell types that may regulate HSCs (Figure 1.3B). Sugiyama and colleagues found that perivascular reticular cells in bone marrow express very high levels of CXCL12, a factor required for the maintenance of HSCs (Sugiyama et al., 2006). They also confirmed that a high proportion of HSCs localize perivascularly, and that the subset of HSCs that localizes to the endosteum or elsewhere is also consistently in contact with CXCL12-expressing reticular cells. The presence of CXCL12-secreting reticular cells in both the perivascular and endosteal environments provides a potential unifying mechanism for the maintenance of HSCs in both sites, and reminds us that the most morphologically recognizable cell types in a particular environment (such as osteoblasts and endothelial cells) need not be the cells that are most functionally important for the creation of a niche. Nonetheless, there are no genetic data confirming that these reticular cells are a more functionally important source of CXCL12 for the maintenance of HSCs than osteoblasts or osteoclasts, so the argument that they create a niche for HSCs is not yet conclusive. Indeed, endothelial cells themselves internalize circulating CXCL12 and resecrete it into the bone marrow (Dar et al., 2005), raising the possibility that endothelial cells could also be an important source of CXCL12 for HSCs.

Taken together, these data highlight the potential complexity of the cell types and factors responsible for the cell-extrinsic regulation of HSCs. Both perivascular cells and endosteal cells are likely to be important contributors to the extrinsic regulation of HSCs.

Table 1.1: Cell-extrinsic factors genetically necessary for normal HSC maintenance in vivo.

Factor	Genetic evidence for regulation of HSCs in vivo
Angiopoietin (Ang-1)	Combined loss of Tie2 (the receptor for Ang-1) as well as Tie1, leads to defects in postnatal HSCs (Puri and Bernstein, 2003). Ang-1 appears to promote the maintenance of quiescent HSCs (Arai et al., 2004)
Calcium ions	Deletion of the calcium sensing receptor leads to reduced bone marrow cellularity and HSC content with increased progenitor cell mobilization into the circulation and spleen (Adams et al., 2006)
CXCL12	Mice deficient in the chemokine CXCL12 or its receptor CXCR4 show disrupted colonization of the bone marrow while conditional deletion of CXCL4 in adult mice leads to reductions in HSC number in the bone marrow and reduced HSC activity upon transplantation (Ara et al., 2003; Nagasawa et al., 1996; Sugiyama et al., 2006; Zou et al., 1998)
Hedgehog (Hh)	Patched (the Hh receptor) heterozygous HSCs have reduced long-term reconstituting activity, suggesting that a member of the Hh family of ligands can negatively regulate HSC self-renewal, at least when the pathway is over-activated (Trowbridge et al., 2006)
Osteopontin (OPN)	The matrix glycoprotein OPN is expressed at the endosteum by bone-lining cells and negatively regulates HSC numbers. OPN deficient mice have moderately increased HSC numbers in the marrow (Nilsson et al., 2005; Stier et al., 2005)
Stem cell factor (SCF, Sl, Steel Factor)	Mice with mutations in SCF (Sl/Sl^d, steel-Dickie mutants) or in its receptor c-kit (W/W^v, dominant spotting mutants) have fewer HSCs and exhibit less HSC function (Barker, 1994; McCarthy et al., 1977)
Thrombopoietin (Tpo)	Tpo is synthesized in the liver and is transported into the bone marrow through the blood. Mice deficient in Tpo or the Tpo receptor c-Mpl have profound reductions in HSC numbers (Kaushansky, 2003; Solar et al., 1998)

In addition to these factors that are genetically necessary for the maintenance of normal numbers of HSCs in the bone marrow, there are additional factors that are likely to regulate to regulate HSC maintenance based on over-expression experiments or experiments performed in culture, but which have not yet been fully tested for necessity in vivo.
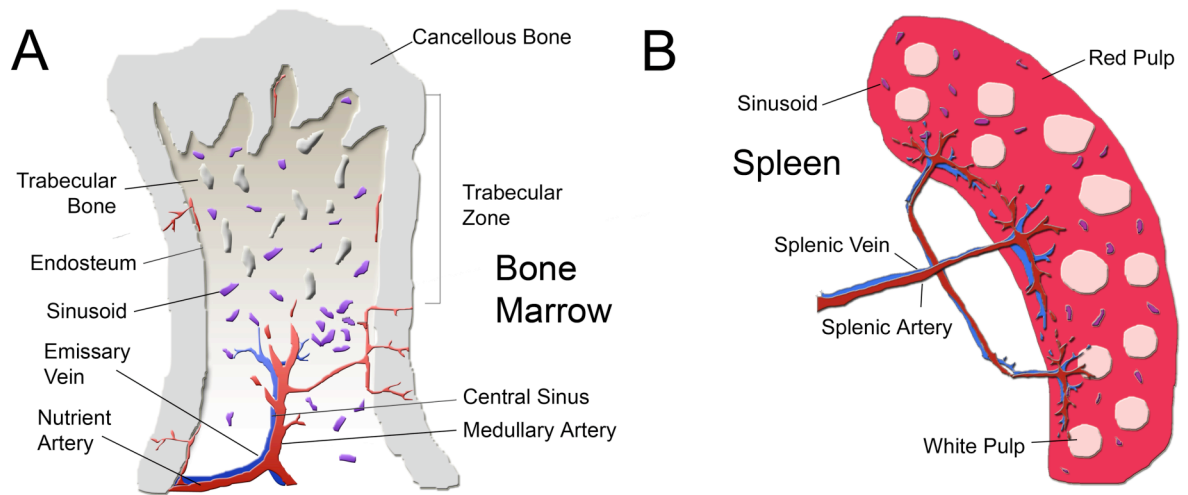
Figure 1.1: Anatomy of the adult haematopoietic organs, bone marrow and spleen.
A) Haematopoietic stem cells (HSCs) reside primarily within bone marrow during adulthood. Bone marrow is a complex organ containing many different haematopoietic and non-haematopoietic cell types. Haematopoiesis occurs within the medullary cavity, surrounded by a shell of vascularized and innervated cancellous bone. Minute projections of bone (trabeculae) are found throughout the trabecular zone of bone, such that many cells in this region are close to bone surface. The interface of bone and bone marrow is known as the endosteum, which is covered by bone-lining cells that can differentiate into bone-forming osteoblasts. Bone-resorbing osteoclasts are also present at the endosteum. Arteries carry oxygen, nutrients, and haematopoietic growth factors such as thrombopoietin (Kaushansky and Drachman, 2002; Wolber and Jelkmann, 2002) into the bone marrow, before feeding into capillaries and then sinusoids, which coalesce to form the venous circulation. Sinusoids are specialized venuoles that form a reticular network of fenestrated vessels that allow cells to pass in and out of circulation.   B) HSCs can also be found at low levels in extramedullary tissues like the spleen (shown) and liver throughout adult life. When bone marrow haematopoiesis is impaired by age, cancer, or myeloablation, expanded numbers of HSCs can engage in extramedullary haematopoiesis in the spleen. HSCs reside around sinusoids in the red pulp of the spleen (Kiel et al., 2005), but not within the white pulp, which contains lymphocytes and antigen-presenting cells.
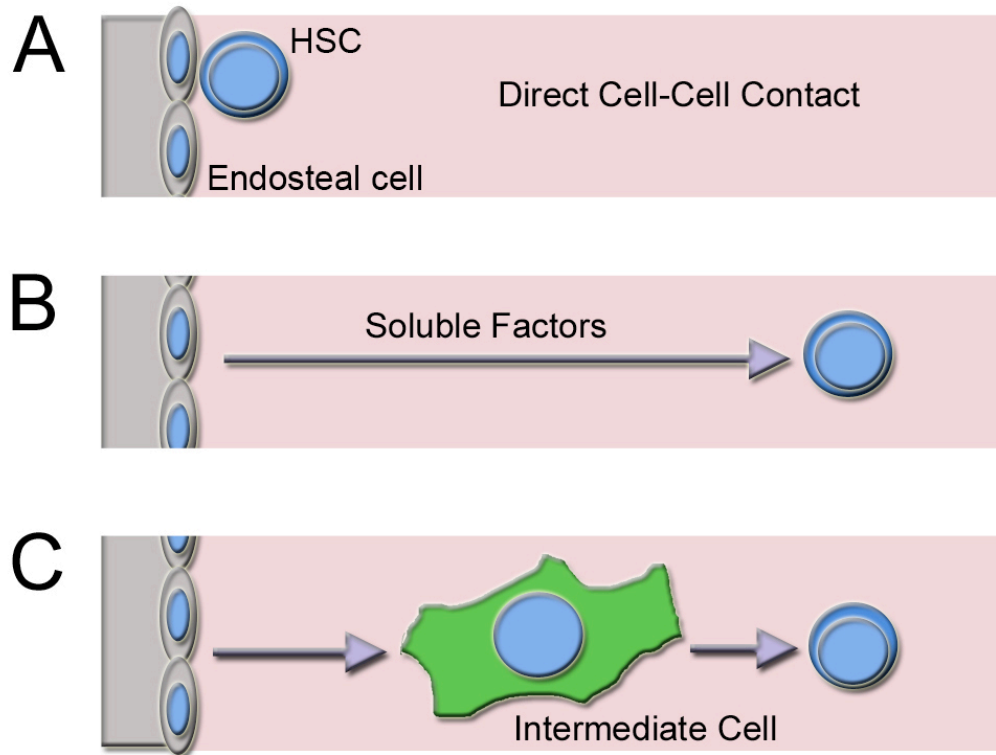
Figure 1.2: Possible mechanisms by which endosteal cells contribute to the formation of HSC niches. Genetic manipulations that increase osteoblast numbers also increase haematopoietic stem cell (HSC) numbers (Calvi et al., 2003; Zhang et al., 2003), and endosteal cells release a variety of factors that are thought to regulate HSC maintenance and localization (Adams et al., 2006; Adams and Scadden, 2006; Arai et al., 2004; Kollet et al., 2006). Existing data are compatible with a variety of ways in which endosteal cells could contribute to HSC niches: A) One possibility is that the endosteum itself is a niche, with HSCs that reside in direct contact with osteoblasts (Adams and Scadden, 2006; Li and Xie, 2005; Suda et al., 2005). This possibility has been favoured based on the idea that HSCs are regulated by N-cadherin and Notch signals from osteoblasts, though more recent studies have questioned whether N-cadherin (Kiel et al., 2007) or Notch (Mancini et al., 2005) regulate HSC maintenance under steady-state conditions. B) An alternative possibility is that factors secreted from endosteal cells diffuse, or are actively transported, to regulate HSC niches that are close to, but not at, the endosteum. C) A third possibility is that endosteal cells promote HSC maintenance by regulating the function of third-party (or intermediate) cells that in turn create HSC niches away from the endosteum. For example, osteoblasts are known to regulate the recruitment of vasculature to bone marrow (Street et al., 2002; Tombran-Tink and Barnstable, 2004).
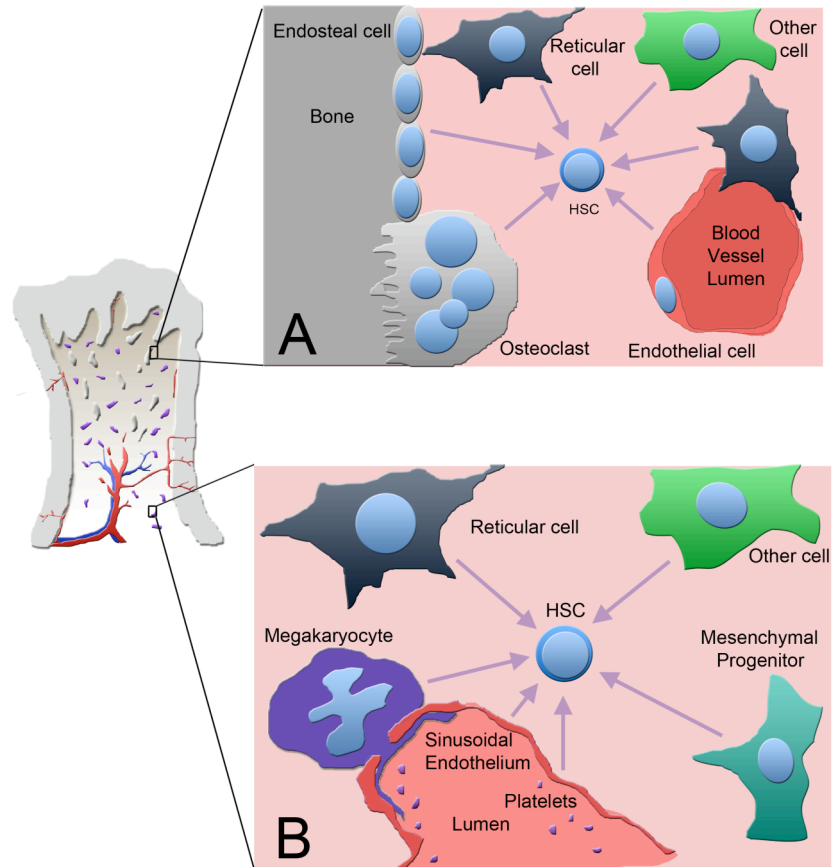
Figure 1.3: Many different cell types may contribute to formation of HSC niches near the endosteum and around sinusoids. A) Osteoblasts and osteoclasts secrete a variety of factors that have been implicated in the regulation of haematopoietic stem cells (HSCs) (Adams and Scadden, 2006; Kollet et al., 2006). Furthermore, bone remodelling by these cell types elevates the concentration of calcium ions locally and in circulation, influencing HSC localization and maintenance through the calcium-sensing receptor (Adams et al., 2006). Sympathetic-nervous-system activity also regulates HSC localization in the bone marrow, perhaps by innervating the endosteum (Katayama et al., 2006). The endosteal surface is also heavily vascularized by arteries that nourish bone in some locations and by the pervasive presence of sinusoids. Vascular and perivascular cells might contribute to the formation of HSC niches at or near the endosteum (Ara et al., 2003; Sugiyama et al., 2006). As yet unidentified cells could also contribute to these niches. B) Perivascular sites are likely to maintain fetal HSCs in placenta, spleen, and liver. During adulthood, the presence of HSCs around sinusoids throughout haematopoietic tissues (Kiel et al., 2005), the ability of endothelial cells to promote HSC maintenance in culture (Li et al., 2004; Ohneda et al., 1998), and the secretion of HSC regulatory factors by perivascular reticular cells and mesenchymal progenitors (Li et al., 2001; Sacchetti et al., 2007; Sugiyama et al., 2006), raises the possibility of perivascular niches for HSCs (Kopp et al., 2005) in the bone marrow and in extramedullary tissues such as the spleen. If such niches exist, a wide variety of perivascular cells could conceivably contribute to such niches, including endothelial cells, megakaryocytes, perivascular reticular cells, mesenchymal progenitors, and other cell types.

A Permanent Residence in Perivascular Niche

Endothelial cell

HSC

Sinusoid

Endosteal cells

B Residence in Endosteal Niche with Migration through Perivascular Sites

C Residence in Distinct Endosteal and Perivascular Niches
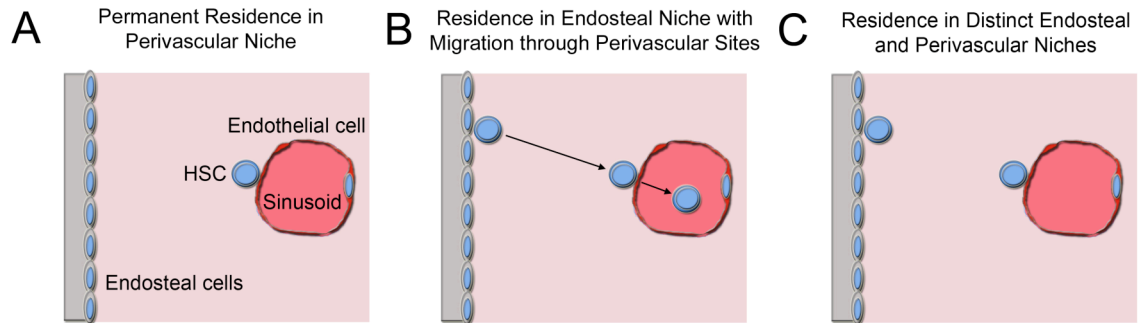
Figure 1.4: Possible reasons for the observation of HSCs in perivascular sites.
When the localization of HSCs (blue) is systematically examined using validated markers in bone marrow and in extramedullary tissues, many HSCs are found adjacent to sinusoids (Kiel et al., 2005). Possible reasons for perisinusoidal localization include: A) HSCs may reside in perivascular niches (Kopp et al., 2005; Sugiyama et al., 2006).   B) HSCs may reside in endosteal niches but their frequent migration through circulation (Wright et al., 2001) means that they are often observed in perivascular sites on their way in or out of the circulation.   C) HSCs may reside in spatially distinct endosteal and perivascular niches, that are either functionally redundant or that have different roles in the regulation of HSCs.

BIBLIOGRAPHY

Adams, G.B., Chabner, K.T., Alley, I.R., Olson, D.P., Szczepiorkowski, Z.M., Poznansky, M.C., Kos, C.H., Pollak, M.R., Brown, E.M., and Scadden, D.T. (2006). Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. Nature *439*, 599-603.

Adams, G.B., and Scadden, D.T. (2006). The hematopoietic stem cell in its place. Nature Immunology *7*, 333-337.

Ara, T., Tokoyoda, K., Sugiyama, T., Egawa, T., Kawabata, K., and Nagasawa, T. (2003). Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. Immunity *19*, 257-267.

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell *118*, 149-161.

Balazs, A.B., Fabian, A.J., Esmon, C.T., and Mulligan, R.C. (2006). Endothelial protein C receptor (CD201) explicitly identifies hematopoietic stem cells in murine bone marrow. Blood *107*, 2317-2321.

Barker, J.E. (1994). Sl/Sld hematopoietic progenitors are deficient in situ. Exp Hematol *22*, 174-177.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R.*, et al.* (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. Nature *425*, 841-846.

Cancelas, J.A., Lee, A.W., Prabhakar, R., Stringer, K.F., Zheng, Y., and Williams, D.A. (2005). Rac GTPases differentially integrate signals regulating hematopoietic stem cell localization. Nat Med *11*, 886-891.

Chen, C.Z., Li, L., Li, M., and Lodish, H.F. (2003). The endoglin(positive) sca-1(positive) rhodamine(low) phenotype defines a near-homogeneous population of long-term repopulating hematopoietic stem cells. Immunity *19*, 525-533.

Chen, C.Z., Li, M., de Graaf, D., Monti, S., Gottgens, B., Sanchez, M.J., Lander, E.S., Golub, T.R., Green, A.R., and Lodish, H.F. (2002). Identification of endoglin as a functional marker that defines long-term repopulating hematopoietic stem cells. Proc Natl Acad Sci U S A *99*, 15468-15473.

Cumano, A., Dieterlen-Lievre, F., and Godin, I. (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. Cell *86*, 907-916.

Dar, A., Goichberg, P., Shinder, V., Kalinkovich, A., Kollet, O., Netzer, N., Margalit, R., Zsak, M., Nagler, A., Hardan, I.*, et al.* (2005). Chemokine receptor CXCR4-dependent internalization and resecretion of functional chemokine SDF-1 by bone marrow endothelial and stromal cells. Nat Immunol *6*, 1038-1046.

de Bruijn, M.F., Ma, X., Robin, C., Ottersbach, K., Sanchez, M.J., and Dzierzak, E. (2002). Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. Immunity *16*, 673-683.

De Bruyn, P.P., Breen, P.C., and Thomas, T.B. (1970). The microcirculation of the bone marrow. Anat Rec *168*, 55-68.

Franz-Odendaal, T.A., Hall, B.K., and Witten, P.E. (2006). Buried alive: how osteoblasts become osteocytes. Dev Dyn *235*, 176-190.

Gekas, C., Dieterlen-Lievre, F., Orkin, S.H., and Mikkola, H.K. (2005). The placenta is a niche for hematopoietic stem cells. Dev Cell *8*, 365-375.

Gong, J.K. (1978). Endosteal marrow: a rich source of hematopoietic stem cells. Science *199*, 1443-1445.

Gu, Y., Filippi, M.D., Cancelas, J.A., Siefring, J.E., Williams, E.P., Jasti, A.C., Harris, C.E., Lee, A.W., Prabhakar, R., Atkinson, S.J.*, et al.* (2003). Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. Science *302*, 445-449.

Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N.R., Crystal, R.G., Besmer, P., Lyden, D., Moore, M.A.*, et al.* (2002). Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell *109*, 625-637.

Huber, T.L., Kouskoff, V., Fehling, H.J., Palis, J., and Keller, G. (2004). Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. Nature *432*, 625-630.

Johnson, R.S., Spiegelman, B.M., and Papaioannou, V. (1992). Pleiotropic effects of a null mutation in the c-fos proto-oncogene. Cell *71*, 577-586.

Katayama, Y., Battista, M., Kao, W.M., Hidalgo, A., Peired, A.J., Thomas, S.A., and Frenette, P.S. (2006). Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell *124*, 407-421.

Kaushansky, K. (2003). Thrombopoietin: accumulating evidence for an important biological effect on the hematopoietic stem cell. Ann N Y Acad Sci *996*, 39-43.

Kaushansky, K., and Drachman, J.G. (2002). The molecular and cellular biology of thrombopoietin: the primary regulator of platelet production. Oncogene *21*, 3359-3367.

Kennedy, M., Firpo, M., Choi, K., Wall, C., Robertson, S., Kabrun, N., and Keller, G. (1997). A common precursor for primitive erythropoiesis and definitive haematopoiesis. Nature *386*, 488-493.

Kiel, M.J., Radice, G.L., and Morrison, S.J. (2007). Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. Cell stem cell *1*, 204-217.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells. Cell *121*, 1109-1121.

Kollet, O., Dar, A., Shivtiel, S., Kalinkovich, A., Lapid, K., Sztainberg, Y., Tesio, M., Samstein, R.M., Goichberg, P., Spiegel, A.*, et al.* (2006). Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nat Med *12*, 657-664.

Koni, P.A., Joshi, S.K., Temann, U.A., Olson, D., Burkly, L., and Flavell, R.A. (2001). Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. J Exp Med *193*, 741-754.

Kopp, H.G., Avecilla, S.T., Hooper, A.T., and Rafii, S. (2005). The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiology (Bethesda) *20*, 349-356.

Laterveer, L., Lindley, I.J., Hamilton, M.S., Willemze, R., and Fibbe, W.E. (1995). Interleukin-8 induces rapid mobilization of hematopoietic stem cells with

radioprotective capacity and long-term myelolymphoid repopulating ability. Blood *85*, 2269-2275.

Li, J.J., Huang, Y.Q., Basch, R., and Karpatkin, S. (2001). Thrombin induces the release of angiopoietin-1 from platelets. Thromb Haemost *85*, 204-206.

Li, L., and Xie, T. (2005). Stem cell niche: structure and function. Annu Rev Cell Dev Biol *21*, 605-631.

Li, W., Johnson, S.A., Shelley, W.C., and Yoder, M.C. (2004). Hematopoietic stem cell repopulating ability can be maintained in vitro by some primary endothelial cells. Exp Hematol *32*, 1226-1237.

Lord, B.I., Testa, N.G., and Hendry, J.H. (1975). The relative spatial distributions of CFUs and CFUc in the normal mouse femur. Blood *46*, 65-72.

Maloney, M.A., Lamela, R.A., Dorie, M.J., and Patt, H.M. (1978). Concentration gradient of blood stem cells in mouse bone marrow--an open question. Blood *51*, 521-525.

Mancini, S.J., Mantei, N., Dumortier, A., Suter, U., Macdonald, H.R., and Radtke, F. (2005). Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. Blood *105*, 2340-2342.

McCarthy, K.F., Ledney, G.D., and Mitchell, R. (1977). A deficiency of hematopoietic stem cells in steel mice. Cell Tissue Kinet *10*, 121-126.

Medvinsky, A., and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. Cell *86*, 897-906.

Mikkola, H.K., and Orkin, S.H. (2006). The journey of developing hematopoietic stem cells. Development *133*, 3733-3744.

Molofsky, A.V., Pardal, R., and Morrison, S.J. (2004). Diverse mechanisms regulate stem cell self-renewal. Current Opinions in Cell Biology *16*, 700-707.

Morrison, S.J., and Weissman, I.L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity *1*, 661-673.

Murayama, E., Kissa, K., Zapata, A., Mordelet, E., Briolat, V., Lin, H.F., Handin, R.I., and Herbomel, P. (2006). Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. Immunity *25*, 963-975.

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S.-I., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature *382*, 635-638.

Nilsson, S.K., Johnston, H.M., and Coverdale, J.A. (2001a). Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. Blood *97*, 2293-2299.

Nilsson, S.K., Johnston, H.M., and Coverdale, J.A. (2001b). Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. Blood *97*, 2293-2299.

Nilsson, S.K., Johnston, H.M., Whitty, G.A., Williams, B., Webb, R.J., Denhardt, D.T., Bertoncello, I., Bendall, L.J., Simmons, P.J., and Haylock, D.N. (2005). Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood *106*, 1232-1239.

North, T.E., de Bruijn, M.F., Stacy, T., Talebian, L., Lind, E., Robin, C., Binder, M., Dzierzak, E., and Speck, N.A. (2002). Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. Immunity *16*, 661-672.

Ohneda, O., Fennie, C., Zheng, Z., Donahue, C., La, H., Villacorta, R., Cairns, B., and Lasky, L.A. (1998). Hematopoietic stem cell maintenance and differentiation are supported by embryonic aorta-gonad-mesonephros region-derived endothelium. Blood *92*, 908-919.

Ottersbach, K., and Dzierzak, E. (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. Dev Cell *8*, 377-387.

Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L.*, et al.* (1999). Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science *283*, 845-848.

Puri, M.C., and Bernstein, A. (2003). Requirement for the TIE family of receptor tyrosine kinases in adult but not fetal hematopoiesis. Proc Natl Acad Sci U S A *100*, 12753-12758.

Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P.G., Riminucci, M.*, et al.* (2007). Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell *131*, 324-336.

Sanchez, M.J., Holmes, A., Miles, C., and Dzierzak, E. (1996). Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. Immunity *5*, 513-525.

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells *4*, 7-25.

Seeman, E., and Delmas, P.D. (2006). Bone quality--the material and structural basis of bone strength and fragility. N Engl J Med *354*, 2250-2261.

Solar, G.P., Kerr, W.G., Zeigler, F.C., Hess, D., Donahue, C., Sauvage, F.J.d., and Eaton, D.L. (1998). Role of c-mpl in early hematopoiesi. Blood *92*, 4-10.

Spangrude, G.J., Brooks, D.M., and Tumas, D.B. (1995). Long-term repopulation of irradiated mice with limiting numbers of purified hematopoietic stem cells: in vivo expansion of stem cell phenotype but not function. Blood *85*, 1006-1016.

Stier, S., Ko, Y., Forkert, R., Lutz, C., Neuhaus, T., Grunewald, E., Cheng, T., Dombkowski, D., Calvi, L.M., Rittling, S.R.*, et al.* (2005). Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J Exp Med *201*, 1781-1791.

Street, J., Bao, M., deGuzman, L., Bunting, S., Peale, F.V., Jr., Ferrara, N., Steinmetz, H., Hoeffel, J., Cleland, J.L., Daugherty, A.*, et al.* (2002). Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc Natl Acad Sci U S A *99*, 9656-9661.

Suda, T., Arai, F., and Hirao, A. (2005). Hematopoietic stem cells and their niche. Trends Immunol *26*, 426-433.

Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity *25*, 977-988.

Suzuki, N., Ohneda, O., Minegishi, N., Nishikawa, M., Ohta, T., Takahashi, S., Engel, J.D., and Yamamoto, M. (2006). Combinatorial Gata2 and Sca1 expression defines hematopoietic stem cells in the bone marrow niche. Proc Natl Acad Sci U S A *103*, 2202-2207.

Taichman, R.S., and Emerson, S.G. (1994). Human osteoblasts support hematopoiesis through the production of granulocyte colony-stimulating factor. J Exp Med *179*, 1677-1682.

Taniguchi, H., Toyoshima, T., Fukao, K., and Nakauchi, H. (1996). Presence of hematopoietic stem cells in the adult liver. Nature Medicine *2*, 198-203.

Tombran-Tink, J., and Barnstable, C.J. (2004). Osteoblasts and osteoclasts express PEDF, VEGF-A isoforms, and VEGF receptors: possible mediators of angiogenesis and matrix remodeling in the bone. Biochem Biophys Res Commun *316*, 573-579.

Trowbridge, J.J., Scott, M.P., and Bhatia, M. (2006). Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration. Proc Natl Acad Sci U S A *103*, 14134-14139.

Visnjic, D., Kalajzic, Z., Rowe, D.W., Katavic, V., Lorenzo, J., and Aguila, H.L. (2004). Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. Blood *103*, 3258-3264.

Wilson, A., Murphy, M.J., Oskarsson, T., Kaloulis, K., Bettess, M.D., Oser, G.M., Pasche, A.C., Knabenhans, C., Macdonald, H.R., and Trumpp, A. (2004). c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev *18*, 2747-2763.

Wolber, E.M., and Jelkmann, W. (2002). Thrombopoietin: the novel hepatic hormone. News Physiol Sci *17*, 6-10.

Wolf, N.S., Kone, A., Priestley, G.V., and Bartelmez, S.H. (1993). In vivo and in vitro characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. Experimental Hematology *21*, 614-622.

Wright, D.E., Wagers, A.J., Gulati, A.P., Johnson, F.L., and Weissman, I.L. (2001). Physiological migration of hematopoietic stem and progenitor cells. Science *294*, 1933-1936.

Yang, B., Kirby, S., Lewis, J., Detloff, P.J., Maeda, N., and Smithies, O. (1995). A mouse model for beta 0-thalassemia. Proc Natl Acad Sci U S A *92*, 11608-11612.

Yao, L., Yokota, T., Xia, L., Kincade, P.W., and McEver, R.P. (2005). Bone marrow dysfunction in mice lacking the cytokine receptor gp130 in endothelial cells. Blood *106*, 4093-4101.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., *et al.* (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.

Zhu, J., Garrett, R., Jung, Y., Zhang, Y., Kim, N., Wang, J., Joe, G.J., Hexner, E., Choi, Y., Taichman, R., *et al.* (2007). Osteoblasts support B lymphocyte commitment and differentiation from hematopoietic stem cells. Blood *109*, 3706-3712.

Zon, L.I. (1995). Developmental biology of hematopoiesis. Blood *86*, 2876-2891.

Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I., and Littman, D.R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature *393*, 595-599.

CHAPTER 2

DIFFERENTIAL EXPRESSION OF SLAM FAMILY MEMBERS DISTINGUISHES STEM AND PROGENITOR CELLS IN THE HEMATOPOIETIC SYSTEM AND REVEALS ENDOTHELIAL NICHES FOR STEM CELLS[1]

SUMMARY

To improve our ability to identify HSCs and their localization in vivo we compared the gene expression profiles of highly purified hematopoietic stem cells (HSCs) and non-self-renewing multipotent hematopoietic progenitors (MPPs). Cell surface receptors of the SLAM family, including CD150, CD244, and CD48, were differentially expressed among functionally distinct progenitors. HSCs were highly purified as CD150+CD244-CD48- cells while MPPs were CD244+CD150-CD48- and most restricted progenitors were CD48+CD244+CD150-. The primitiveness of hematopoietic progenitors could thus be predicted based on the combination of SLAM family members they expressed. This is the first family of receptors whose combinatorial expression precisely distinguishes stem and progenitor cells. The ability to purify HSCs based on a simple combination of SLAM receptors allowed us to identify HSCs in tissue sections. Many HSCs were associated with sinusoidal endothelium in spleen and bone

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marrow, though some HSCs were associated with endosteum. HSCs thus occupy multiple niches, including sinusoidal endothelium in diverse tissues.

INTRODUCTION

Two central and related questions in stem cell biology involve the identification of markers that distinguish stem cells from other progenitors, and the identification of microenvironments ('niches') in which stem cells reside (Morrison et al., 1997a; Spradling et al., 2001). These issues have been studied extensively in the hematopoietic system (Hackney et al., 2002; Ivanova et al., 2002; Lemischka, 1997; Phillips et al., 2000; Ramalho-Santos et al., 2002), but our inability to rigorously identify HSCs using simple combinations of markers has compromised our ability to study HSC microenvironments in vivo.

The locations and identities of differentiated cells are often defined by the differential expression of individual families of cell surface receptors. The locations and identities of olfactory sensory neurons and chemosensory neurons are distinguished by their differential expression of olfactory receptors (Buck and Axel, 1991), and Mrg family receptors (Dong et al., 2001) respectively. Although the gene expression profiles of multiple stem cell populations have been described (Easterday et al., 2003; Evsikov and Solter, 2003; Fortunel et al., 2003; Ivanova et al., 2002; Iwashita et al., 2003; Ramalho-Santos et al., 2002), no single family of cell surface receptors has yet been found in which members are differentially expressed in a way that correlates with primitiveness or developmental potential.

In the absence of simple combinations of markers that reliably purify HSCs, it has been necessary to use complex combinations of markers. HSCs have been highly enriched as Thy-1loSca-1+Lineage-c-kit+ cells or CD34-Sca-1+Lineage-c-kit+ cells using combinations of 10 to 12 surface markers (Morrison and Weissman, 1994; Osawa et al., 1996; Spangrude et al., 1988). But even using these complicated sets of markers, only 20% of intravenously injected cells gave long-term multilineage reconstitution in most studies (Morrison et al., 1995; Osawa et al., 1996; Spangrude et al., 1995; Wagers et al., 2002). By gating more restrictively on existing HSC markers, or by combining these with Hoechst exclusion, nearly homogeneous subsets of bone marrow HSCs have been isolated (Matsuzaki et al., 2004; Takano et al., 2004; Uchida et al., 2003) but these markers remain too complex for the identification of HSCs in tissue sections.

Studies of various transgenic mice have demonstrated the functional importance of osteoblasts in regulating bone marrow HSCs (Calvi et al., 2003; Visnjic et al., 2004; Zhang et al., 2003). Immunofluorescence studies with markers of primitive hematopoietic progenitors have suggested that HSCs interact with osteoblasts at the endosteum of bone marrow (Arai et al., 2004; Wilson et al., 2004; Zhang et al., 2003). However, not all HSCs can be associated with osteoblasts. The ability of cytokines to mobilize HSCs into circulation within minutes (Laterveer et al., 1995) has suggested that a subset of HSCs must be closely associated with blood vessels in the bone marrow (Heissig et al., 2002). Moreover, there are no osteoblasts in sites of extramedullary hematopoiesis, like the liver and spleen, where HSCs are maintained throughout adult life (Taniguchi et al., 1996). These observations indicate that the endosteum/osteoblast microenvironment is unlikely to be the sole supportive niche for HSCs.

Endothelial cells also regulate HSC function and could contribute to the creation of HSC niches. Endothelial cells are capable of maintaining HSCs in culture (Cardier and Barbera-Guillem, 1997; Li et al., 2004; Ohneda et al., 1998), and ablation of endothelial cells in vivo by administration of anti-VE-cadherin antibody leads to hematopoietic failure (Avecilla et al., 2004). Endothelial cells also create stem cell niches in other tissues such as the nervous system (Capela and Temple, 2002; Louissaint et al., 2002; Palmer et al., 2000; Shen et al., 2004). Simple combinations of markers that identify HSCs with high reliability would make it possible to test whether HSCs interact with endothelial cells in vivo.

We have found that SLAM family receptors are differentially expressed among hematopoietic progenitors in a way that correlates with progenitor primitiveness. The SLAM family is a group of 10 to 11 cell surface receptors that are tandemly arrayed at a single locus on chromosome 1 (Engel et al., 2003; Sidorenko and Clark, 2003). SLAM family members regulate the proliferation and activation of lymphocytes (Howie et al., 2002; Wang et al., 2004) but were not known to be expressed by hematopoietic progenitors. Our ability to purify HSCs using simple combinations of SLAM family members made it possible to image HSCs in tissue sections using markers that had been validated as yielding high HSC purity in functional assays. Many HSCs within the bone marrow and spleen were associated with sinusoidal endothelium. This indicates that HSC niches are heterogeneous and reveals the importance of sinusoidal endothelial cells for the localization of HSCs throughout hematopoietic tissues.

RESULTS

The purity of cells used for gene expression profiling

To identify genes that are tightly associated with HSC identity we have compared the gene expression profiles of highly enriched populations of HSCs and transiently reconstituting multipotent progenitors (MPPs) (Morrison et al., 1997b; Morrison and Weissman, 1994). HSCs were isolated as Thy-1loSca-1+Lineage-c-kit+ cells. One out of every 4.9±2.5 (20%) intravenously injected Thy-1loSca-1+Lineage-c-kit+ cells long-term multilineage reconstituted irradiated mice in limit dilution competitive reconstitution assays (Table 2.3).

Thy-1loSca-1+Lineage-c-kit+ HSCs give rise to non-self-renewing Thy-1loSca-1+Mac-1loCD4lo MPPs in vivo (Morrison et al., 1997b). We have enhanced the purity of this cell population by further selecting the B220- subset of Thy-1loSca-1+Mac-1loCD4lo cells (Table 2.4). 1 out of every 4.0 intravenously injected Thy-1loSca-1+Mac-1loCD4loB220- cells (25%) reconstituted irradiated mice in competitive reconstitution assays, usually giving transient multilineage reconstitution (Table 2.4). Thy-1loSca-1+Mac-1loCD4loB220- cells are more highly enriched for MPPs than any previously characterized cell population.

Genes that are tightly linked to HSC identity

We isolated 3 independent 5,000 cell aliquots of HSCs or MPPs or 8,000 CD45+ bone marrow cells (which include nearly all hematopoietic cells; more of these cells were used because they have a lower RNA content), and independently extracted and amplified RNA from each aliquot for gene expression profiling (Iwashita et al., 2003).

The gene expression profiles were compared using Affymetrix oligonucleotide arrays (Table 2.5). Variability was low among samples of the same type: Pearson correlation coefficient, R^2=0.988 to 0.991 for untransformed data (Table 2.5). However, the variability between samples of different types (HSC versus MPP, R^2=0.798±0.024; HSC versus CD45+, R^2=0.558±0.009) was significantly higher (P<0.0005). Transcript expression was detected (present calls) at 46% of probe sets for HSCs, 46% of probe sets for MPPs, and 41% of probe sets for CD45$^+$ cells.

We identified genes for which signal intensities were at least 3-fold higher in HSCs, the difference was statistically significant (P<0.05), and signals were significantly above background in at least one HSC sample (non-zero present call). We identified 1151 probe sets that satisfied these criteria for being upregulated in HSCs as compared to CD45+ cells, and 46 probe sets in HSCs as compared to MPPs (out of 36,701 probe sets total). Twenty-seven of these 46 probe sets were expressed at higher levels in HSCs as compared to both MPPs and CD45+ cells (Table 2.1). To further evaluate these genes, we compared their expression by quantitative (real-time) RT-PCR in at least two independent samples of HSCs, MPPs, and whole bone marrow cells. Of the 25 genes against which qPCR primers could be designed, all were confirmed as being expressed at >1.9-fold higher levels in HSCs as compared to MPPs and CD45+ cells (Table 2.1).

CD150 (SLAM) is expressed by HSCs but not by MPPs

One of these genes encodes the homotypic cell surface receptor CD150 (SLAM), which was not previously identified as being expressed in stem cells or other hematopoietic progenitors. CD150 is the founding member of the SLAM family of cell

surface receptors (Engel et al., 2003; Sidorenko and Clark, 2003). CD150 appeared to be 4 to 17-fold upregulated in HSCs as compared to MPPs and CD45+ bone marrow cells by microarray analysis and qPCR (Table 2.1). Only 6.6±1.7% of whole bone marrow cells were CD150+ by flow-cytometry (Figure 2.1A). Consistent with the trends observed at the RNA level (Table 2.1), CD150 was expressed by 46±12% of cells within the Thy-1loSca-1+Lineage-c-kit+ HSC population but by only 0.9±0.5% of cells in the Thy-1loSca-1+Mac-1loCD4loB220- MPP population (Figure 2.1B,C).

To test whether CD150+ cells include HSCs, we performed competitive reconstitution assays in which CD150+ or CD150- donor bone marrow cells were transplanted into lethally irradiated recipient mice along with a radioprotective dose of recipient-type whole bone marrow cells (Figure 2.1D). In each of two independent experiments, recipients of the CD150+ cells were long-term multilineage reconstituted by donor cells (6/6 mice) while recipients of CD150- cells almost always (8/9 mice) exhibited transient multilineage reconstitution (1/9 mice was long-term multilineage reconstituted). These data indicate that HSCs are enriched in the CD150+ fraction and depleted in the CD150- fraction of bone marrow cells. Recipients of CD150+ bone marrow cells were always able to transfer long-term multilineage donor cell reconstitution to secondary recipients, while recipients of CD150- cells were not able to transfer donor cell reconstitution to secondary recipients (data not shown). These results identify CD150 as a new marker of HSCs.

CD244 is expressed by transiently reconstituting MPPs but not by HSCs

To test whether other SLAM family members might be differentially expressed between hematopoietic progenitors we examined the SLAM family member CD244 which was not detected in HSCs by microarray analysis (Table 2.1). At the protein level, only $8.9\pm0.6\%$ of bone marrow cells expressed CD244 by flow-cytometry (Figure 2.2A). Little or no CD244 staining was detected among Thy-1loSca-1+Lineage-c-kit+ HSCs (Figure 2.2B) but $33\pm10\%$ of cells in the Thy-1loSca-1+Mac-1loCD4lo MPP population were CD244+ (Fig. 1.2C). This suggested that at least some MPPs expressed CD244.

We performed two independent competitive reconstitution assays in which CD244+ or CD244- donor bone marrow cells were transplanted into lethally irradiated recipient mice along with a radioprotective dose of recipient-type whole bone marrow cells (Figure 2.2D). Recipients of the CD244- cells were always long-term multilineage reconstituted by donor cells (8/8 mice) while recipients of the CD244+ cells were always transiently multilineage reconstituted (8/8 mice). Consistent with this, recipients of CD244- cells were always able to transfer long-term multilineage reconstitution to secondary recipients, while recipients of CD244+ cells were never able to transfer donor cell reconstitution to secondary recipients (data not shown; transplants were performed 16 weeks after reconstitution of primary recipients). These data indicate that HSCs are contained within the CD244- fraction, while at least some transiently reconstituting MPPs are contained within the CD244+ fraction of bone marrow cells.

CD48 is expressed by restricted progenitors but not by HSCs or MPPs

By microarray analysis, the SLAM family member CD48 was expressed at significantly higher levels on CD45+ cells as compared to HSCs or MPPs (fold change>3). CD48 is a ligand for CD244 (Engel et al., 2003). To test whether CD48+ cells were depleted of HSC activity, we performed competitive reconstitution assays in which CD48+ or CD48- donor bone marrow cells were transplanted into lethally irradiated recipient mice along with a radioprotective dose of recipient-type whole bone marrow cells (Figure 1.3). Recipients of the CD48- cells were always long-term multilineage reconstituted by donor cells (5/5 mice) while recipients of the CD48+ cells were always reconstituted by B cells (6/6 mice), but never by myeloid cells (0/6) and rarely by T cells (1/6). These data indicate that CD48+ cells include restricted B cell progenitors, but that all HSCs and MPPs are CD48-.

Although CD48 was not expressed by multipotent progenitors, it was expressed by most progenitors that formed myeloerythroid colonies in culture in addition to B lineage progenitors (Figure 2.3D, E). 83.4±3.7% of all colony-forming progenitors from bone marrow fell within the CD48+CD244+CD150- population, which accounts for only 5.1±0.4% of bone marrow cells (Figure 2.3E). Some additional erythroid (BFU-E) and megakaryocyte (CFU-Meg) progenitors fell within the CD48+CD244+CD150- population (Figure 2.3E). While CD150 is expressed by HSCs, and CD244 is expressed by transiently reconstituting MPPs, most colony-forming progenitors express CD48. Each of these markers is thus expressed at a different stage of the hematopoiesis hierarchy.

A SLAM code for hematopoietic stem cells

These results raised the possibility that HSCs and other progenitors could be isolated based on combinations of SLAM family members (SLAM codes). HSC activity was contained in the CD150+ but rarely in the CD150- fraction (Figure 2.1D), the CD244- but not the CD244+ fraction (Figure 2.2D), and the CD48- but not the CD48+ fraction (Figure 2.3D). The CD150+CD48-CD244- fraction of bone marrow cells represented only 0.0084±0.0028% of whole bone marrow cells. Since CD150+CD48- cells were uniformly CD244- (Figure 2.4A,B), we tested the reconstituting potential of CD150+CD48- cells. On average, 1 out of every 4.8±2.7 (21%) injected cells engrafted and yielded long-term multilineage reconstitution (Figure 1.4C; Table 1.6). These results are similar to those obtained with Thy-1loSca-1+Lineage-c-kit+ cells (1 in 4.9±2.5 engrafted and yielded long-term multilineage reconstitution; Table 2.3), indicating that the simple combination of CD150 and CD48 can highly enrich HSCs.

To test whether the combination of CD150 and CD48 with other markers might yield an even more highly enriched population of HSCs we competitively reconstituted irradiated mice with single CD150+CD48-Sca-1+Lineage-c-kit+ cells in five independent experiments (Table 2.2). Only 0.0058±0.0012% of bone marrow cells were CD150+CD48-Sca-1+Lineage-c-kit+. One CD150+CD48-Sca-1+Lineage-c-kit+ cell was sorted per well and then the contents of each well were individually injected into the recipient mice. We visually confirmed the presence of a single cell per well prior to injection, and functionally confirmed the presence of a single cell per well in control studies (Figure 2.8). 1 out of every 2.1 CD150+CD48-Sca-1+Lineage-c-kit+ cells (47%) engrafted and gave long-term multilineage reconstitution. In contrast, the CD150- subset

of Thy-1loSca-1+Lineage-c-kit+ cells (Figure 2.1B) did not give long-term multilineage reconstitution (data not shown). The combination of CD150 and CD48 with previously identified HSC markers significantly increased HSC purity.

The HSC SLAM code is conserved among mouse strains

One impediment in the identification of HSCs is that some of the best markers, including Thy-1 and Sca-1, are not conserved among mouse strains (Spangrude and Brooks, 1992, 1993). To test whether the HSC SLAM markers are conserved among mouse strains, we isolated CD150+CD48- cells from the bone marrow of Balb/c and DBA/2 mice, which arise from distinct breeding lineages as compared to C57BL mice (Laboratory, 1997). CD150+CD48- cells were also rare in Balb/c and DBA/2 mice, representing 0.016±0.002% or 0.028±0.007% of bone marrow cells respectively (Figure 2.5 A, B). While Balb/c mice have a similar frequency of HSCs in their bone marrow as C57BL mice, DBA/2 mice have a 2 to 3 fold increase in HSC frequency (deHaan et al., 1997).

Ten male CD150+CD48- cells from either strain were injected into lethally irradiated female recipients, along with a radioprotective dose of 200,000 female bone marrow cells. Blood chimerism was tested by quantitative (real-time) PCR using primers that amplify *SRY*, a Y-chromosome (donor) marker. Sixteen weeks after transplantation, 11 out of 16 Balb/c recipients were reconstituted (>1.5%) by male cells, averaging 10.8±14.3% of blood cells (Figure 2.5E). Seven out of 15 DBA/2 recipients were clearly reconstituted (>0.6%) by male cells, averaging 3.5±3.8% of blood cells (Figure 2.5F). Lower levels of DBA/2 reconstitution were expected given that DBA/2 mice have more

competing HSCs in co-transplanted female bone marrow. Two additional DBA/2 mice
exhibited very low levels of male cells (~0.2%) and were not considered HSC
reconstituted.

To confirm that these mice exhibited multilineage reconstitution, 3 reconstituted
mice and 1 unreconstituted mouse from each strain were sacrificed and myeloid (Mac-
1+B220-CD3-), B (B220+CD3-Mac-1-), and T (CD3+Mac-1-B220-) cells were isolated
from their spleens. In each case, the reconstituted mice had male cells in all 3 lineages
while the unreconstituted mice did not (Figure 2.5E,F). These results correspond to 1 out
of 9.1 Balb/c CD150+CD48- cells (11%) and 1 out of 16.4 DBA CD150+CD48- cells
(6%) giving long-term multilineage reconstitution. HSCs from Balb/c and DBA/2 mice
are also highly enriched within the CD150+CD48- population.

Identifying HSCs using simple markers that yield high purity in functional assays

1 out of 4.8 (21%) CD150+CD48- cells from C57BL mice gave long-term
multilineage reconstitution (Figure 2.4, Table 2.6). This raised the possibility of
identifying HSCs in tissue sections using a simple two-color stain. Initial studies revealed
that a subset of megakaryocytes also appeared CD150+CD48- in sections (data not
shown), partially explaining why not every CD150+CD48- cell gave long-term
multilineage reconstitution. To enhance our ability to reliably identify HSCs in tissue
sections, we sought an additional marker that would distinguish HSCs from
megakaryocytes. CD41 is a commonly used marker of megakaryocyte lineage cells (Na
Nakorn et al., 2003; Phillips et al., 1988). Although CD41 is expressed by primitive
HSCs, CD41 is downregulated by HSCs during the transition to definitive hematopoiesis

and most adult HSCs do not express CD41 (Ferkowicz et al., 2003; Mikkola et al., 2003). By flow-cytometry, 37±5% of CD150+CD48- cells were CD41+ (Figure 2.6A), and megakaryocytes (which can be recognized by their unique size and morphology) were excluded when sections were examined for CD150+CD48-CD41- cells (Figure 2.7C). The exclusion of CD41+ cells thus further enhances the purity of CD150+CD48- HSCs.

To confirm that adult HSCs were CD41-, even in extramedullary tissues, we sorted CD41+ and CD41- cells from the spleen after cyclophosphamide/G-CSF treatment (Figure 2.6B). Cyclophosphamide/G-CSF treatment leads to the mobilization of HSCs from the bone marrow, increasing the frequency of HSCs in the spleen (Morrison et al., 1997c). While CD41- cells always (5/5) gave long-term multilineage reconstitution in recipient mice, we never (0/5) detected reconstitution from CD41+ cells (Figure 1.6C). Adult HSCs do not express detectable CD41 in mobilized spleen, consistent with previous studies of adult bone marrow.

To test the purity of CD150+CD48-CD41- cells, we isolated this population from normal bone marrow (where they represented 0.0065±0.0009% of cells) and mobilized spleen (where they represented 0.0063±0.0005% of cells), and injected single cells into irradiated mice in competitive reconstitution assays. 1 out of every 2.2 bone marrow CD150+CD48-CD41- cells (45 %) gave long-term multilineage reconstitution (Table 2.2). 1 out of every 3 CD150+CD48-CD41- cells from mobilized spleen (33%; 4/12 mice) gave long-term multilineage reconstitution (Table 2.2), a dramatic increase in purity relative to mobilized Thy-1loSca-1+Lineage-c-kit+ cells (Morrison et al., 1997c). This two-color stain thus simplifies and improves HSC isolation.

Extramedullary HSCs associate with sinusoidal endothelium

Prior studies have imaged the interaction of primitive hematopoietic progenitors with osteoblasts in the endosteum of bone marrow (Arai et al., 2004; Wilson et al., 2004; Zhang et al., 2003) (see Figure 2.9 for schematic of bone marrow and spleen architecture) but HSCs have not been imaged in sites of extramedullary hematopoiesis. To address this we have examined the spleens of cyclophosphamide/G-CSF mobilized mice. CD150+CD48-CD41- cells were mainly associated with sinusoidal endothelium in parafollicular areas (red pulp) of the mobilized spleen. To be sure that rare differentiated cells could not appear to be CD150+CD48-CD41- by immunohistochemistry we further stained these cells with lineage markers (Gr-1, Mac-1, B220, CD2, CD3, CD4, and CD8) to exclude myeloid, B, and T cells. Only 0.0059±0.005% of cells in sections were CD150+CD48-CD41-Lineage- (identified by scanning 633,000 cells in sections from 3 independent spleens; a frequency comparable to the frequency detected by flow-cytometry) and all of these were found in the parafollicular red pulp. Moreover, these cells expressed Sca-1 and CD45, just like the CD150+CD48-CD41- cells identified by flow-cytometry (Figure 2.10). Of the 37 CD150+CD48-CD41-Lineage- cells that were observed, 23 (62%) were in contact with sinusoidal endothelial cells (Figure 2.7A). Another 14 CD150+CD48-CD41-Lineage- cells (38%) were not visibly in contact with endothelium, though they were always near (<10 cell diameters) sinusoids in the red pulp (Figure 2.7B). Compared to other cells in red pulp, CD150+CD48-CD41-Lineage- cells were more than 3-fold more likely to be in contact with sinusoids, as 18.2±2.2% of all nucleated cells in the red pulp were in contact with sinusoids. The proportion of cells

associated with sinusoids in white pulp was much lower. These data suggest that most HSCs within the mobilized spleen are associated with sinusoidal endothelium.

Bone marrow HSCs associate with sinusoidal endothelium in addition to endosteum

We observed a total of 35 CD150+CD48-CD41-Lineage- cells in sections from three femurs. These cells represented 0.0067±0.0016% of cells in the sections (identified by scanning 522,000 cells in sections from 3 independent bones; a frequency comparable to the frequency detected by flow-cytometry). Like in the spleen, the CD150+CD48-CD41-Lineage- cells identified in sections also expressed Sca-1 and CD45 (Figure 2.10). Most of these cells (20/35; 57%) were located in the trabecular zone, and the remaining cells were distributed throughout the diaphysis (shaft). Five of the cells (14%) were associated with endosteum, consistent with prior studies (Arai et al., 2004; Wilson et al., 2004; Zhang et al., 2003) and another 9 cells were not associated with recognizable cell types. Most of the CD150+CD48-CD41-Lineage- cells that we observed (21 of 35; 60%) were in contact with sinusoidal endothelium (Figure 2.7C). Only 10.1±1.4% of all nucleated bone marrow cells were in contact with sinusoids, indicating that CD150+CD48-CD41-Lineage- cells were 6-fold more likely to be located in contact with sinusoids. These data may underestimate the fraction of HSCs near endosteum because some bone fragments peeled away from slides after sectioning, raising the possibility that some HSCs may have been lost. Nonetheless, many HSCs appear to localize to sinusoidal endothelium.

DISCUSSION

By comparing the gene expression profiles of highly purified Thy-1loSca-1+Lineage-c-kit+ HSCs (Table 2.3), Thy-1loSca-1+Mac-1loCD4lo MPPs (Table 2.4), and CD45+ bone marrow cells we found that SLAM family receptors were differentially expressed among hematopoietic stem and progenitor cells. CD150 was expressed by HSCs but not by MPPs, and not by restricted hematopoietic progenitors (Figures 2.1, 2.3). CD244 was expressed by MPPs and by some restricted progenitors but not by HSCs (Figure 2.2). CD48 was expressed by restricted B lineage and myeloerythroid lineage progenitors, but not by multipotent progenitors (Figure 2.3). This demonstrates that SLAM family members are differentially expressed among hematopoietic progenitors in a way that correlates with primitiveness. This is the first example of a single family of cell surface receptors that is precisely differentially expressed among stem and progenitor cells in a way that predicts developmental potential.

Improving HSC purification

SLAM family members are so precisely differentially expressed that HSCs are very highly enriched within the CD150+CD48- cell population, which represents only 0.0084±0.0028% of C57BL bone marrow cells (Figure 2.4). 21% of CD150+CD48- cells (1 in 4.8) that were intravenously injected into irradiated mice gave long-term multilineage reconstitution in limit dilution reconstitution assays (Table 2.6). This demonstrates that two SLAM family markers yield HSC enrichments that are comparable to much more complex combinations of markers (Benveniste et al., 2003; Chen et al., 2003; Morrison et al., 1995; Osawa et al., 1996; Spangrude et al., 1995; Wagers et al.,

2002). Moreover, 47% of single CD150+CD48-Sca-1+Lineage-c-kit+cells (1 in 2.1) and

45% of single CD150+CD48-CD41- cells (1 in 2.2) yield long-term multilineage

reconstitution in irradiated mice (Table 2.2). SLAM family markers simplify and enhance

HSC purification, and are conserved among mouse strains (Figure 2.5).

Most of the best protocols for HSC isolation yield populations from which 20% of

intravenously injected cells engraft and give long-term multilineage reconstitution

(Benveniste et al., 2003; Chen et al., 2003; Morrison et al., 1995; Osawa et al., 1996;

Spangrude et al., 1995; Wagers et al., 2002). This raised the question of whether the

maximum efficiency with which HSCs are able to engraft after intravenous

transplantation is only around 20% (1 in 5), or whether available markers only yield

populations of HSCs that are 20% pure (Benveniste et al., 2003). Recently, HSC purities

of 40 to 96% were achieved by gating more restrictively on previously identified markers

(Matsuzaki et al., 2004; Takano et al., 2004). However, many HSCs are excluded from

these very restrictive gates, raising the possibility that highly efficient engraftment is a

property of only a subset of HSCs (Christopherson et al., 2004). The fact that we have

been able to achieve 47% functional HSC purity using new markers that include most or

all bone marrow HSCs (this study and (Uchida and Weissman, 1992)) suggests that

highly efficient engraftment is a property of nearly all HSCs in young adult bone marrow.

The ability of SLAM family members to improve HSC purity is even more

dramatic in the mobilized spleen. Only 1% of Thy-1loSca-1+Lin-c-kit+ cells (1 in 100)

from cyclophosphamide/G-CSF mobilized spleen give long-term multilineage

reconstitution, raising the possibility that mobilized HSCs might engraft less efficiently

than HSCs from normal bone marrow (Morrison et al., 1997c). The fact that 33% of

CD150+CD48-CD41- cells (1 in 3.0) from the mobilized spleen were able to give long-term multilineage reconstitution indicates that even mobilized HSCs are able to reconstitute with high efficiency.

We have not yet detected an HSC defect in *CD150*-deficient mice (Wang et al., 2004). Hematopoiesis appeared normal in *CD150*$^{-/-}$ mice as the cellularity and composition of the bone marrow, blood, spleen, and thymus were grossly normal (Figure 2.11). We did not observe any differences between *CD150*$^{-/-}$ mice and littermate controls in terms of complete blood cell counts (data not shown) or the frequency of colony forming progenitors in the bone marrow (Figure 2.11). We also did not detect any difference in HSC frequency or reconstituting potential upon transplantation into irradiated mice (Figure 2.11C, D). Thus CD150 is not required for HSC maintenance or function in young adult mice, but could regulate more subtle aspects of HSC biology.

The mechanism by which the tandemly arrayed genes at the SLAM locus are differentially expressed among primitive hematopoietic progenitors is uncertain. Loss of *CD150* did not detectably affect the expression pattern of CD48 or CD244 on HSCs or on bone marrow as a whole (data not shown). Thus signaling by CD150 did not appear to repress CD48 or CD244. Understanding the mechanisms responsible for the differential expression of SLAM genes might provide important insights into the determination of stem cell identity given that SLAM receptors are differentially expressed in a way that correlates with primitiveness.

Identifying HSC niches in tissue sections

The identification of a simple combination of markers that includes most HSCs made it possible for the first time to examine HSC localization in bone marrow and in extramedullary tissues using markers that were validated in functional assays to yield high HSC purity. Many HSCs appeared to be in contact with sinusoidal endothelium in bone marrow, while other HSCs appeared to be associated with endosteum (Figure 2.7C). The precise proportion of bone marrow HSCs in each location is uncertain given that half of CD150+CD48-CD41- cells failed to give long-term multilineage reconstitution in irradiated mice. HSCs that localized to endosteum were presumably associated with osteoblasts, consistent with prior studies (Arai et al., 2004; Calvi et al., 2003; Visnjic et al., 2004; Zhang et al., 2003). Since bone marrow cells intravasate into circulation through sinusoids, the fact that many HSCs were associated with sinusoidal endothelium explains how HSCs could be mobilized into circulation within minutes of treatment with certain cytokines (Laterveer et al., 1995). Bone marrow HSCs appear to localize to at least two distinct niches, defined by the association of HSCs with sinusoidal endothelial cells and osteoblasts in different locations within the bone marrow.

We found no heterogeneity within the CD150+CD48-CD41- HSC population that correlated with the difference in localization. For example, only 3.8% of CD150+CD48-CD41- cells in normal bone marrow were in S/G2/M phases of the cell cycle (Figure 2.12), consistent with previous studies reporting that HSCs are mainly quiescent (Cheshier et al., 1999; Morrison and Weissman, 1994). This means that the vast majority of CD150+CD48-CD41- cells associated with both sinusoids and endosteum must be in G0/G1 phase of the cell cycle.

HSCs in the spleens of mice treated with cyclophosphamide/G-CSF appeared to usually associate with sinusoidal endothelium as well (Figure 2.7A). However, since two-thirds of single CD150+CD48-CD41- cells failed to give long-term multilineage reconstitution in irradiated mice, it is not possible to infer the precise proportion of HSCs associated with sinusoids as compared to other sites. These results suggest that sinusoidal endothelial cells create a niche that sustains HSCs in extramedullary tissues. These HSCs are unlikely to be migrating into circulation because the number of HSCs in the spleen continues to increase for several days after the onset of mobilization (Morrison et al., 1997c). Moreover, most of the CD150+CD48-CD41-Lineage- cells we observed in normal adult bone marrow were also in contact with endothelial cells. The association of many HSCs with sinusoidal endothelium during steady state hematopoiesis suggests that this is not a transient interaction.

The observation that HSCs interact with sinusoidal endothelial cells in bone marrow and extramedullary tissues is consistent with observations that endothelial cells express factors that regulate HSC maintenance and function. Definitive HSCs first arise during embryonic development among endothelial cells in the dorsal aorta, and have a very close developmental relationship with the endothelial lineage (Kennedy et al., 1997; Kubo and Alitalo, 2003; North et al., 2002; Oberlin et al., 2002). Co-culture of HSCs with vascular endothelial cells from a variety of hematopoietic and non-hematopoietic tissues maintains the repopulating capacity of HSCs under conditions in which HSCs would otherwise differentiate or die (Cardier and Barbera-Guillem, 1997; Li et al., 2004; Ohneda et al., 1998). This indicates that endothelial cells express factors that promote the maintenance of HSCs. Endothelial cells appear to regulate the function of primitive

hematopoietic progenitors via multiple mechanisms in vivo (Avecilla et al., 2004; Heissig et al., 2002). These observations suggest that endothelial cells create a niche in hematopoietic tissues that sustains a substantial fraction of the HSC pool.

Neural stem cells are also thought to localize to vascular niches (Capela and Temple, 2002; Louissaint et al., 2002; Palmer et al., 2000), and endothelial cells can support the self-renewal of neural stem cells in culture (Shen et al., 2004). This raises the possibility that endothelial cells are generally important in the construction of mammalian stem cell niches and that sinusoidal endothelium represents a specialization adapted for the maintenance of HSCs.

SLAM family markers represent an important new resource for studying HSC biology. The fact that a single family of tandemly arrayed receptors can be differentially expressed among progenitors in a way that correlates with primitiveness supports the idea that stem cell identity is partially determined at the level of transcriptional regulation. SLAM family markers simplify and enhance HSC purification, demonstrating that even mobilized HSCs are able to engraft mice with high efficiency. The ability to reliably identify HSCs using a two-color stain made it possible to identify HSCs in tissue sections using functionally validated markers. This analysis revealed that a subset of HSCs associate with sinusoidal endothelial cells in bone marrow and mobilized spleen. The use of SLAM markers in future studies should refine our understanding of stem cell identity and the role of the environment in regulating HSCs function in vivo.

MATERIALS AND METHODS

All mice used in this study were housed in the Unit for Laboratory Animal Medicine at the University of Michigan. Donor hematopoietic cells were obtained from adult (6-8 week-old) C57BL/Ka-CD45.2:Thy-1.1 mice. Recipient mice in reconstitution assays were adult C57BL/Ka-CD45.1:Thy-1.2 mice.

Flow-cytometric isolation of stem and progenitor cells

Bone marrow cells were flushed from the long bones 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 (45um, Sefar America, Kansas City MO) to obtain a single cell suspension.

Thy-1loSca-1+Lineage-c-kit+ HSC and Thy-1loSca-1+Mac-1loCD4loB220-MPPs were isolated as previously described (Morrison et al., 1997b; Morrison and Weissman, 1994). For isolation of Thy-1loSca-1+Lineage-c-kit+ cells, whole bone marrow cells were incubated with unconjugated monoclonal antibodies to lineage markers including B220 (6B2), CD3 (KT31.1), CD5 (53-7.3), CD8 (53-6.7), Gr-1 (8C5) and Ter119. Following dilution, pelleted cells were resuspended in anti-rat IgG specific F(ab)$_2$ conjugated to phycoerythrin (PE; Jackson ImmunoResearch, West Grove PA). Cells were then stained with directly conjugated antibodies to Sca-1 (Ly6A/E-APC), c-kit (2B8-biotin), Thy-1.1 (19XE5-FITC), Mac-1 (M1/70-PE) and CD4 (GK1.5-PE). Progenitors were often enriched by pre-selecting for Sca-1$^+$ or c-kit$^+$ cells using paramagnetic microbeads (Miltenyi Biotec, Auburn CA) and autoMACS. For isolation

of Thy-1loSca-1+Mac-1loCD4loB220- MPPs the directly conjugated antibodies described above were combined with anti-B220-Tricolor (6B2, Caltag Burlingame CA).

Cells sorted based on CD150 expression were incubated with unconjugated antibody to CD150 (26D12; DNAX, Palo Alto CA), and subsequently stained with goat anti-rat IgG F(ab)$_2$ fragment conjugated to FITC, PE or APC (Jackson ImmunoResearch). When CD150 was combined with lineage markers, directly conjugated antibodies were used to stain lineage markers. Cells sorted according to CD41, CD48 or CD244 expression were stained with directly conjugated anti-CD41 (MWReg30-FITC), anti-CD48 (HH48-1-FITC or PE) or with directly conjugated CD244.2 (2B4-FITC). Cells were resuspended in 2μg/ml 7-AAD (Molecular Probes) or DAPI to discriminate live from dead cells. All flow-cytometry was performed on a FACS Vantage dual laser flow-cytometer (Becton-Dickinson, San Jose CA).

Long-term competitive reconstitution assays

Adult recipient mice were irradiated with an Orthovoltage x-ray source delivering approximately 300 rads/min. C57BL and DBA/2 recipient mice received two doses of 550-570rad, delivered at least two hours apart. Balb/c recipients received two doses of 495rad. When HSCs/MPPs were tested for reconstituting potential the donor (CD45.2+) population was sorted, and the number of cells to be injected per mouse were re-sorted into individual wells of a 96-well plate containing 200,000 CD45.1+ whole bone marrow cells in HBSS+. The contents of individual wells were injected into the retro-orbital venous sinus of individual lethally irradiated CD45.1+ recipients. For at least 16 weeks after transplantation, blood was obtained from the tail veins of recipient mice, subjected

to ammonium-chloride potassium red cell lysis (Morrison and Weissman, 1994), and stained with directly conjugated antibodies to CD45.2 (104, FITC), B220 (6B2), Mac-1 (M1/70), CD3 (KT31.1) and Gr-1 (8C5) to monitor engraftment.

Immunofluorescence analysis of hematopoietic tissue sections

Femurs from 6-12 week old wild-type mice were embedded in 8% gelatin (Sigma) in phosphate buffer and snap frozen in -80°C N-methylbutane chilled in a slurry of ethanol and dry ice. 7 um sections were generated using the CryoJane system (Instrumedics, Hackensack NJ) with coated slides and a tungsten carbide blade. Details of the staining procedure can be found in Supplemental Methods. To obtain spleens from cyclophosphamide/G-CSF mobilized mice, mice were injected intra-peritoneally with 4mg of cyclophosphamide (~200 mg/kg; Bristol-Myers Squibb) and then on successive days with 5µg of human G-CSF by subcutaneous injection (~250 µg/kg per day; Amgen Biologicals). Mice were sacrificed after four days of G-CSF treatment, and their spleens were dissected, sectioned, and fixed.

RNA amplification for microarray analysis

RNA extraction, amplification, and microarray analysis were performed as described (Iwashita et al., 2003).

Methylcellulose culture

Unfractionated bone marrow cells, or single resorted hematopoietic progenitors were plated in wells of 96-well plates (Corning, Corning NY) containing 100µl 1.0%

methylcellulose (Stem Cell Technologies, Vancouver BC) as previously described (Morrison et al., 1996). The methylcellulose was supplemented with 20% charcoal absorbed fetal bovine serum (Cocalico, Reamstown PA), 1% BSA (Sigma), 1% penicillin/streptomycin (Gibco) 50ng/ml stem cell factor (SCF), 10ng/ml interleukin-3 (IL-3), 10ng/ml interleukin-6 (IL-6), 3U/ml erythropoietin (Epo), 10ng/ml Flt-3 and 10ng/ml thrombopoietin (Tpo). All cytokines were obtained from R&D Systems (Minneapolis MN). Colonies were maintained at $37^{o}C$ in humidified chambers containing 6% CO_2. Colony formation was scored after 10-14 days of culture.

RNA amplification for microarray analysis

Methods for RNA extraction, amplification, and microarray analysis were as described (Iwashita et al., 2003). Briefly, total RNA was extracted from 3 independent, freshly isolated aliquots of 5,000 Thy-1loSca-1+Lineage-c-kit+ cells, 5,000 Thy-1loSca-1+Mac-1loCD4loB220- cells, or 8,000 CD45+ cells using Trizol with 250µg/ml glycogen (Roche Diagnostic Corporation, Indianapolis IN). The extracted RNA (30µl volume) was treated for 20min at 37°C with 2µl of RNase-free DNaseI (2U/µl; Ambion, Austin TX) in the presence of 2µl of RNase inhibitor (10U/µl) (Invitrogen). The RNA was then purified with RNeasy Mini Kit (Qiagen, Valencia CA) according to the manufacturer's instructions and washed 3 times with 500µl of RNase-free water in a Microcon YM-100 (Millipore, Bedford MA). After adding 0.025µg T7-d(T)$_{24}$ primer (containing a T7 RNA polymerase binding sequence; 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG(T)24,; Proligo, Boulder CO), the RNA was dried down to 2.5µl. RNA was amplified through two

consecutive rounds of amplification using a modified version of the method of Baugh et al. (Baugh et al., 2001). To make cDNA, first strand was synthesized using T7-d(T)$_{24}$ primer. After second strand synthesis, complementary RNA (cRNA) was generated by T7 RNA polymerase (Promega, Madison WI). For the second round of amplification, first strand cDNA was synthesized using random hexamers and second strand was synthesized using the T7-d(T)$_{24}$ primer. The double stranded cDNA was resuspended with 22μl RNase-free water and transcribed to cRNA with the biotin labeling kit (BioArray Highyield RNA transcript labeling kit (T7), Enzo Diagnostics, Farmingdale NY) for 12 hours. cRNA was purified using the RNeasy Mini Kit. 60μg of biotinylated cRNA were obtained from two rounds of RNA amplification from 5,000 cells.

Hybridization and data analysis

After fragmentation, 15μg of HSC cRNA were hybridized per chip to Mouse Genome U74 Arrays (version 2 Chips A, B and C; Affymetrix). The chips were hybridized and scanned according to the manufacturer's instructions. Signal intensities were analyzed as described previously (Iwashita et al., 2003). To measure fold changes, all values less than 100 were set to 100. The significance of differences in signal intensity for each probe set was evaluated by T-test using the log$_{10}$ transformed values from 3 independent replicates per cell type.

Quantitative real time-PCR (qPCR)

2,000 to 10,000 cells were directly sorted into 400ul Trizol (Ambion, Austin TX) containing 250ug/ml glycogen (Roche, Indianapolis IN). RNA was extracted according

to manufacturer's instructions. The extracted RNA (30ul volume) was treated for 20 minutes at 37°C with 2ul RNase-free DNase-1 (2U/ul; Ambion) in the presence of 2ul RNase inhibitor (10U/ul; Invitrogen). The RNA was then purified using an RNeasy Mini Kit (Qiagen, Valencia CA) according to manufacturer's instructions and washed three times with 500ul RNase-free water. The RNA was used for making cDNA by reverse transcription with 1ug random hexamer. The cDNA was extracted with phenol-chloroform and precipitated with 20ug glycogen. After dissolving the cDNA with RNase-free water, cDNA equivalent to 200 cells was used for each PCR reaction. qPCR was performed using at least two independent cell samples. Primers were designed to have a Tm of ~59°C and to generate short amplicons (100-150bp). The PCR reactions were performed using a LightCycler (Roche Diagnostic Corporation) according to the manufacturer's instructions. The RNA content of samples compared by qPCR was normalized based on the amplification of hypoxanthine phosphoribosyl transferase (HPRT). In addition to confirming the specificity of the qPCR reactions by examining the melting curves of the products, qPCR products were separated in 2% agarose gels to confirm the presence of a single band of the expected size. To estimate the difference in the expression levels of individual RNAs between samples, we assumed that one cycle difference in the timing of amplification by qPCR was equivalent to a 1.9-fold difference in expression level (90% amplification efficiency), a typical value (Fink et al., 1998).

Experiments to examine the engraftment of female Balb/c and DBA/2 mice by male HSCs involved quantitative PCR of extracted genomic DNA rather than cDNA but otherwise similar methods as described above were employed. Genomic DNA was extracted from blood cells or splenocytes and resuspended at 10-50ng/ul. The level of

male cell chimerism was tested by qPCR using primers that amplified genomic *SRY* and

ß-actin (to normalize DNA content). Known dilutions of male DNA into female DNA

were used to establish standards. Individual samples were normalized to the 100% male

reference standard according to the following formula: 1.9 exp (C_T SRY$^{100\%}$ - C_T

SRY)/1.9 exp(C_T actin$^{100\%}$- C_T actin), where C_T SRY$^{100\%}$ represents the *SRY* crossing

point for the reference sample and C_T actin$^{100\%}$ the *ß-actin* crossing point for the

reference sample. Level of chimerism in experimental animals was estimated by linear

regression using all control samples.

CD150-deficient mice

CD150-deficient mice (Wang et al., 2004) were backcrossed for more than ten

generations onto the C57BL/6 background. Genotyping was performed using the

primers: *CD150$^{+/+}$*-F (5'CAC CCC AGG CAC TTC ACC AAG TCC CAG AGC) and

CD150$^{+/+}$-R (5'GCT GGC TGT GAA CTC CCA TCC CAT CCT TG); *CD150$^{-/-}$*-

F(5'GAA AAT TGG GTC AGG AAG TAA ACG CAG) and *CD150$^{-/-}$*-R(5'GGG CCA

GCT CAT TCC TCC CAC).

Immunofluorescence analysis of HSC localization in tissue sections

Freshly dissected undecalcified femurs from 6-12 week old wild-type mice or

spleens from 6-12 week old mice treated with CY/G-CSF were embedded in 8% gelatin

(Sigma) in 0.1M phosphate buffer pH7.4 and snap-frozen in N-methylbutane chilled in a

slurry of ethanol and dry ice. Sections were generated using the CryoJane tape transfer

system (Instrumedics, Hackensack NJ) with methacrylate coated slides, a tungsten

carbide blade (Diamond Knives, Wilmington DE) and Bright Cryostat at -24$^{\text{O}}$C. The

7um sections were air-dried overnight at room temperature and subsequently fixed in -

20°C acetone for 15 minutes. Slides were then blocked with 20% goat serum in 0.1M

phosphate buffer (pH 7.4) for 40 minutes prior to antibody staining. Slides were first

incubated in 0.03ug/ul 26D12 anti-CD150 antibody (DNAX, Palo Alto CA) for 1 hour

and rinsed. Goat anti-rat IgG conjugated to Alexa555 (Molecular Probes) was added at

1/200 dilution and rinsed. Slides were then incubated in 0.1ug/ul rat IgG (Sigma-

Aldrich) for 10min and rinsed. To avoid the problem of other cells in the tissue sections

appearing to be CD150+CD48-CD41- in a way that was not detected by flow-cytometry

we included the additional panel of lineage markers in the tissue immunofluorescence

stains. By themselves these lineage markers should exclude differentiated cells,

providing redundancy within the panel of markers for the exclusion of non-HSCs.

Unconjugated anti-CD48 and FITC-conjugated anti-CD41 antibodies (each 1/100

dilution) as well as FITC-conjugated lineage markers including anti-B220 (stains B

cells), CD2 (T cells), CD4 (T cells), CD8a (T cells), Gr-1 (myeloid cells) and CD11b

(myeloid cells) antibodies (each 1/200 dilution), and biotinylated pan-endothelial cell

antigen (MECA-32) at a 1/25 dilution were then incubated for 1hr in 0.1ug/ul rat IgG and

rinsed. CD48 was visualized by incubation with FITC-conjugated goat anti-Armenian

hamster (Jackson ImmunoResearch) at a 1/200 dilution for 30min. Pan-endothelial cell

antigen was visualized with Alexa647-conjugated streptavidin (Molecular Probes) at a

1/200 dilution. Finally, slides were rinsed twice for 5 minutes each and mounted without

drying using Antifade (Molecular Probes). To ensure the accuracy of antibody labeling

of HSCs, control slides were separately stained as above without addition of CD150

primary antibody. All antibody incubations took place in blocking buffer. All rinse steps

were 2 times for 3 minutes each with blocking buffer unless otherwise indicated. All

antibodies were purchased from Becton Dickinson unless otherwise noted. Microscopy

was performed using an Olympus BX51 florescence microscope or an Olympus FV-500

confocal microscope.

**Controls to ensure that the CD150+CD48-CD41-Lineage- cells identified in tissue
sections were the same as the CD150+CD48-CD41- cells isolated by flow-cytometry
and functionally characterized**

We performed a number of controls to confirm that the CD150+CD48-CD41-

Lineage- cells identified by immunofluorescence in tissue sections were the same as the

CD150+CD48-CD41- HSCs isolated by flow-cytometry. If non-stem cells were included

in the CD150+CD48-CD41-Lineage- population identified in sections, then the

frequency of these cells in sections should be higher than the frequency of

CD150+CD48-CD41- cells identified by flow-cytometry. The CD150+CD48-CD41-

population in mobilized spleen by flow-cytometry was 0.0063±0.0005% whereas the

frequency of CD150+CD48-CD41-Lineage- cells in spleen sections was 0.0059%. The

frequency of CD150+CD48-CD41- cells in bone marrow by flow-cytometry

(0.0065±0.0009%) also did not differ from the frequency of CD150+CD48-CD41-

Lineage- cells identified within bone marrow sections (0.0067±0.0016%). These results

demonstrate that the frequency of CD150+CD48-CD41-Lineage- cells in sections was

not higher than the frequency of CD150+CD48-CD41- cells identified by flow-

cytometry.

To test whether the CD150+CD48-CD41- cells identified by flow-cytometry

represent the same population identified in tissue sections as CD150+CD48-CD41-

Lineage- cells we examined two independent markers of HSC, Sca-1 and CD45. The HSC activity within the CD150+CD48- population was also Sca-1+Lineage-c-kit+ (Table 2.2), consistent with prior studies of HSC marker expression. By flow-cytometry, we found that 88±3% of CD150+CD48-CD41- cells were Sca-1+ and in tissue sections we found that 71% of CD150+CD48-CD41-Lineage- cells were detectably Sca-1+ (Figure 2.10). This may underestimate the true frequency of Sca-1+ cells in tissue sections since the flow-cytometer is more sensitive than the eye to lower levels of staining, and the Sca-1 stain that we were forced to use in this multi-color analysis was dim. Furthermore, essentially all of the CD150+CD48-CD41- cells identified by flow-cytometry as well as the CD150+CD48-CD41-Lineage- cells identified in tissue sections were CD45+ (Figure 2.10). This demonstrates that most of the cells identified in tissue sections expressed two independent HSC markers also seen on the CD150+CD48-CD41- population by flow-cytometry.

ACKNOWLEDGEMENTS

Table 2.1: Genes that were expressed at higher levels in HSCs as compared to MPPs and CD45+ cells by both microarray analysis and quantitative PCR. cRNA from Thy-1loSca-1+Lineage-c-kit+ HSCs, Thy-1loSca-1+Mac-1loCD4loB220- MPPs, or CD45+ bone marrow cells were hybridized to oligonucleotide arrays. The average untransformed probe intensities from three independent samples were used to calculate fold-change (HSC/MPP; HSC/CD45+). The table lists all of the genes that were expressed at significantly higher levels in HSCs as compared to MPPs and CD45+ cells by both microarray analysis (fold change>3.0) and quantitative (real-time) PCR (fold change>1.9).

Table 2.1: Genes that were expressed at higher levels in HSCs as compared to MPPs and CD45+ cells by both microarray analysis and quantitative PCR.

Gene symbol	Unigene Title	Genbank accession	Microarray		qPCR	
			HSC/ MPP	HSC/ CD45	HSC/ MPP	HSC/ WBM
Clca1	chloride channel calcium activated 1	AF047838	8.3	9.1	32.8	3.3
Cpne8	Copine VIII	AV240111	7.0	11.0	3.8	29.9
Sdpr	serum deprivation response	AI839175	7.0	7.0	116.7	40.3
Catnal1	catenin alpha-like 1	AI152317	6.9	8.8	4.0	26.2
Prkcm	Protein kinase C mu	AV297026	6.4	7.0	ND	ND
Vwf	Von Willebrand factor homolog	AI843063	6.2	17.2	5.2	4.2
Est	RIKEN full-length library, clone:E330020H17	AI853427	5.7	5.7	4.9	2.8
Mjd	Machado-Joseph disease homolog	AV262417	5.2	5.9	1.9	2.3
Ly64	lymphocyte antigen 64	AV204260	4.8	13.7	9.0	53.8
D10Ertd 755e	DNA segment, Chr 10, ERATO Doi 755, expressed	AU019706	4.7	4.7	ND	ND
C530008 M17Rik	RIKEN cDNA C530008M17 gene	AI851362	4.7	7.4	15.3	2.9
Est	Mus musculus transcribed sequences	AI666656	4.1	10.7	1.9	2.8
Slam	signaling lymphocyte activation molecule	AI120008	4.0	4.0	5.8	17.0
Est	Mus musculus transcribed sequences	AV236645	3.6	9.7	5.6	7.0
Rnf125	ring finger protein 125	AV361188	3.6	6.0	2.4	2.9
Peg12	paternally expressed 12	AI413890	3.6	3.8	9.2	22.9
Bgn	biglycan	AV016619	3.5	64.1	7.7	52.6
Exosc1	exosome component 1	AI592141	3.4	7.8	2.5	2.6
Stub1	STIP1 homology and U-box containing protein 1	AW046544	3.4	5.0	2.5	2.2
Gemin4	gem (nuclear organelle) associated protein 4	AV341751	3.4	5.4	2.8	5.9
Tfpi	tissue factor pathway inhibitor	AI480514	3.2	4.9	4.6	9.2
Est	Mus musculus transcribed sequences	AI482323	3.2	16.9	3.4	10.2
pbx1	Pre B-cell leukemia transcription factor 1	AI845678	3.1	4.3	3.5	15.6
Sdsl	serine dehydratase-like	AI504310	3.1	14.7	7.0	6.1
Ppap2b	phosphatidic acid phosphatase type 2B	AV346092	3.1	3.7	2.5	2.9
Armcx1	armadillo repeat containing, X-linked 1	AI846227	3.0	3.1	5.6	34.0
Phactr2	phosphatase and actin regulator 2	AW123926	3.0	4.0	1.9	3.0

Table 2.2: **Competitive reconstitution of irradiated mice with single cells from various HSC populations reveals that diverse HSC populations are able to engraft with high efficiency in lethally irradiated mice.**

HSC population	Experiment	Cells that engrafted	Engrafted mice with long-term multilineage reconstitution	Cells that long-term multilineage reconstituted
BM CD150+CD48-Sca-1+Lineage- c-kit+	1	67% (10/15)	70% (7/10)	47% (7/15)
	2	67% (6/9)	66% (4/6)	44% (4/9)
	3	44% (8/18)	88% (7/8)	39% (7/18)
	4	53% (8/15)	88% (7/8)	47% (7/15)
	5	58% (7/12)	100% (7/7)	58% (7/12)
	Mean±SD	58±10%	82±14%	47±7% (1 in 2.1)
BM CD150+CD48-CD41-	1	30% (3/10)	100% (3/3)	30% (3/10)
	2	100% (5/5)	60% (3/5)	60% (3/5)
	Mean±SD	65%	80%	45% (1 in 2.2)
Mobilized spleen CD150+CD48-CD41-	1	42% (5/12)	80% (4/5)	33% (1 in 3)

Single cells were sorted into different wells of 96 well plates, and the wells were visually inspected to confirm that only a single cell was sorted (see Figure 2.8). Then the contents of each well were individually injected along with 300,000 recipient CD150⁻ bone marrow cells.

Table 2.3: Thy-1loSca-1+Lineage-c-kit+ cells are highly enriched for long-term reconstituting, multipotent HSCs.

Cells injected	Mice that engrafted	Frequency of cells that engrafted	Engrafted mice with long-term multilineage reconstitution	Frequency of cells that long-term multilineage reconstituted
10	24/28	1 in 5.7	88% (21/24)	1 in 7.7 (21/28)
5	13/15	1 in 3.0	100% (13/13)	1 in 3.0 (13/15)
4	25/34	1 in 3.5	92% (23/25)	1 in 4.1 (23/34)
Mean±SD	82±7%	1 in 4.1±1.4	93±6%	1 in 4.9±2.5 (20%)

The indicated number of donor-type (CD45.2+) Thy-1loSca-1+Lineage-c-kit+ cells was transplanted intravenously into lethally irradiated recipients (CD45.1+) along with 200,000 recipient-type (CD45.1+) whole bone marrow cells for radioprotection. Recipients were considered engrafted by donor cells if any CD45.2+ cells were detected in their peripheral blood (above background: >0.1-0.3% of myeloid cells or >0.1-0.15% of lymphoid cells). The frequency of cells that engrafted was calculated based on limit-dilution (Poisson) statistics (Smith et al., 1991). Mice were considered long-term multilineage reconstituted if donor-type myeloid, B, and T cells were present for more than 16 weeks after reconstitution.

Table 2.4: Thy-1loSca-1+ Mac-1loCD4loB220- cells are highly enriched for transiently reconstituting, multipotent progenitors.

Cells injected	Mice that engrafted	Frequency of cells that engrafted	Engrafted mice with short-term multilineage reconstitution	Engrafted mice with only B and T lineage reconstitution	Engrafted mice with B-only lineage reconstitution
4	69% (11/16)	1 in 4.0	64% (7/11)	27% (3/11)	9% (1/11)

In previous studies, only 26% of Thy-1loSca-1+Mac-1loCD4lo cells formed myeloerythroid colonies in methylcellulose cultures, and many clones gave rise to only B lineage reconstitution in vivo (Morrison et al., 1997b; Morrison and Weissman, 1994). This raised the question of whether this population contained multipotent progenitors, or a mixture of multipotent and lymphoid committed progenitors. To resolve this question we searched for sources of heterogeneity within the Thy-1loSca-1+Mac-1loCD4lo population, and found that 55±14% of these cells expressed the B cell marker B220. The B220+ subset of Thy-1loSca-1+Mac-1loCD4lo cells lacked the ability to form colonies in methylcellulose or to give multilineage reconstitution in vivo (data not shown), while the B220- subset was more highly enriched for transiently reconstituting multipotent progenitors.

Table 2.5: **Summary of the microarray analyses of Thy-1loSca-1+Lineage-c-kit+ HSCs, Thy-1loSca-1+Mac-1loCD4lo MPPs, and CD45+ bone marrow cells.**

A. Genes that were differentially expressed among HSCs, MPPs, and CD45+ cells

based on an analysis of oligonucleotide arrays containing 36,701 probe sets

Probe sets with fold change>3, P<0.05, non zero present call

HSC>MPP	47 probe sets
HSC<MPP	236 probe sets
HSC>CD45+	1151 probe sets
HSC<CD45+	835 probe sets

B. Squared Pearson correlation coefficients (R^2 values) between similar or different samples

		Mean±SD
Among replicate HSC samples, N=3	log transformed	0.894±0.012
	non-log transformed	0.991±0.002
Among replicate MPP samples, N=3	log transformed	0.894±0.003
	non-log transformed	0.988±0.003
Among replicate CD45+ samples, N=3	log transformed	0.859±0.012
	non-log transformed	0.991±0.0004
Between HSCs and MPPs, N=9	log transformed	0.851±0.011
	non-log transformed	0.798±0.024
Between HSCs and CD45+ samples, N=9	log transformed	0.686±0.013
	non-log transformed	0.558±0.009

To calculate the squared Pearson's correlation coefficient between two groups, we transformed each value to the base 10 logarithm ($\log_{10}$). $\log_{10}$ transformation is statistically preferred because the Pearson's correlation coefficient should be calculated based on normally distributed data, and the untransformed data are not normally distributed.

Table 2.6: CD150+CD48- bone marrow cells are highly enriched for long-term self-renewing, multipotent HSCs based on the ability of 3 or 5 CD150+CD48- cells to competitively reconstitute lethally irradiated mice.

Cell dose	Mice that engrafted	Frequency of cells that engrafted	Engrafted mice with long-term multilineage reconstitution	Frequency of cells that long-term multilineage reconstituted (HSCs)
5	14/15	1 in 2.4	93% (13/14)	1 in 3.1 (13/15)
3	4/6	1 in 3.2	50% (2/4)	1 in 7.9 (2/6)
3	9/14	1 in 3.4	100% (9/9)	1 in 3.4 (9/14)
Mean±SD		1 in 3.0±0.5	81±27%	1 in 4.8±2.7 (21%)
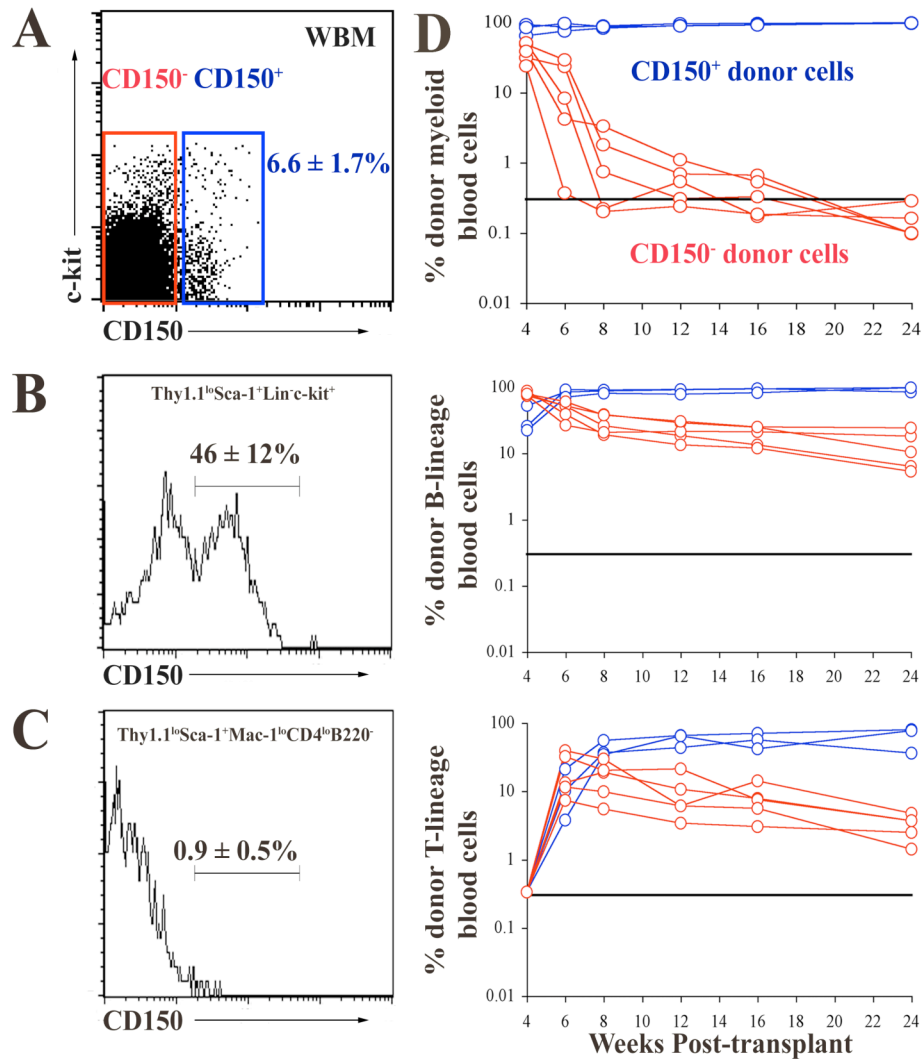
Figure 2.1: CD150+ cells are enriched for HSCs while CD150- cells include transiently reconstituting MPPs. Only 6.6% of bone marrow cells express CD150 (A). CD150 expression was detected among Thy-1lowSca-1+Lineage-c-kit+ HSCs (B) but not among Thy-1lowSca-1+Mac-1loCD4loB220- MPPs (C). 20,000 CD150+ bone marrow cells gave rise to long-term multilineage reconstitution in all recipients (D, blue lines), while 180,000 CD150- bone marrow cells gave transient multilineage reconstitution (D, red lines). Cell doses were based on the fraction of 200,000 whole bone marrow cells that were CD150+ or CD150- as in prior HSC marker studies (Morrison et al., 1995; Uchida and Weissman, 1992). The black line at 0.3% represents the background threshold, meaning that reconstitution could not be detected in mice falling below this line. Data are from one of two independent experiments that gave similar results. Each line represents the frequency of donor-derived myeloid, B, or T cells in a single mouse.

63
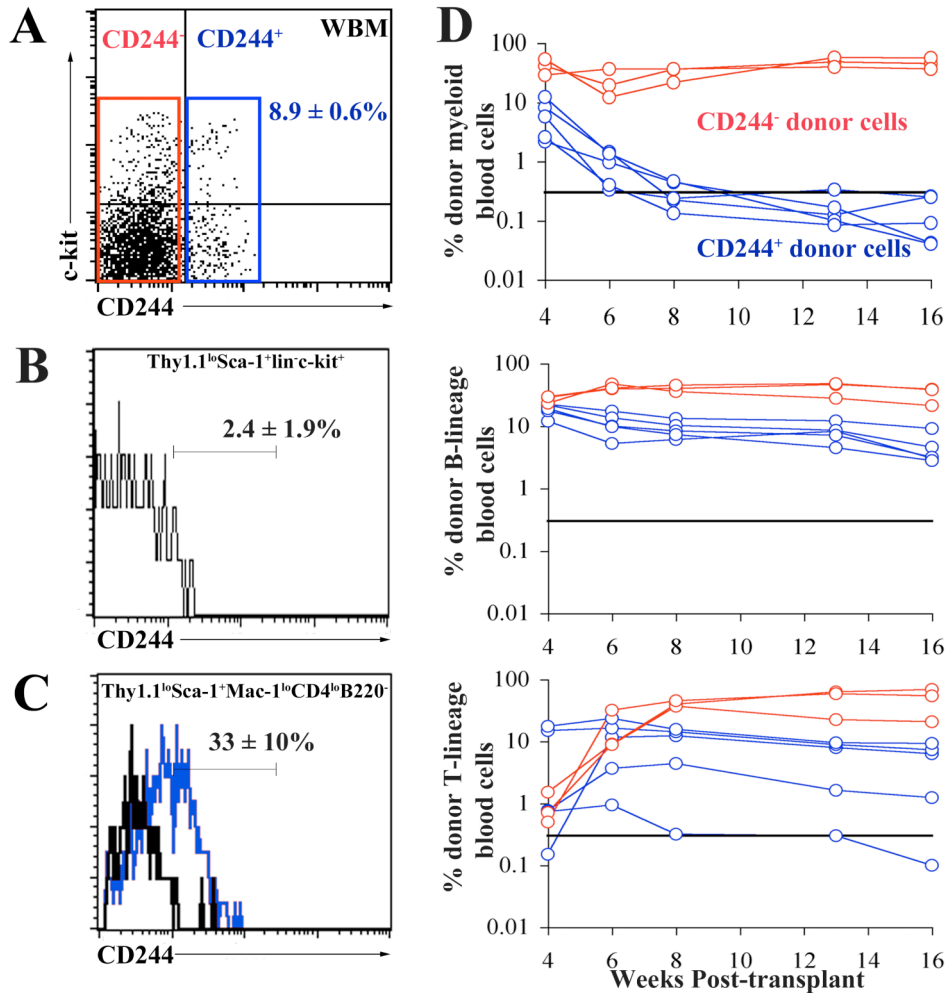
Figure 2.2: HSCs are CD244- while transiently reconstituting multipotent progenitors are CD244+. Only 8.9% of bone marrow cells express CD244 (A). CD244 expression was not detected within the Thy-1lowSca-1+Lineage-c-kit+ HSC population (B) but was detected on approximately 33% of Thy-1lowSca-1+Mac-1loCD4loB220- MPPs (C). Note that the black histogram represents background fluorescence while the blue histogram represents staining with the directly conjugated anti-CD244 antibody. 20,000 CD244+ bone marrow cells gave transient multilineage reconstitution in all recipients (blue lines), while 180,000 CD244- bone marrow cells gave long-term multilineage reconstitution in all recipients (red lines). The data are from one of two independent experiments that gave similar results.
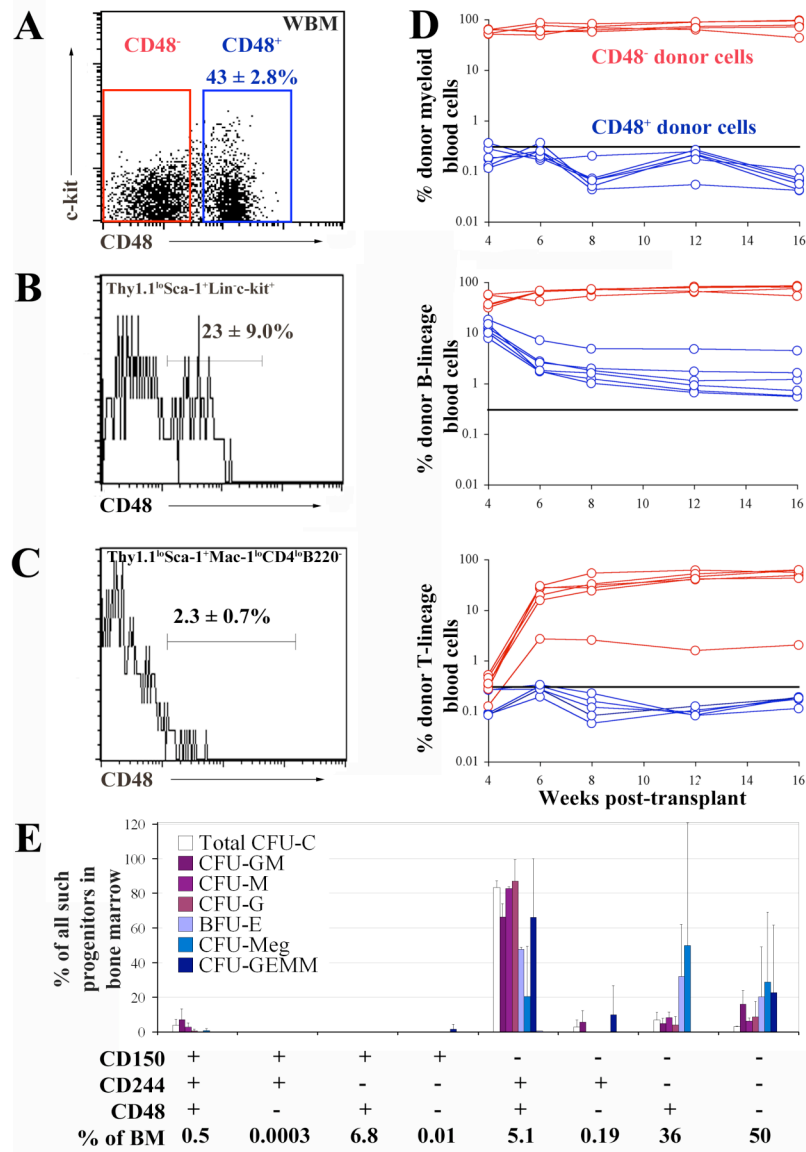
Figure 2.3: HSCs and MPPs are contained within the CD48- fraction while colony-forming progenitors are mainly in the CD48+ fraction of bone marrow cells. 43% of bone marrow cells express CD48 (A). CD48 expression was detected in the Thy-1loSca-1+Lineage-c-kit+ HSC population (B) but not in Thy-1loSca-1+Mac-1loCD4loB220- MPPs (C). All multilineage reconstituting activity was contained within the CD48- cell fraction (D). 80,000 CD48+ bone marrow cells gave rise only to B cells in all recipients (blue lines), while 120,000 CD48- bone marrow cells gave long-term multilineage reconstitution in all recipients (red lines). CD48-c-kit+ cells are highly enriched HSC activity but are not visible in panel A because they represent only 0.1% of bone marrow cells. The vast majority of bone marrow cells that formed myeloerythroid colonies in methylcellulose were CD48+CD244+CD150- (E). Each bar represents the percentage of total colony forming progenitors from unfractionated bone marrow that are contained in each cell population. For example, 83.4% of all colonies (CFU-C, white bar) formed by bone marrow cells originate from the CD48+CD244+CD150- population.
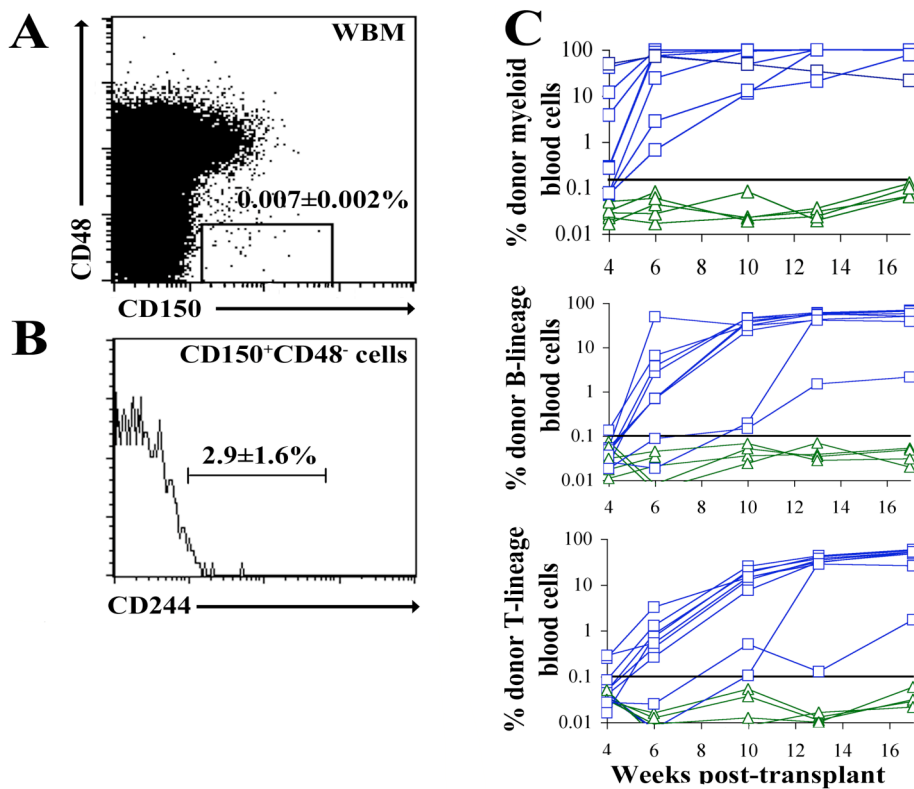
Figure 2.4: CD150+CD48-CD244- cells are highly enriched for long-term reconstituting HSCs. CD150+CD48- cells represent only 0.0084% of bone marrow cells (A), and these cells were uniformly negative for CD244 expression (B). Injection of 3 donor-type CD150+CD48- cells into lethally irradiated recipient mice in a competitive reconstitution assay lead to long-term multilineage reconstitution by donor cells in nine of fourteen mice (blue lines) and no reconstitution in five of fourteen mice (green lines).
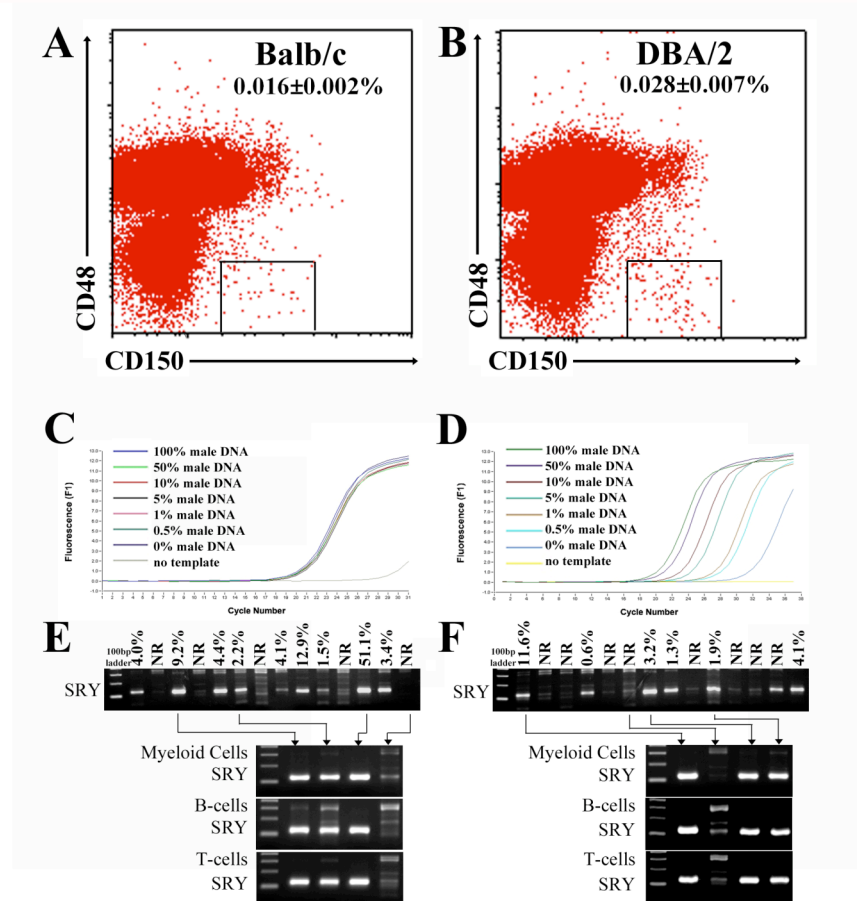
Figure 2.5: Balb/c and DBA/2 HSCs are enriched in the CD150+CD48- population.
CD150+CD48- cells are rare in Balb/c (A) and DBA/2 (B) bone marrow. Ten
CD150+CD48- cells from male donors were injected into lethally irradiated female
recipients in competitive reconstitution assays. At week 16, DNA from peripheral
leukocytes was extracted and subjected to quantitative PCR using primers specific for
genomic SRY to determine the relative contribution of male cells to the peripheral blood
of female recipients. Control DNA from untreated male mice was diluted into control
DNA from untreated female mice as indicated (C and D) to construct a standard curve.
DNA content in each sample was normalized based on genomic ß-actin amplification
(C), and the level of male DNA (SRY) in each sample was determined by qPCR (D). To
be considered reconstituted, the male contribution to peripheral blood had to be clearly
detectable by qPCR, and the amplified product had to be specific based on melting curve
analysis, and electrophoresis. Eleven out of 16 Balb/c recipients were clearly
reconstituted by male cells (E; only 14 mice are shown). Seven out of 15 DBA/2
recipients were cleary reconstituted by male cells (F; only 14 mice are shown). Splenic
myeloid (Mac-1+B220-CD3-), B (B220+CD3-Mac-1-), and T (CD3+Mac-1-B220-) cells
were isolated and examined for donor cell chimerism in 3 reconstituted mice and 1
unreconstituted mouse from each strain. In each case, the reconstituted mice showed
multilineage reconstitution.
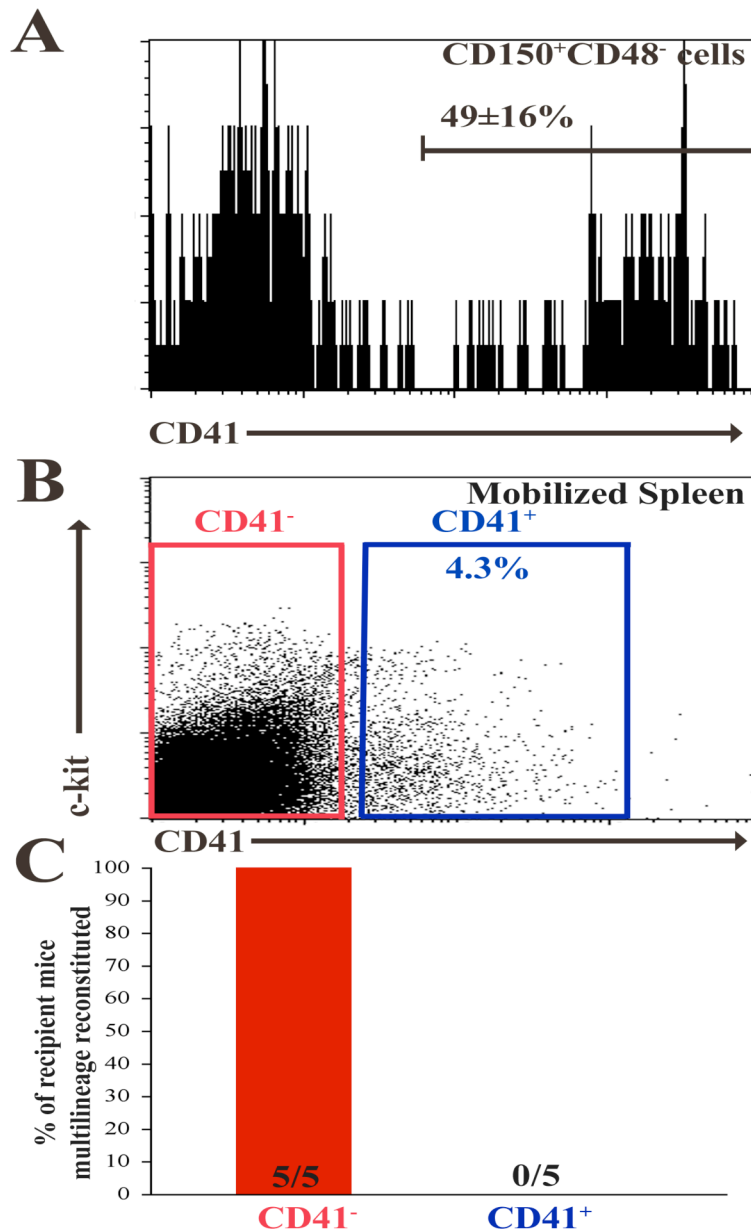
Figure 2.6: Excluding CD41+ cells increases the purity of HSCs in the CD150+CD48- fraction. (**A**) 37±5% of CD150+CD48- bone marrow cells express CD41. (**B**) Only 4.3% of splenocytes in cyclophosphamide/G-CSF mobilized mice express CD41. (**C**) 10,000 CD41+ mobilized splenocytes gave no detectable reconstitution in any recipients (0 of 5 mice, blue), while 200,000 CD41- mobilized splenocytes gave long-term multilineage reconstitution in all recipients (5 of 5 mice, red).
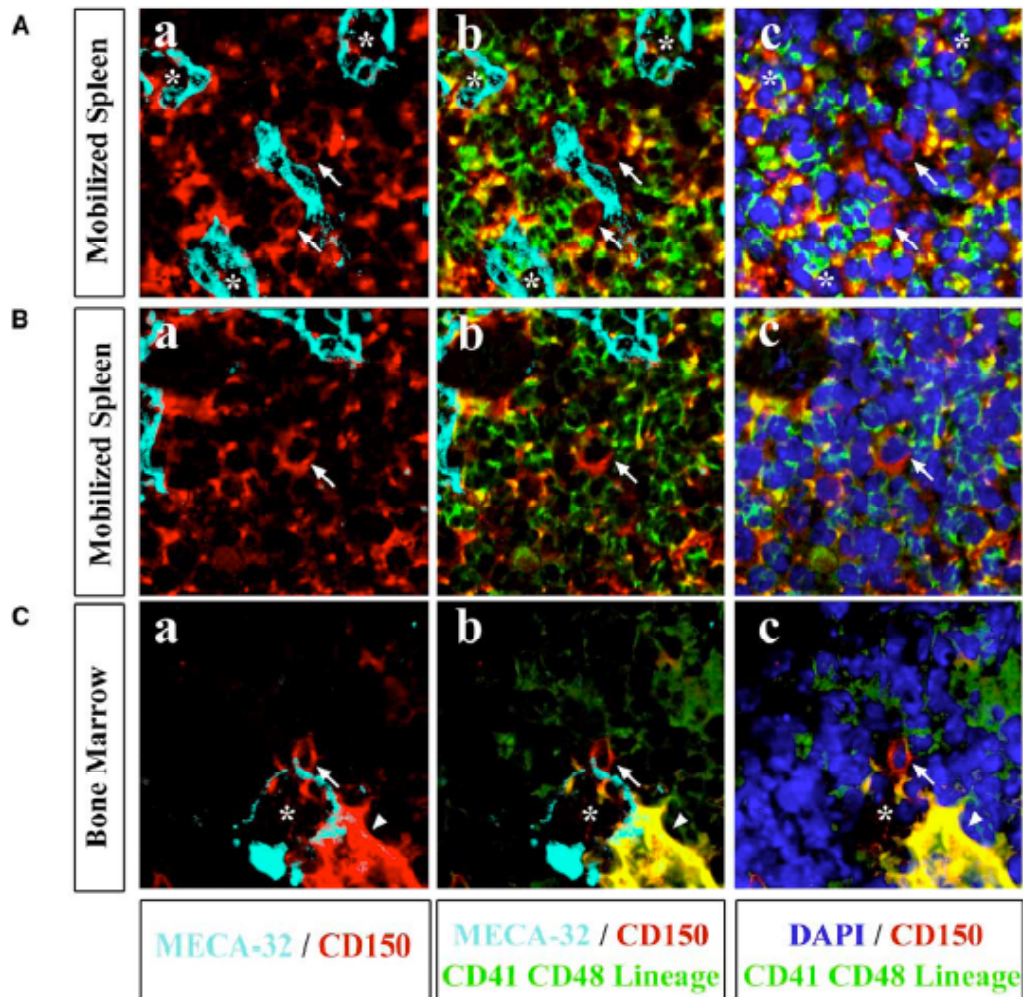
Figure 2.7: HSCs are associated with sinusoidal endothelial cells in the spleen (A, arrows) and bone marrow (C; arrow). In the cyclophosphamide/G-CSF mobilized spleen, CD150+CD48-CD41-Lineage- cells represented 0.0059±0.005 of cells in sections. 62% (23/37) of these cells were in contact with sinusoidal endothelial cells (A, arrows; * indicates the lumen of the sinusoids). Another 38% (14/37) of CD150+CD48-CD41-Lineage- cells were located in parafollicular regions that were often near sinusoids but not visibly in contact (B, arrow). It is unclear whether these cells are migrating to/from sinusoids or whether there are multiple niches within the spleen. In normal bone marrow, CD150+CD48-CD41-Lineage- cells represented 0.0067±0.0016% of cells in sections. Some of these cells were closely associated with endosteum (not shown). However, most of these cells contacted sinusoidal endothelium (C, arrow). Note the large megakaryocyte that was also associated with the sinusoid (C, arrowhead). Each of these images represents a single optical section, but a series of images through each cell is shown in Figure 2.13.
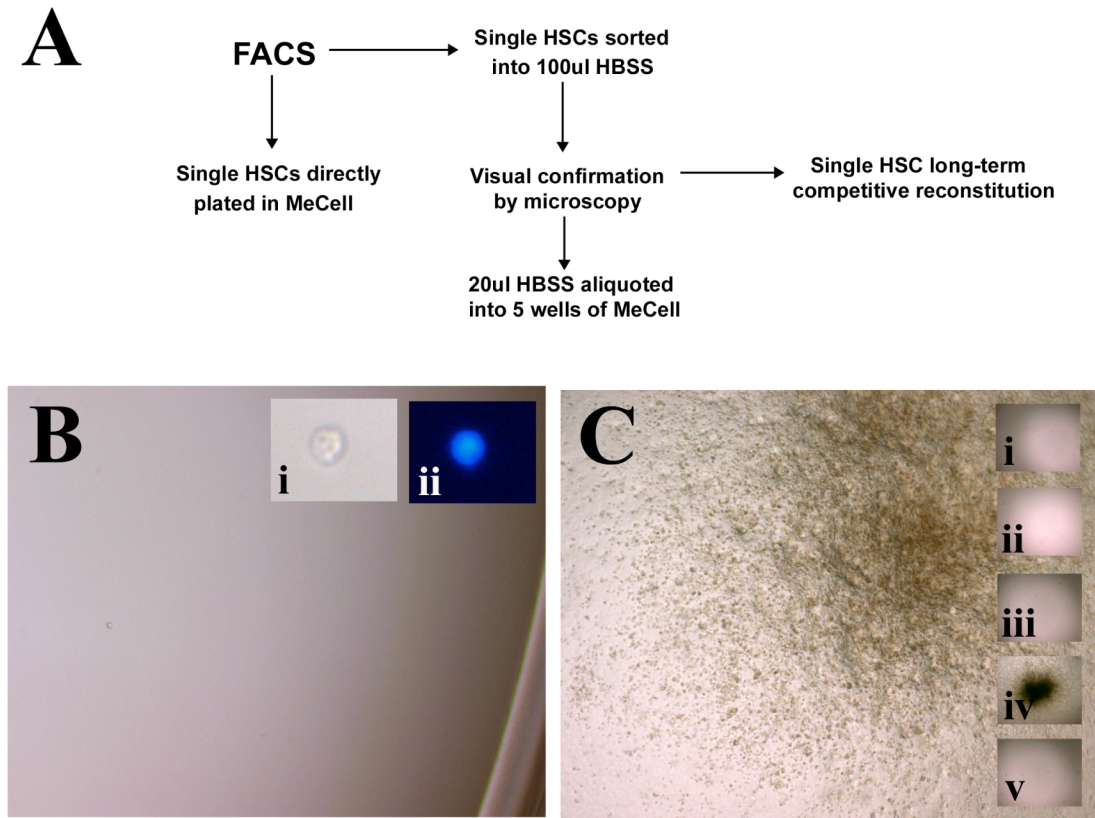
Figure 2.8: Visualization of single HSCs prior to transplantation. Single CD150+Sca-1+Lineage-CD48-c-kit+ HSCs were sorted and then resorted by flow-cytometry (FACSVantage SE) on counter mode using doublet discrimination (A). Single cells were deposited into 100ul of sterile HBSS buffer containing 2.5ug/ml Hoechst 33342 (Sigma) and 5.0ug/ml verapamil (Sigma) in individual wells of a 96-well plate (B). After the presence of a single cell was assessed using light microscopy (i) and confirmed using UV florescence microscopy (ii), the contents of each well were injected into lethally irradiated recipients along with a radioprotective dose of 300,000 recipient-type CD150$^-$ bone marrow cells. In none of more than 150 wells inspected was more than one cell seen. In control studies to functionally test whether there was only a single cell per well, the contents of each well were divided into five equal volumes and plated into separate wells of methylcellulose and allowed to form colonies (C). In three separate experiments, 90.0±10.0% of the sets of five methylcellulose wells contained a single hematopoietic colony and in no circumstance (0/48) was more than one colony observed. When single cells were directly plated into methylcellulose, 93.3±7.6% of single cells formed hematopoietic colonies. There was thus no difference between the clonogenicity of directly plated HSCs or of diluted HSCs (p=0.67). The contents of a representative set of five methylcellulose cultures is shown in the inset (i-v). These results confirm that only a single cell was sorted per well in these experiments.
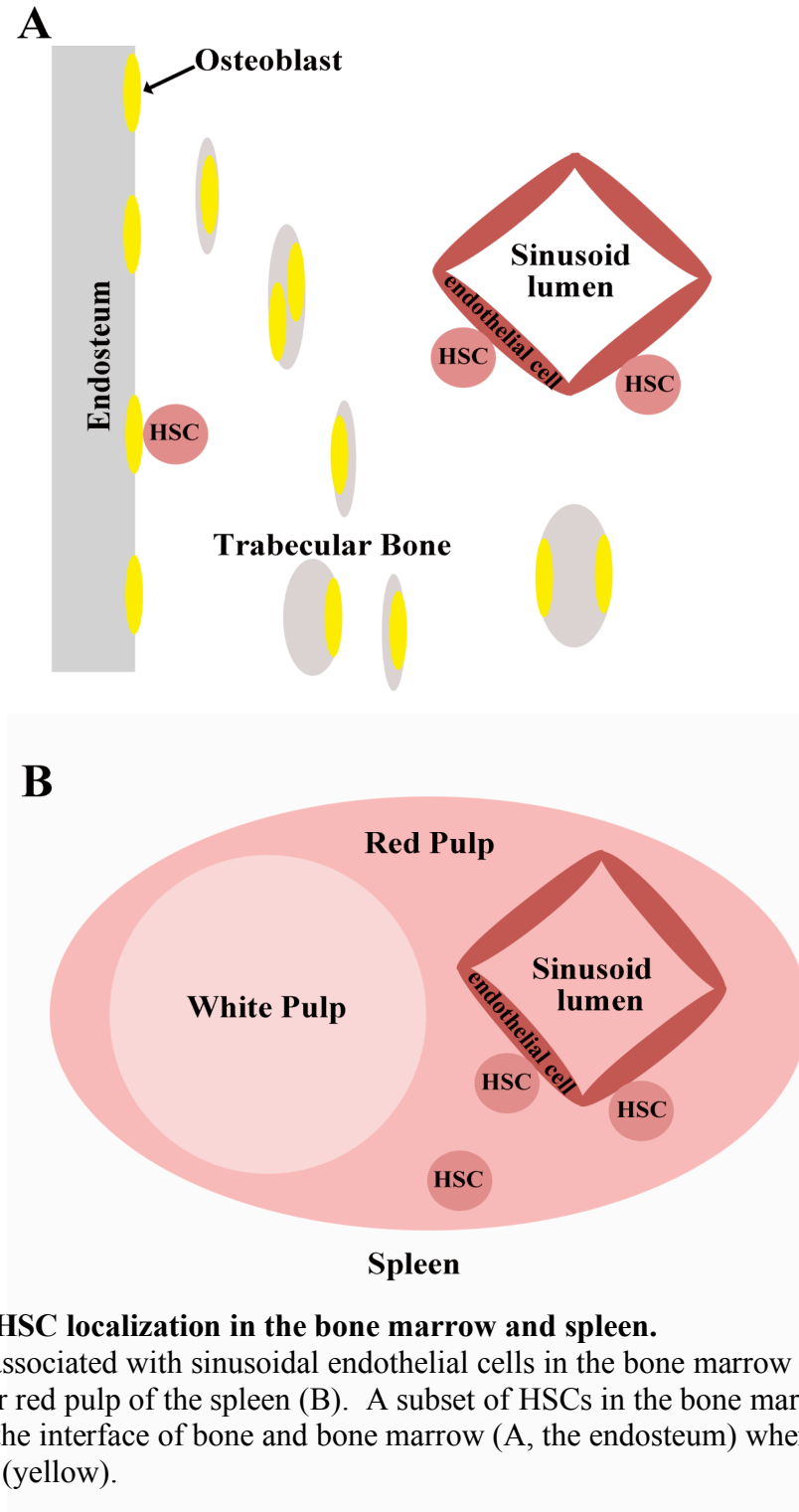
Figure 2.9: HSC localization in the bone marrow and spleen.
HSCs were associated with sinusoidal endothelial cells in the bone marrow (A) and in the parafollicular red pulp of the spleen (B). A subset of HSCs in the bone marrow also localized to the interface of bone and bone marrow (A, the endosteum) where osteoblasts also localize (yellow).
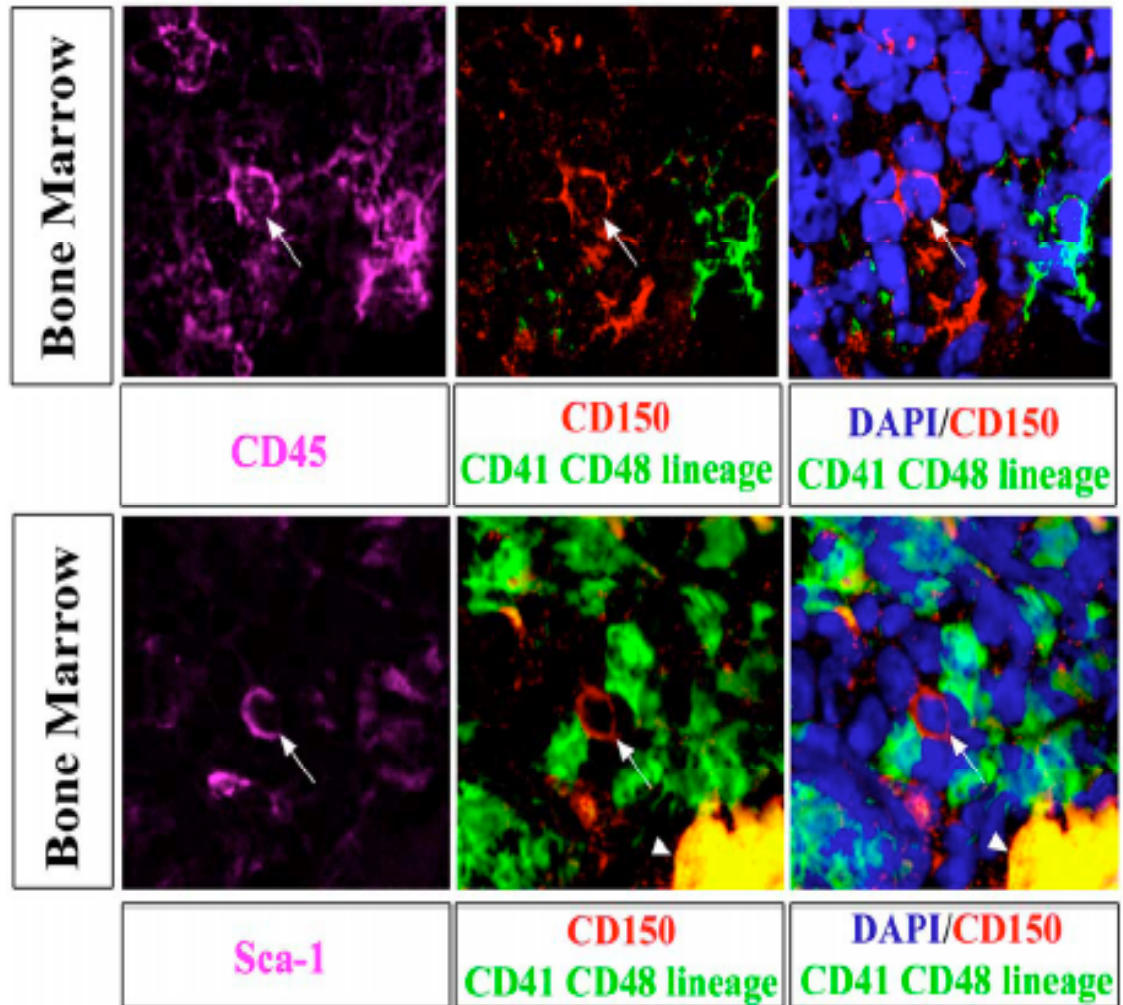
**Figure 2.10: CD150+CD48-CD41- cells express CD45 and Sca-1 in tissue sections
and by flow-cytometry**. CD150+CD48-CD41- cells isolated by flow-cytometry were
highly enriched for long-term multilineage reconstituting HSCs, and were present at very
similar frequencies as CD150+CD48-CD41-Lineage- cells identified in tissue sections.
Bone marrow CD150+CD48-CD41- cells isolated by flow-cytometry expressed both
CD45 (98% CD45+) and Sca-1 (89±3.5% Sca-1+). CD150+CD48-CD41-Lineage- cells
identified in bone marrow sections also expressed CD45 (100% CD45+; A) and Sca-1
(71% Sca-1+; B). CD150+CD48-CD41-Lineage- cells identified in spleen sections also
expressed CD45 (100% CD45+) and Sca-1 (80% Sca-1+) (data not shown). The co-
expression of these additional HSC markers on both the flow-cytometrically isolated cells
and on the cells identified in tissue sections strongly supports the conclusion that this is
the same cell population identified by both methods.

A

		Bone Marrow		Spleen	
		$CD150^{+/+}$	$CD150^{-/-}$	$CD150^{+/+}$	$CD150^{-/-}$
Cellularity	[$x10^7$ cells]	3.2±0.9	3.0±0.4	12±7	11±1
B cells	B220+sIgM+ (%)	9.8±2.3	10.3±2.3	30.0±1.4	30.8±2.2
	B220+sIgM- (%)	13.6±4.1	14.6±3.5	2.2±0.5	2±0.5
pro-B cells	B220+sIgM-CD43lo (%)	5.3±2.2	5.8±1.4		
pre-B cells	B220+sIgM-CD43- (%)	3.1±1.3	3.4±1.2		
T cells	CD3+ (%)	1.7±0.4	1.6±0.3	6.4±0.7	7.3±0.6
Erythroid Progenitors	Ter119+ (%)	22.2±4.9	18.1±2.7	4.4±4.6	2.7±1.5
Myeloid Progenitors	Mac-1+Gr-1+ (%)	28.2±7.1	29.8±4.6	0.7±0.2	0.7±0.2
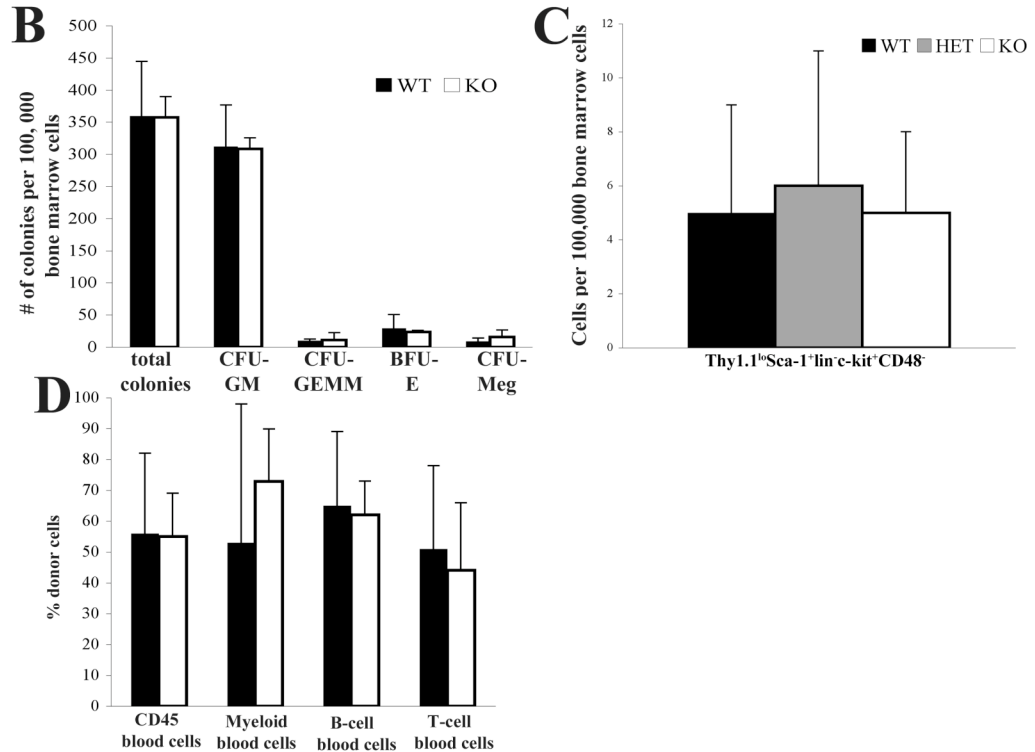
Figure 2.11: *CD150*-deficient mice exhibit normal hematopoiesis, colony-forming (CFU-C) progenitor activity, and HSC frequency and function.
(A) No statistically significant differences were observed in overall cellularity or in the frequencies of myeloid, B, T, or erythroid populations in the bone marrow or spleens of adult *CD150*-deficient mice. There was also no difference in the overall cellularity or frequency of progenitor populations within the thymus (data not shown). Each statistic represents mean ± standard deviation for five mice per genotype. (B) Colony forming assays reveal no differences between $CD150^{+/+}$ and $CD150^{-/-}$ littermates in the frequency of restricted hematopoietic progenitors in the bone marrow (4 independent experiments). (C) No statistically significant difference in the frequency of Thy-1loSca-1+lin-c-kit+CD48- HSCs in $CD150^{+/+}$ (black bars), $CD150^{+/-}$ (grey bars), and $CD150^{-/-}$ (white bars) littermates (n=5 to 7 for each genotype). (D) 200,000 $CD150^{+/+}$ or $CD150^{-/-}$ donor type bone marrow cells were transplanted into irradiated recipient mice along with 200,000 recipient-type bone marrow cells. No statistically significant differences were observed at 16 weeks post-transplantation in the level of donor-type CD45, myeloid, B-cell, or T-cells in recipient mice that received $CD150^{+/+}$ or $CD150^{-/-}$ bone marrow cells.
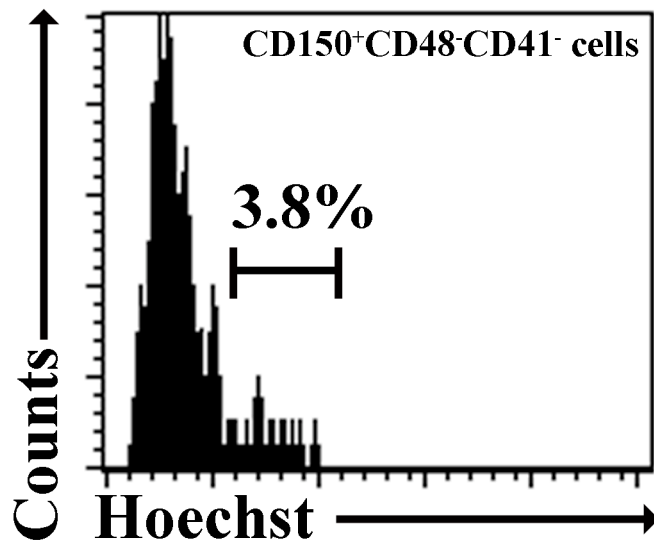
Figure 2.12: Few CD150+CD48-CD41- cells are in cycle.
Only 3.8% of bone marrow CD150+CD48-CD41- cells were in S/G2/M phases of the cell cycle based on Hoechst 33342 staining of DNA content. This is consistent with prior studies that found most adult bone marrow HSCs are quiescent (Cheshier et al., 1999; Morrison and Weissman, 1994).

Figure 2.13: Serial optical sections through the cells shown in Figure 2.7.   A series of optical sections through the images from Figures 2.7A-C are shown such that each row represents a single optical section. White arrows point to the CD150+CD48-CD41-Lineage- cells in each image. The spleen contains a higher frequency of CD150+ cells than the bone marrow as some lymphocytes, in addition to HSCs, express CD150. Each image, particularly from the spleen, contained 2 to 4 µm particles lacking nuclei that stained for both CD150 (red) and CD41 (green). These appear to be platelets, which are CD150+CD41+ and are present throughout hematopoietic tissues, particularly in the spleen. Note that HSCs in the bone marrow were sometimes adjacent to clusters of cells that failed to stain with lineage markers (* in C), presumably reflecting clusters of other early hematopoietic progenitors near HSC niches.

| DAPI | CD150 | CD48 CD41 lineage | Merge |

Figure 2.13A

| DAPI | CD150 | CD48 CD41 lineage | Merge |

Figure 2.13B

| **DAPI** | **CD150** | **CD48 CD41 lineage** | **Merge** |
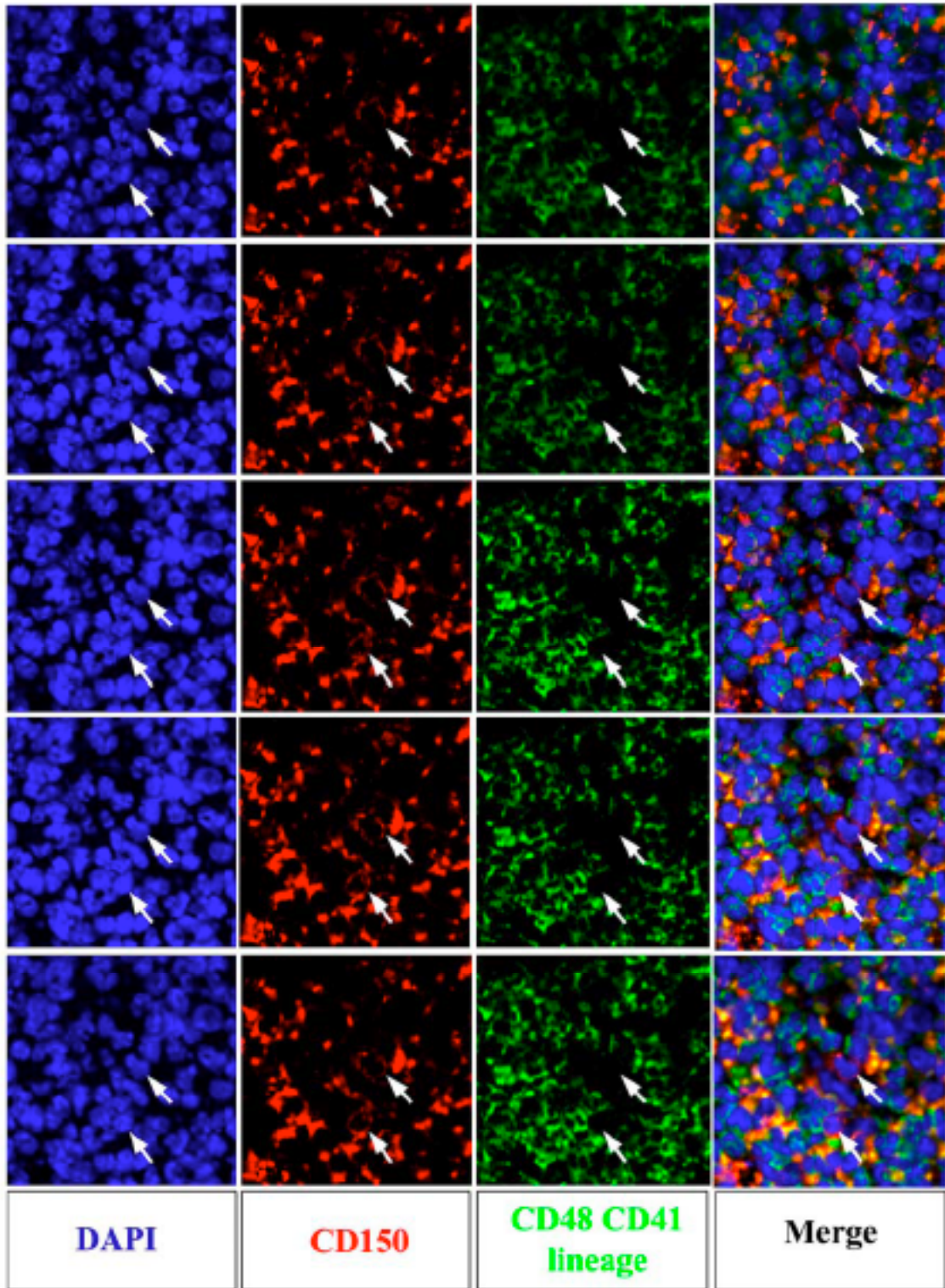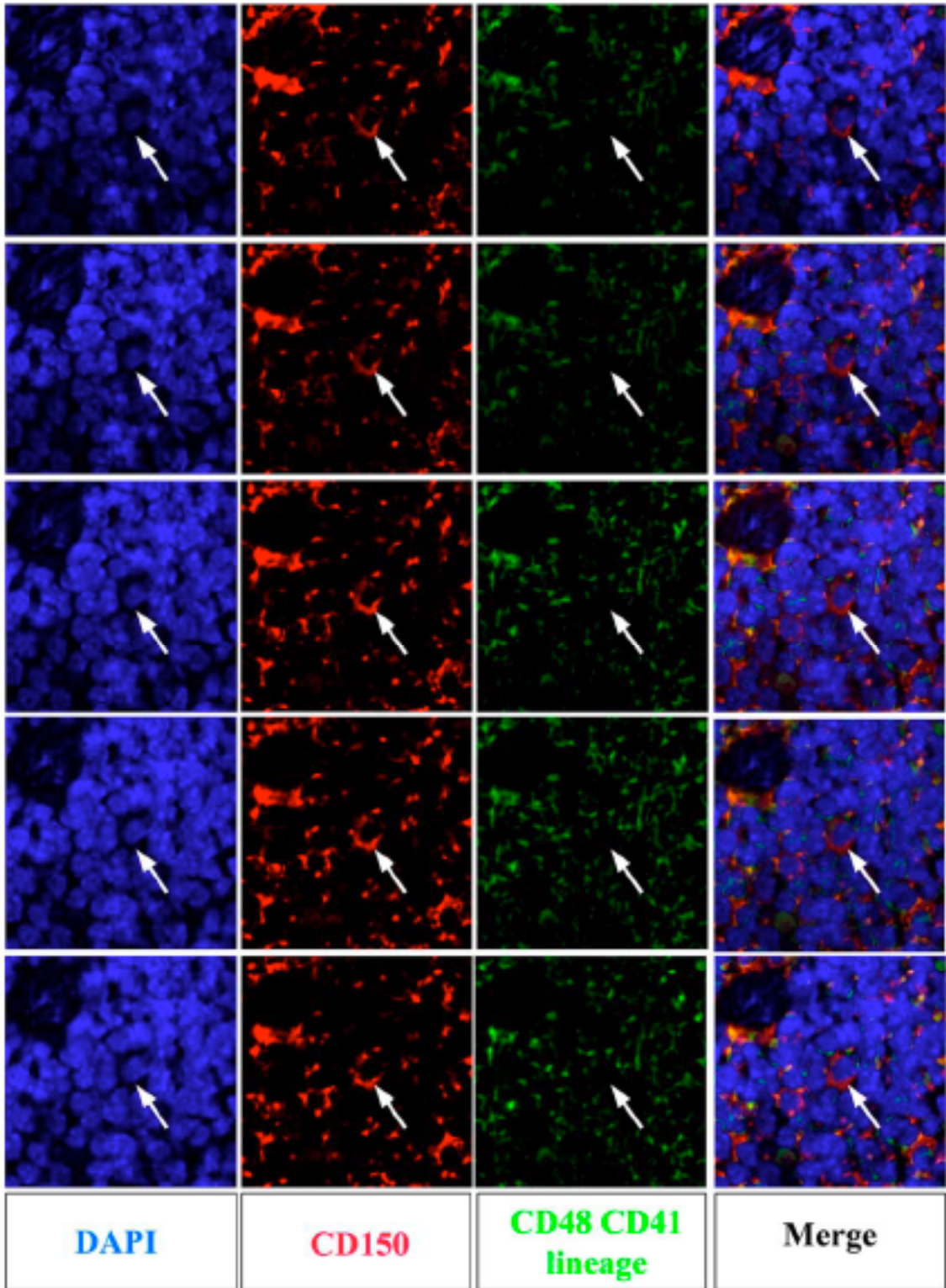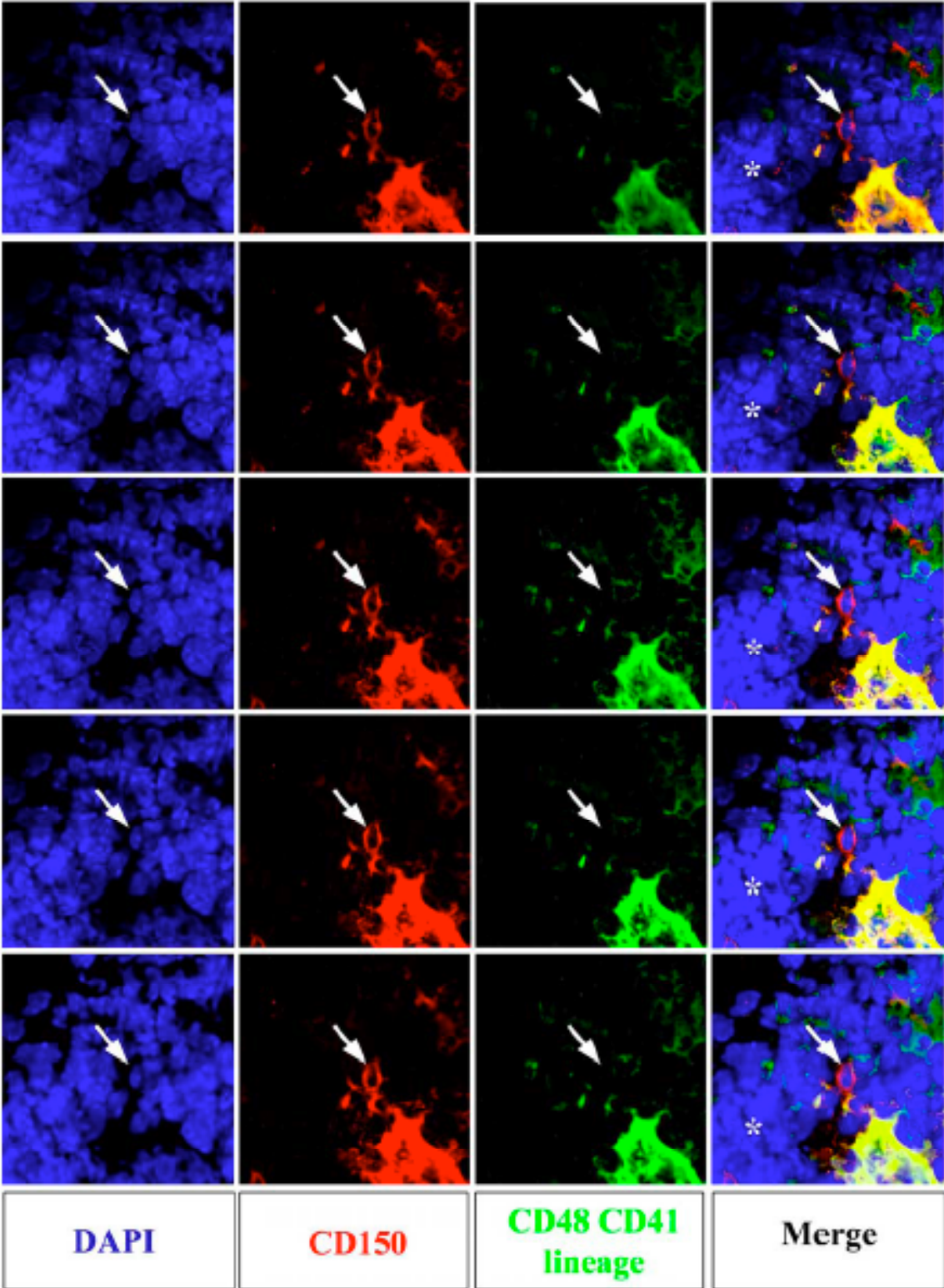
Figure 2.13C

BIBLIOGRAPHY

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell *118*, 149-161.

Avecilla, S.T., Hattori, K., Heissig, B., Tejada, R., Liao, F., Shido, K., Jin, D.K., Dias, S., Zhang, F., Hartman, T.E.*, et al.* (2004). Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. Nat Med *10*, 64-71.

Baugh, L.R., Hill, A.A., Brown, E.L., and Hunter, C.P. (2001). Quantitative analysis of mRNA amplification by in vitro transcription. Nucleic Acids Res *29*, E29.

Benveniste, P., Cantin, C., Hyam, D., and Iscove, N.N. (2003). Hematopoietic stem cells engraft in mice with absolute efficiency. Nat Immunol *4*, 708-713.

Buck, L., and Axel, R. (1991). A novel multigene family may encode odorant receptors -- a molecular basis for odor recognition. Cell *65*, 175-187.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R.*, et al.* (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. Nature *425*, 841-846.

Capela, A., and Temple, S. (2002). LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. Neuron *35*, 865-875.

Cardier, J.E., and Barbera-Guillem, E. (1997). Extramedullary hematopoiesis in the adult mouse liver is associated with specific hepatic sinusoidal endothelial cells. Hepatology *26*, 165-175.

Chen, C.Z., Li, L., Li, M., and Lodish, H.F. (2003). The endoglin(positive) sca-1(positive) rhodamine(low) phenotype defines a near-homogeneous population of long-term repopulating hematopoietic stem cells. Immunity *19*, 525-533.

Cheshier, S., Morrison, S.J., Liao, X., and Weissman, I.L. (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proceedings of the National Academy of Sciences USA *96*, 3120-3125.

Christopherson, K.W., 2nd, Hangoc, G., Mantel, C.R., and Broxmeyer, H.E. (2004). Modulation of hematopoietic stem cell homing and engraftment by CD26. Science *305*, 1000-1003.

deHaan, G., Nijhof, W., and VanZant, G. (1997). Mouse strain-dependent changes in frequency and proliferation of hematopoietic stem cells during aging: correlation between lifespan and cycling activity. Blood *89*, 1543-1550.

Dong, X., Han, S., Zylka, M.J., Simon, M.I., and Anderson, D.J. (2001). A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. Cell *106*, 619-632.

Easterday, M.C., Dougherty, J.D., Jackson, R.L., Ou, J., Nakano, I., Paucar, A.A., Roobini, B., Dianati, M., Irvin, D.K., Weissman, I.L.*, et al.* (2003). Neural progenitor genes. Germinal zone expression and analysis of genetic overlap in stem cell populations. Dev Biol *264*, 309-322.

Engel, P., Eck, M.J., and Terhorst, C. (2003). The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. Nat Rev Immunol *3*, 813-821.

Evsikov, A.V., and Solter, D. (2003). Comment on " 'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". Science *302*, 393; author reply 393.

Ferkowicz, M.J., Starr, M., Xie, X., Li, W., Johnson, S.A., Shelley, W.C., Morrison, P.R., and Yoder, M.C. (2003). CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. Development *130*, 4393-4403.

Fink, L., Seeger, W., Ermert, L., Hanze, J., Stahl, U., Grimminger, F., Kummer, W., and Bohle, R.M. (1998). Real-time quantitative RT-PCR after laser-assisted cell picking. Nature Medicine *4*, 1329-1333.

Fortunel, N.O., Otu, H.H., Ng, H.H., Chen, J., Mu, X., Chevassut, T., Li, X., Joseph, M., Bailey, C., Hatzfeld, J.A.*, et al.* (2003). Comment on " 'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". Science *302*, 393; author reply 393.

Hackney, J.A., Charbord, P., Brunk, B.P., Stoeckert, C.J., Lemischka, I.R., and Moore, K.A. (2002). A molecular profile of a hematopoietic stem cell niche. Proc Natl Acad Sci U S A *99*, 13061-13066.

Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N.R., Crystal, R.G., Besmer, P., Lyden, D., Moore, M.A.*, et al.* (2002). Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell *109*, 625-637.

Howie, D., Okamoto, S., Rietdijk, S., Clarke, K., Wang, N., Gullo, C., Bruggeman, J.P., Manning, S., Coyle, A.J., Greenfield, E.*, et al.* (2002). The role of SAP in murine CD150 (SLAM)-mediated T-cell proliferation and interferon gamma production. Blood *100*, 2899-2907.

Ivanova, N.B., Dimos, J.T., Schaniel, C., Hackney, J.A., Moore, K.A., and Lemischka, I.R. (2002). A stem cell molecular signature. Science *298*, 601-604.

Iwashita, T., Kruger, G.M., Pardal, R., Kiel, M.J., and Morrison, S.J. (2003). Hirschsprung disease is linked to defects in neural crest stem cell function. Science *301*, 972-976.

Kennedy, M., Firpo, M., Choi, K., Wall, C., Robertson, S., Kabrun, N., and Keller, G. (1997). A common precursor for primitive erythropoiesis and definitive haematopoiesis. Nature *386*, 488-493.

Kubo, H., and Alitalo, K. (2003). The bloody fate of endothelial stem cells. Genes Dev *17*, 322-329.

Laboratory, J. (1997). Definitions of inbred strains, substrains, sublines, and F1 hybrids. In Handbook on Genetically Standardized JAX mice, R.R. Fox, and B.A. Witham, eds. (Bar Harbor, Jackson Laboratory), p. 148.

Laterveer, L., Lindley, I.J., Hamilton, M.S., Willemze, R., and Fibbe, W.E. (1995). Interleukin-8 induces rapid mobilization of hematopoietic stem cells with radioprotective capacity and long-term myelolymphoid repopulating ability. Blood *85*, 2269-2275.

Lemischka, I.R. (1997). Microenvironmental regulation of hematopoietic stem cells. Stem Cells *15 Suppl 1*, 63-68.

Li, W., Johnson, S.A., Shelley, W.C., and Yoder, M.C. (2004). Hematopoietic stem cell repopulating ability can be maintained in vitro by some primary endothelial cells. Exp Hematol *32*, 1226-1237.

Louissaint, A., Jr., Rao, S., Leventhal, C., and Goldman, S.A. (2002). Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. Neuron *34*, 945-960.

Matsuzaki, Y., Kinjo, K., Mulligan, R.C., and Okano, H. (2004). Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. Immunity *20*, 87-93.

Mikkola, H.K., Fujiwara, Y., Schlaeger, T.M., Traver, D., and Orkin, S.H. (2003). Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo. Blood *101*, 508-516.

Morrison, S.J., Hemmati, H.D., Wandycz, A.M., and Weissman, I.L. (1995). The purification and characterization of fetal liver hematopoietic stem cells. Proc Natl Acad Sci USA *92*, 10302-10306.

Morrison, S.J., Shah, N.M., and Anderson, D.J. (1997a). Regulatory mechanisms in stem cell biology. Cell *88*, 287-298.

Morrison, S.J., Wandycz, A.M., Akashi, K., Globerson, A., and Weissman, I.L. (1996). The aging of hematopoietic stem cells. Nature Medicine *2*, 1011-1016.

Morrison, S.J., Wandycz, A.M., Hemmati, H.D., Wright, D.E., and Weissman, I.L. (1997b). Identification of a lineage of multipotent hematopoietic progenitors. Development *124*, 1929-1939.

Morrison, S.J., and Weissman, I.L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity *1*, 661-673.

Morrison, S.J., Wright, D., and Weissman, I.L. (1997c). Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. Proc Natl Acad Sci USA *94*, 1908-1913.

Na Nakorn, T., Miyamoto, T., and Weissman, I.L. (2003). Characterization of mouse clonogenic megakaryocyte progenitors. Proc Natl Acad Sci U S A *100*, 205-210.

North, T.E., de Bruijn, M.F., Stacy, T., Talebian, L., Lind, E., Robin, C., Binder, M., Dzierzak, E., and Speck, N.A. (2002). Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. Immunity *16*, 661-672.

Oberlin, E., Tavian, M., Blazsek, I., and Peault, B. (2002). Blood-forming potential of vascular endothelium in the human embryo. Development *129*, 4147-4157.

Ohneda, O., Fennie, C., Zheng, Z., Donahue, C., La, H., Villacorta, R., Cairns, B., and Lasky, L.A. (1998). Hematopoietic stem cell maintenance and differentiation are supported by embryonic aorta-gonad-mesonephros region-derived endothelium. Blood *92*, 908-919.

Osawa, M., Hanada, K.-I., Hamada, H., and Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science *273*, 242-245.

Palmer, T.D., Willhoite, A.R., and Gage, F.H. (2000). Vascular niche for adult hippocampal neurogenesis. J Comp Neurol *425*, 479-494.

Phillips, D.R., Charo, I.F., Parise, L.V., and Fitzgerald, L.A. (1988). The platelet membrane glycoprotein IIb-IIIa complex. Blood *71*, 831-843.

Phillips, R.L., Ernst, R.E., Brunk, B., Ivanova, N., Mahan, M.A., Deanehan, J.K., Moore, K.A., Overton, G.C., and Lemischka, I.R. (2000). The genetic program of hematopoietic stem cells. Science *288*, 1635-1640.

Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R.C., and Melton, D.A. (2002). "Stemness": transcriptional profiling of embryonic and adult stem cells. Science *298*, 597-600.

Shen, Q., Goderie, S.K., Jin, L., Karanth, N., Sun, Y., Abramova, N., Vincent, P., Pumiglia, K., and Temple, S. (2004). Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science *304*, 1338-1340.

Sidorenko, S.P., and Clark, E.A. (2003). The dual-function CD150 receptor subfamily: the viral attraction. Nat Immunol *4*, 19-24.

Smith, L.G., Weissman, I.L., and Heimfeld, S. (1991). Clonal analysis of hematopoietic stem-cell differentiation in vivo. Proc Natl Acad Sci U S A *88*, 2788-2792.

Spangrude, G.J., and Brooks, D.M. (1992). Phenotypic analysis of mouse hematopoietic stem cells shows a Thy-1-negative subset. Blood *80*, 1957-1964.

Spangrude, G.J., and Brooks, D.M. (1993). Mouse strain variability in the expression of the hematopoietic stem cell antigen Ly-6A/E by bone marrow cells. Blood *82*, 3327-3332.

Spangrude, G.J., Brooks, D.M., and Tumas, D.B. (1995). Long-term repopulation of irradiated mice with limiting numbers of purified hematopoietic stem cells: in vivo expansion of stem cell phenotype but not function. Blood *85*, 1006-1016.

Spangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. Science *241*, 58-62.

Spradling, A., Drummond-Barbosa, D., and Kai, T. (2001). Stem cells find their niche. Nature *414*, 98-104.

Takano, H., Ema, H., Sudo, K., and Nakauchi, H. (2004). Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med *199*, 295-302.

Taniguchi, H., Toyoshima, T., Fukao, K., and Nakauchi, H. (1996). Presence of hematopoietic stem cells in the adult liver. Nature Medicine *2*, 198-203.

Uchida, N., Dykstra, B., Lyons, K.J., Leung, F.Y., and Eaves, C.J. (2003). Different in vivo repopulating activities of purified hematopoietic stem cells before and after being stimulated to divide in vitro with the same kinetics. Exp Hematol *31*, 1338-1347.

Uchida, N., and Weissman, I.L. (1992). Searching for hematopoietic stem cells: evidence that Thy-1.1lo Lin- Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. Journal of Experimental Medicine *175*, 175-184.

Visnjic, D., Kalajzic, Z., Rowe, D.W., Katavic, V., Lorenzo, J., and Aguila, H.L. (2004). Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. Blood *103*, 3258-3264.

Wagers, A.J., Sherwood, R.I., Christensen, J.L., and Weissman, I.L. (2002). Little evidence for developmental plasticity of adult hematopoietic stem cells. Science *297*, 2256-2259.

Wang, N., Satoskar, A., Faubion, W., Howie, D., Okamoto, S., Feske, S., Gullo, C., Clarke, K., Sosa, M.R., Sharpe, A.H*., et al.* (2004). The Cell Surface Receptor SLAM Controls T Cell and Macrophage Functions. J Exp Med *199*, 1255-1264.

Wilson, A., Murphy, M.J., Oskarsson, T., Kaloulis, K., Bettess, M.D., Oser, G.M., Pasche, A.C., Knabenhans, C., Macdonald, H.R., and Trumpp, A. (2004). c-Myc

controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev *18*, 2747-2763.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q.*, et al.* (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.

CHAPTER 3

LACK OF EVIDENCE THAT HEMATOPOIETIC STEM CELLS DEPEND ON N-CADHERIN-MEDIATED ADHESION TO OSTEOBLASTS FOR THEIR MAINTENANCE[1]

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, using commercial anti-N-cadherin antibodies, or by ß-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 5 cell diameters of the endosteum. These results question whether significant numbers of HSCs depend on N-cadherin-mediated adhesion to osteoblasts.

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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 that support the maintenance of HSCs (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 (Arai et al., 2004; Nilsson et al., 2005; Stier et al., 2005; Taichman et al., 1996; 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 regulate 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 (Wilson et al., 2004; Zhang et al., 2003). 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-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 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 5 cell diameters of a sinusoid. In contrast, only 8 to 21% of HSCs were within 5 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-1+lineage- HSCs. CD150+CD48-CD41-c-kit+Sca-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) (Figure 3.1A,B). However, we were never able to amplify *N-cadherin* from

aliquots of CD150+CD48-CD41-c-kit+Sca-1+lineage- HSCs (Figure 3.1A,B). We were also unable to amplify *N-cadherin* from aliquots of c-kit+Sca-1+lineage-Flk2- HSCs (Figure 3.1A,B), 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 3.1B). 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 (Wilson et al., 2004; Zhang et al., 2003), 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 3.1C). In contrast, neither antibody showed any staining above background on either CD150+CD48-CD41-c-kit+Sca-1+lineage- HSCs or c-kit+Sca-1+lineage-Flk2- HSCs (Figure 3.1D). 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 Methods) 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 in Figure 3.2 (A-C). 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 3.2B). Nonetheless, there were some locations where residual bone marrow cells were present (Figure 3.2Bii). After scoring the endosteal surface with the bone burr these residual cells were effectively removed (Figure 3.2C). We did not detect any difference in overall viability or in the frequency of CD150+CD48-CD41-c-kit+Sca-1+lineage- HSCs in the burred fraction of bone marrow cells as compared to the flushed fraction of cells (Figure 3.2D). Moreover, the total number of bone marrow cells isolated in the burred fraction was only 1.6% of the bone marrow cells recovered 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 (2 femurs and 2 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 6 recipients of burred bone marrow cells and 7 of 7 recipients of flushed bone marrow cells became long-term multilineage reconstituted by donor cells (Figure 3.2F). There was no statistically significant difference between the levels of donor cells that arose from flushed versus burred bone marrow cells (Figure 3.2F).

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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 ß-galactosidase activity. ß-galactosidase expression in these mice matches the known pattern of N-cadherin expression in 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 ß-galactosidase activity throughout the myocardium (Figure 3.3) as reported previously (Luo et al., 2005). c-kit+Sca-1+lineage-Flk2- HSCs and CD150+CD48-CD41-c-kit+Sca-1+lineage- HSCs from Rosa mice (which ubiquitously express ß-galactosidase) showed ß-galactosidase activity (Figure 3.4A), but not HSCs from *N-cadherin*^lacZ gene trap mice (Figure 3.4C) or littermate controls (Figure 3.4B). 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, 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 ß-galactosidase activity, but not HSCs from *N-cadherin^{lacZ}* gene trap mice or littermate controls (Figure 3.5). We were similarly unable to detect ß-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 readily detected ß-galactosidase activity in HSCs from Rosa mice (Figure 3.5B). 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 3.6A), 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 4,000 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 (Figure 3.6A-C). 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 (Figure 3.6A-C). 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 3.1). 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 3.1). 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 3.1). 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 3.11A). *biglycan–*

deficient mice develop an osteoporosis-like phenotype, with less trabecular bone, fewer

osteoblasts, and fewer osteoblast progenitors (Chen et al., 2002; Xu et al., 1998) (Figure

3.11B-D). This is evident in 3 month-old mice, but worsens with age. We detected no

statistically significant differences among *biglycan–*deficient mice and littermate controls

of any age in the frequency of myeloid, erythroid, B, or T lineage cells in the bone

marrow (Figure 3.12A) or spleen (Figure 3.12B). We also did not detect any differences

in bone marrow cellularity (Figure 3.13A), or the number (Figure 3.13B) or type (Figure

3.13C) 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 to

15, or 24 months of age in the frequency (Figure 3.13D) or absolute number (Figure

3.13E) 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 3.13F). These data

indicate that *biglycan* deficiency had no effect on the level of reconstituting activity in

competitive reconstitution assays, irrespective of whether the donors, or 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 5 cell diameters of, sinusoids or endosteum (Figure 3.14B). While not statistically significant, *biglycan*-deficient mice tended to have a lower proportion of bone marrow cells adjacent to, or within 5 cell diameters of, endosteum (Figure 3.14B), consistent with the reduced trabecular bone in these mice (Figure 3.11B, C).

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 to 0.0049% of cells in bone marrow sections in these experiments (Figure 3.14C). 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 within 5 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 5 cell diameters of the endosteum: only 8-21% of HSCs were visibly within 5 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 they 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 (Figure 3.1A,B), by staining with the YS anti-N-cadherin antibody (Figure 3.1D), by staining with the GC-4 anti-N-cadherin antibody (Figure 3.1D), or by analyzing ß-galactosidase expression in *N-cadherin^{lacZ}* gene trap mice (Figure 3.4C). 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-Flk2- 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 3.6). 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 (Figure 3.6B,C). 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 HSC frequency or function irrespective of age (Figure 3.13). 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 3.7), 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 3.14). Moreover, we extended this observation by finding that almost all bone marrow CD150+CD48-CD41-lineage- cells localize within 5 cell diameters of sinusoids (Figure 3.14). 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 (Li et al., 2004; Ohneda et al., 1998), 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 3.14) and prior studies (Kiel et al., 2005). However, these cells are considerably

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more likely than other bone marrow cells to localize to the endosteum (Figure 3.14B versus C). This is consistent with the suggestion that a subset of HSCs localizes to the endosteum (Arai et al., 2004; Kiel et al., 2005; Nilsson et al., 2001; Sugiyama et al., 2006; Suzuki et al., 2006; Zhang et al., 2003) and that osteoblasts and osteoclasts may regulate the maintenance and migration of at least some HSCs (Adams et al., 2006; Arai et al., 2004; Calvi et al., 2003; Kollet et al., 2006; Zhang et al., 2003). 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.

MATERIALS AND METHODS

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. For analysis of N-cadherin expression in the forebrain, neonatal mice were sacrificed 1 to 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 um, Sefar America, Kansas City, MO) to obtain a single cell suspension. For isolation of c-kit+Sca-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 Flk2 (A2F10.1) was used to isolate Flk2- progenitors. For isolation of CD150+CD48-CD41-c-kit+Sca-1+lineage- HSCs, bone marrow cells were incubated with PE-conjugated anti-CD150 (TC15-12F12.2; BioLegend, San Diego, California), FITC-conjugated anti-CD48 (HM48-1; BioLegend), FITC-conjugated anti-CD41 (MWReg30; BD Pharmingen, San Diego, California), 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-1+ or c-kit+ cells using paramagnetic microbeads and autoMACS (Miltenyi Biotec, Auburn, CA). Non-viable cells were excluded from sorts and analyses using the viability dye DAPI (1ug/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 micro-drill using a

0.5mm 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).

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 minutes 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 100ul 1.0% methylcellulose (Stem Cell Technologies, Vancouver, BC) as previously described. The methylcellulose was supplemented with 1% penicillin/streptomycin (Gibco), 50ng/ml stem cell factor (SCF), 10ng/ml interleukin-3 (IL-3), 10ng/ml interleukin-6 (IL-6) and 3U/ml erythropoietin (Epo). Colonies were maintained at $37^{\circ}C$ in humidified incubators at 6% O_2. Colony formation was scored after 10-14 days of culture.

Long-term competitive repopulation assay

Adult recipient mice (CD45.2) were irradiated with an Orthovoltage X-ray source delivering approximately 300 rad/min in two equal doses of 570 rad, delivered at least 2 hr apart. Cells were injected into the retro-orbital sinus of anesthetized recipients. In addition to the sorted populations of CD45.1 cells, each recipient mouse received 200,000 to 300,000 CD45.2 marrow cells for radioprotection. Beginning at 4 weeks after transplant and continuing until at least 16 weeks following transplantation blood was obtained from the tail veins of recipient mice, subjected to ammonium-chloride potassium red cell lysis, and stained with directly conjugated antibodies to CD45.1 (A20, FITC), B220 (6B2), Mac-1 (M1/70), CD3 (KT31.1), and Gr-1 (8C5) to monitor engraftment.

FDG staining

Analysis of beta-galactosidase expression using fluorescein di-beta-D-galactopyranoside (FDG; Molecular Probes, Invitrogen, Carlsbad CA) was carried out according to manufacturer's instructions. Bone marrow cells, previously stained with antibodies for the isolation of HSCs as described above and enriched for c-kit+ cells, were incubated for 1 to 5 minutes at 37°C in a hypotonic solution comprised of equal parts deionized H_2O and PBS with 2mM FDG. After the incubation, cells were washed and analyzed by flow-cytometry. For these experiments, c-kit+Sca-1+lineage- HSCs were isolated using Sca-1-APC and c-kit-PE-Cy5 antibodies. ß-galactosidase activity in CD150+CD48-CD41-c-kit+Sca-1+lineage- HSCs was assessed by sorting these (FITC

negative) HSCs, then staining with FDG, and analyzing by flow-cytometry. In all cases, cells from a negative control littermate were stained and analyzed at the same time.

X-Gal staining

For X-gal staining, slides were thawed at room temperature for 15 minutes, fixed in 0.25% glutaraldehyde in PBS at 4°C for 30min, rinsed in 0.1M phosphate buffer (PB) twice, incubated in X-Gal staining medium (0.1M PB containing 10 mM $K_3Fe(CN)_6$ and 10 mM $K_4Fe(CN)_6$ along with the beta-galactosidase substrate X-gal (1 mg/ml; Molecular Probes)) at 37 °C for 4-12 hours, then rinsed three times in 0.1M PB at room temperature. Finally, the excess buffer was shaken off and the slides were mounted in 70% glycerol in PBS. Positive (tissue isolated from ROSA mice) and negative controls (tissue isolated from wild-type littermate mice) were always stained and analyzed in parallel with slides from *N-Cadherin^{lacZ}* gene trap mice. Images were gathered using an Olympus Florescence Microscope.

Quantitative PCR

2,000 to 10,000 HSCs or bone marrow cells were directly sorted into 400ul Trizol (Ambion, Austin, TX) containing 250ug/ml glycogen (Roche, Indianapolis IN). RNA was extracted according to manufacturer's instructions. The extracted RNA (30ul volume) was treated for 20min at 37°C with RNase-free DNase-I (2U/ul; Ambion) in the presence of 2ul RNase inhibitor (10U/ul; Invitrogen). The RNA was then purified using an RNeasy Mini Kit (Qiagen, Valencia CA) according to manufacturer's instructions and washed three times with 500ul RNase-free water. The RNA was used for making cDNA

by reverse transcription with 1ug random hexamer. The cDNA was extracted with phenol-chloroform and precipitated with 20ug glycogen. After dissolving the cDNA with RNase-free water, cDNA equivalent to 200 cells was used for each PCR reaction. The PCR reactions were performed using a LightCycler (Roche Diagnostic Corporation) according to manufacturer's instructions. The RNA content of samples was normalized based on the amplification of *hypoxanthine phosphoribosyl transferase* (*HPRT*). In addition to confirming the specificity of the PCR reactions by examining the melting curves of the products, PCR products were separated in 2% agarose gels to confirm the presence of a single band of the expected size.

Primer sequences for amplification of *N-cadherin* were as described previously (Arai et al., 2004): *N-cadherin* F-AGAGGGATCAAAGCCTGGGACGTAT and *N-cadherin* R-TCCACCCTGTTCTCAGGGACTTCTC. Primer sequences for amplification of *HPRT* were as described previously (Kiel et al., 2005): *HPRT* F-CCTCATGGACTGATTATGGACA and *HPRT* R-ATGTAATCCAGCAGGTCAGCAA.

Staining of bone marrow sections

HSC localization in bone marrow sections was performed as previously described (Kiel et al., 2005) with minor modifications. Freshly dissected undecalcified femurs from 9-15 month old wild-type and *biglycan*-deficient mice were embedded in 8% gelatin (Sigma) in phosphate buffer and snap frozen in N-methylbutane chilled in a slurry of ethanol and dry ice. Sections (5 μm) were cut using the CryoJane system (Instrumedics, Hackensack, New Jersey), a tungsten carbide blade (Diamond Knives, Wilmington DE)

and Bright Cryostat (Huntingdon, UK) at -24°C and placed on methacrylate coated slides. The sections were air-dried overnight at room temperature and subsequently fixed in -20°C acetone for 5 minutes. Slides were then blocked with 20% goat serum in 0.1M phosphate buffer (pH 7.4) for 40 minutes prior to antibody staining. Slides were first incubated in 0.03μg/μl 26D12 anti-CD150 antibody (DNAX, Palo Alto CA) for 2 hr and rinsed. Goat anti-rat IgG conjugated to Alexa555 (Molecular Probes) was added at 1/100 dilution and rinsed. Slides were then incubated in 0.1μg/μl rat IgG (Sigma-Aldrich) for 10 minutes and rinsed. FITC-conjugated anti-CD48 and FITC-conjugated anti-CD41 antibodies (each 1/100 dilution) as well as FITC-conjugated lineage markers including anti-B220 (stains B cells), CD2 (T cells), CD4 (T cells), CD8a (T cells), Gr-1 (myeloid cells) and CD11b (myeloid cells) antibodies (1/100 to 1/200 dilution), and biotinylated pan-endothelial cell antigen (MECA-32) at a 1/30 dilution were then incubated for 1 hour in 0.1μg/μl rat IgG and rinsed. Pan-endothelial cell antigen was visualized with Alexa647-conjugated streptavidin (Molecular Probes) at a 1/200 dilution for 1 hour. Finally, slides were rinsed twice for 5 min each and mounted without drying using Prolong Antifade (Molecular Probes). All antibody incubations took place in blocking buffer. All rinse steps were 2 times for 3 min each with blocking buffer unless otherwise indicated. All antibodies were purchased from Becton Dickinson unless otherwise noted. The nuclear dye DAPI was included in all stains to evaluate nuclear morphology and to exclude debris (lacking a nucleus), or dying cells (with fragmented nuclei). Microscopy was performed using an Olympus BX51 florescence microscope or an Olympus FV-500 confocal microscope.

For von Kossa staining of bone marrow sections, slides were equilibrated to room temperature, fixed in acetone at –20ºC for 15 minutes, rinsed in water, incubated in 5% silver nitrate for 1 hour at room temperature in the dark, rinsed, incubated in photographic developer solution for 5 minutes, rinsed, incubated in 0.1% gold chloride, rinsed, fixed in 5% sodium thiosulfate for 5 minutes, rinsed, stained in Wright-Giemsa for 30 seconds and rinsed prior to imaging.

For osteopontin staining of bone marrow, sections were fixed in acetone at –20ºC for 5 minutes, washed in 0.1M phosphate buffer, incubated with primary osteopontin antibody (Chemicon, Billerica MA) for 2-3 hours at room temperature, then incubated in anti-rat IgG Alexa 555 (Molecular Probes, Carlsbad CA) for 2 hours at room temperature, stained with DAPI (Sigma-Aldrich, St. Louis MO) and mounted with Anti-Fade (Molecular Probes). The average number of nucleated bone-lining osteopontin+ cells within the medullary space of the bone was calculated per high power field in the distal third of the femur.

Table 3.1: 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 Ab	N-cad+	0.01±0.02%	0.03±0.08%	0%	0.05±0.11%
	N-cad-	0.41±0.08%	99.97±0.08%	100%	99.95±0.11%
GC-4 Ab	N-cad+	0.2±0.2%	0.7±0.4%	0%	0.8±0.6%
	N-cad-	0.5±0.03%	99.3±0.4%	100%	99.2±0.6%
$N\text{-}cad^{+/+}$	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%
$N\text{-}cad^{lacZ}$	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, the GC-4 monoclonal antibody, or based on FDG staining for ß-galactosidase activity in $N\text{-}cadherin^{+/+}$ wild-type (control) mice or $N\text{-}cadherin^{lacZ}$ gene trap mice. Cells were plated in methylcellulose medium supplemented with SCF, IL-3, IL-6, and Erythropoietin. After 10 to 14 days the number and type of colonies was counted. The data represent 4-6 independent experiments in which an average of 16,000 to 17,500 N-cadherin- bone marrow cells or 3,000 to 5,400 N-cadherin+ bone marrow cells were plated per experiment (based on antibody staining) and 2 independent experiments in which 18,000 to 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 $N\text{-}cadherin^{lacZ}$ 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.
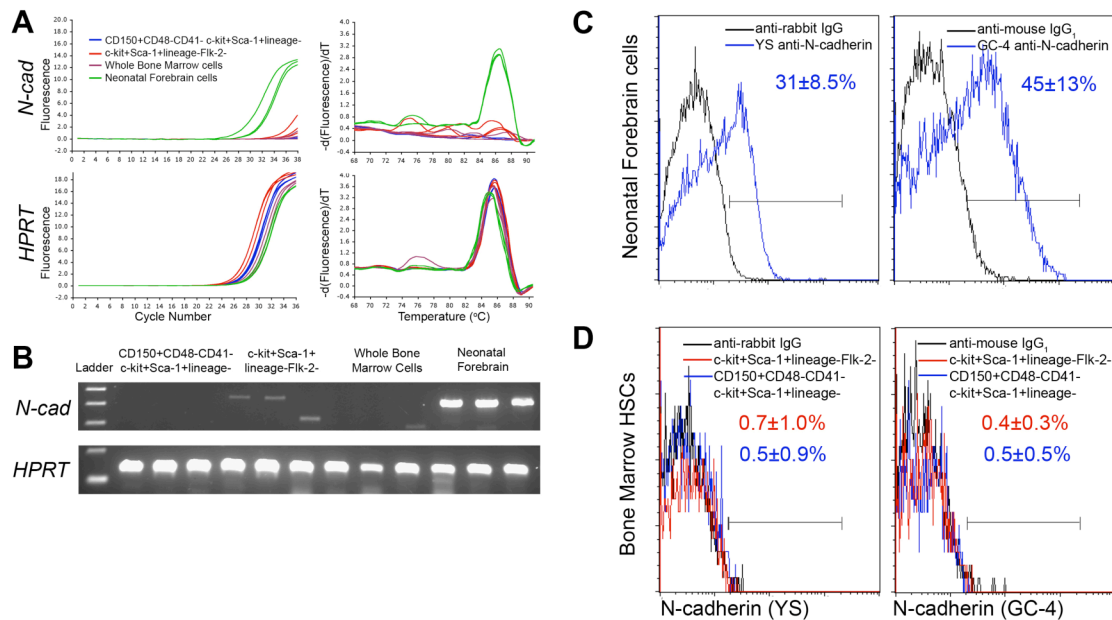
Figure 3.1: Failure to detect *N-cadherin* mRNA or protein expression by highly purified HSCs. (A) *N-cadherin* could be readily amplified from three independent samples of neonatal forebrain cells but not CD150+CD48-CD41-c-kit+Sca-1+lineage-HSCs or c-kit+Sca-1+lineage-Flk2- HSCs by quantitative PCR, 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 4 to 5 independently sorted HSC aliquots. 200 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 non-specific 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 3 to 9 independent experiments.
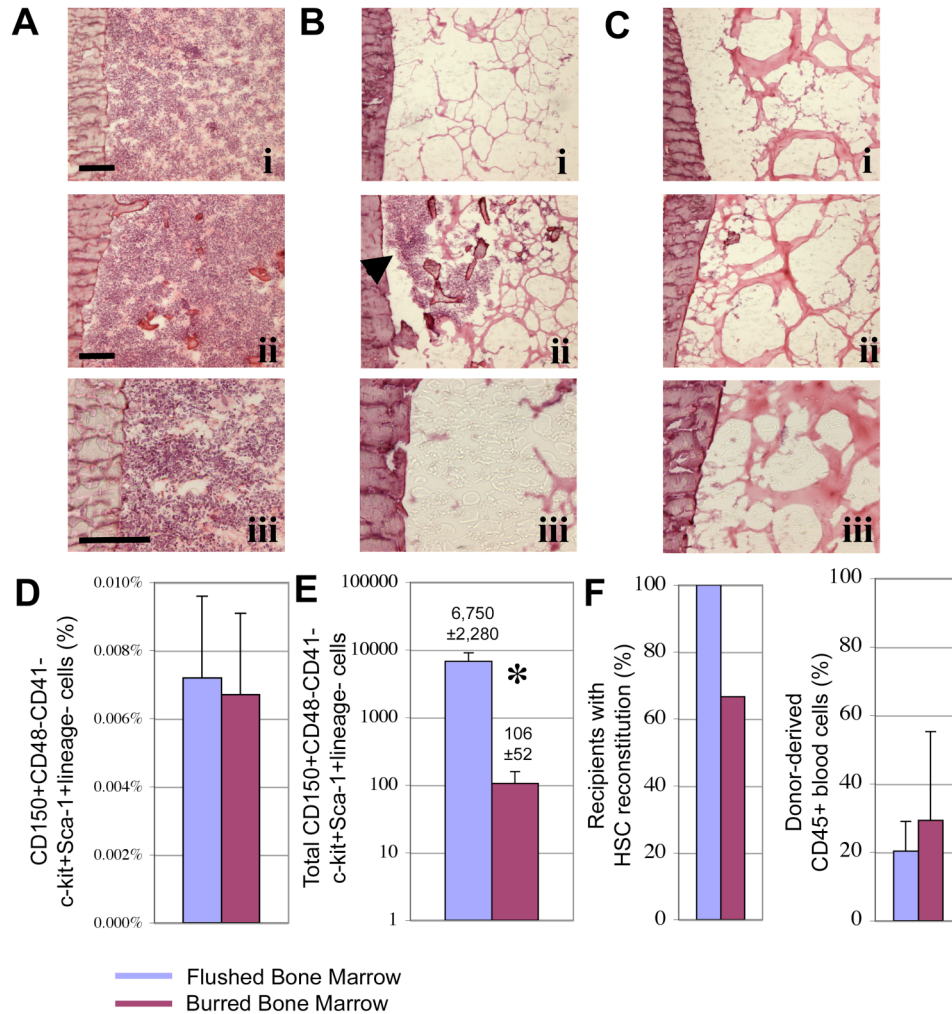
Figure 3.2: Flushing with medium washes the vast majority of bone marrow cells and HSCs out of the bone marrow cavity. Hematoxylin and Eosin stained sections through undecalcified, gelatin embedded femurs (A) or femurs that were "flushed" with medium to remove marrow cells (B) or subsequently "burred" (drilling through the marrow cavity) to remove residual cells (C). Images show diaphysis sections (i) and epiphysis (ii) sections as well as higher magnification images (iii) (scale bar is always 150 μm). Only 1.6% of bone marrow cells were retained within the marrow cavity after flushing (arrowhead), and these residual cells were recovered by burring. D) The frequency of CD150+CD48-CD41-c-kit+Sca-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 98.4% 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, 7/7 recipients of flushed marrow cells and 4/6 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 standard deviation.

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A

N-cad^{+/+}
Negative Littermate

X-Gal

B

N-cad^{lacZ/+}
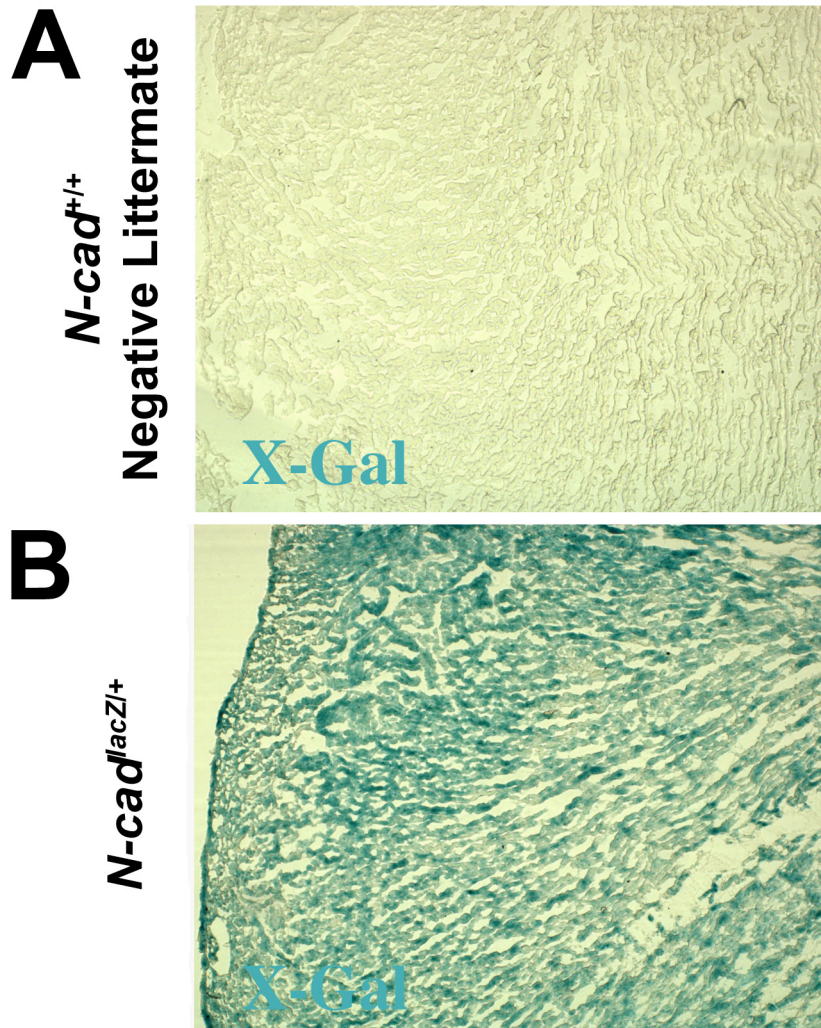
X-Gal

Figure 3.3: ß-galactosidase expression in the adult myocardium from *N-cadherin^{lacZ/+}* gene trap mice. Seven micron sections through the heart of an 8 week old *N-cadherin^{lacZ/+}* gene trap mouse were stained with X-Gal to visualize the ß-galactosidase expression pattern. As expected, X-Gal staining (blue) was detected throughout the myocardium of the *N-cadherin^{lacZ/+}* mouse (*B*) but not *N-cadherin^{+/+}* littermate controls (*A*).
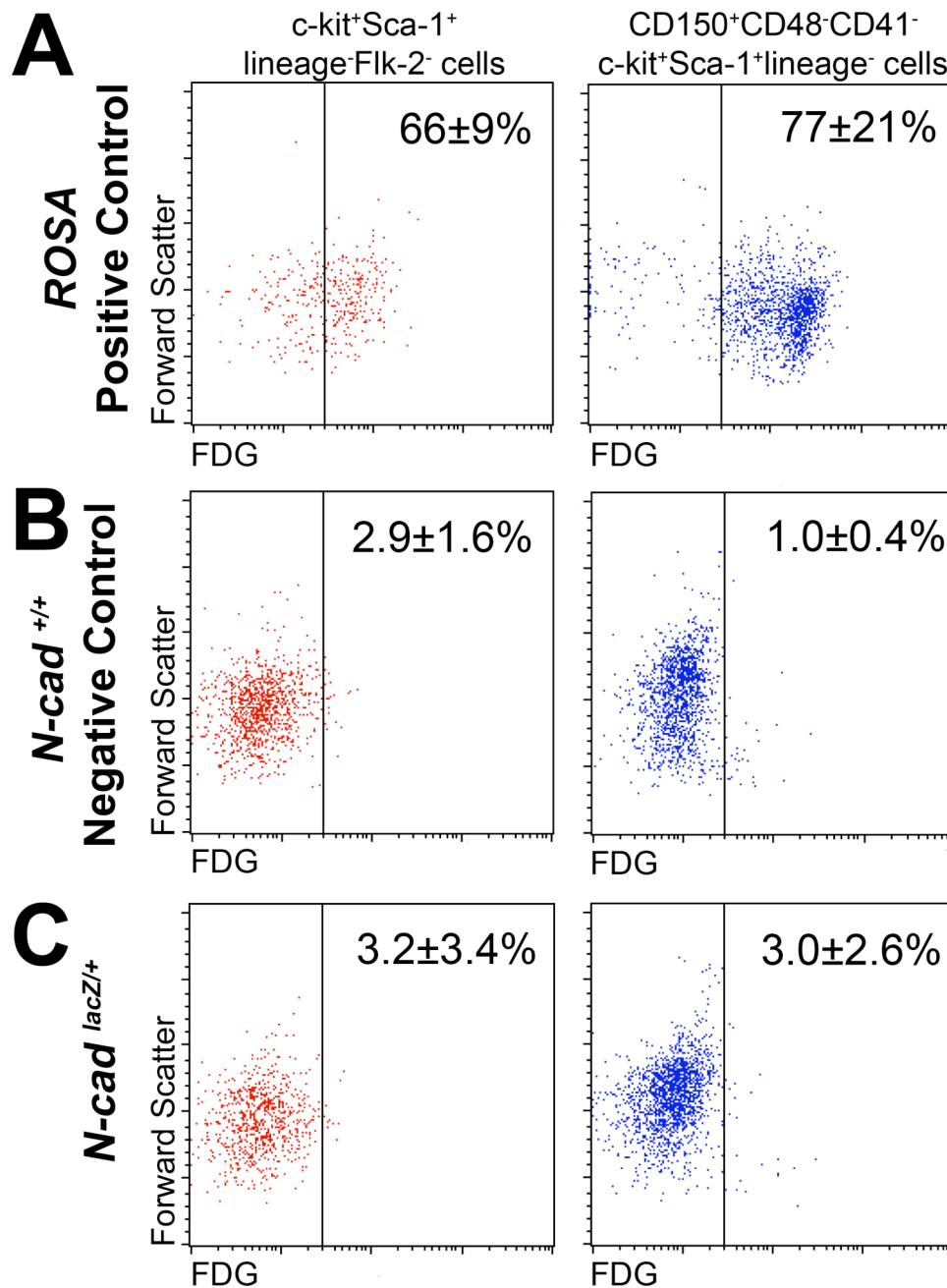
Figure 3.4: N-cadherin is not detectably expressed by HSCs isolated from *N-cadherin*$^{lacZ/+}$ gene trap mice. c-kit+Sca-1+lineage-Flk-2- HSCs or CD150+CD48-CD41-c-kit+Sca-1+lineage- HSCs were isolated from Rosa mice (A), *N-cadherin*$^{lacZ/+}$ gene trap mice (C) or *N-cadherin*$^{+/+}$ (B) littermate controls and stained with FDG to measure ß-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.
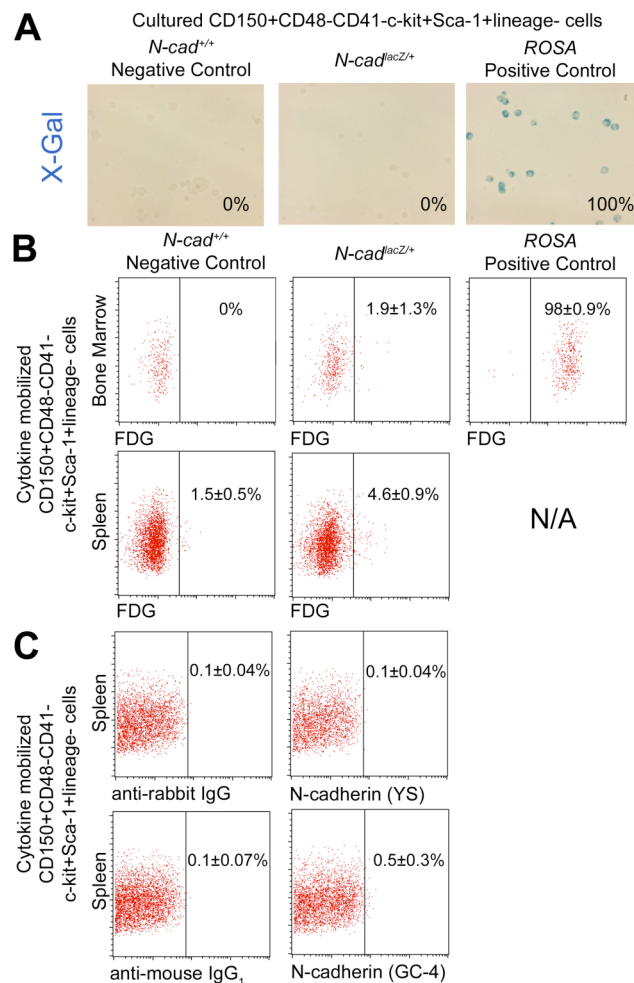
Figure 3.5: Mobilization or culture did not detectably increase N-cadherin expression by HSCs.   A) CD150+CD48-CD41-c-kit+Sca-1+lineage- HSCs from wild-type (negative control) mice, *N-cadherin^{lacZ/+}* gene trap mice, or Rosa (positive control mice) were cultured in the presence of stem cell factor, thrombopoietin, insulin-like growth factor II, and acidic fibroblast growth factor for 4 days (Zhang and Lodish, 2005) and then subjected to X-Gal staining. All Rosa cells stained positively with X-gal but we did not detect staining by *N-cadherin^{lacZ/+}* cells or littermate control cells. B) CD150+CD48-CD41-c-kit+Sca-1+lineage- HSCs were isolated from the bone marrow or spleen of cyclophosphamide/G-CSF mobilized mice (mice were treated with cyclophosphamide followed by five daily injections of G-CSF before being analyzed; see (Morrison et al., 1997) for details). FDG staining was detected from Rosa (positive control) cells but not from *N-cadherin^{lacZ/+}* cells or littermate (negative) control cells. Numbers represent the percentage (mean±SD) of CD150+CD48-CD41-c-kit+Sca-1+lineage- HSCs with FDG expression above background. C) CD150+CD48-CD41-c-kit+Sca-1+lineage- HSCs isolated from the spleens of cyclophosphamide/G-CSF mobilized mice were stained with antibodies against N-cadherin. Secondary only controls are shown for comparison. Numbers represent the percentage (mean±SD) of HSCs with staining above background. Data were collected from 2 independent experiments. There were no statistically significant differences between controls and antibody stained samples or between wild-type controls and *N-cadherin^{lacZ/+}* samples (p>0.15).
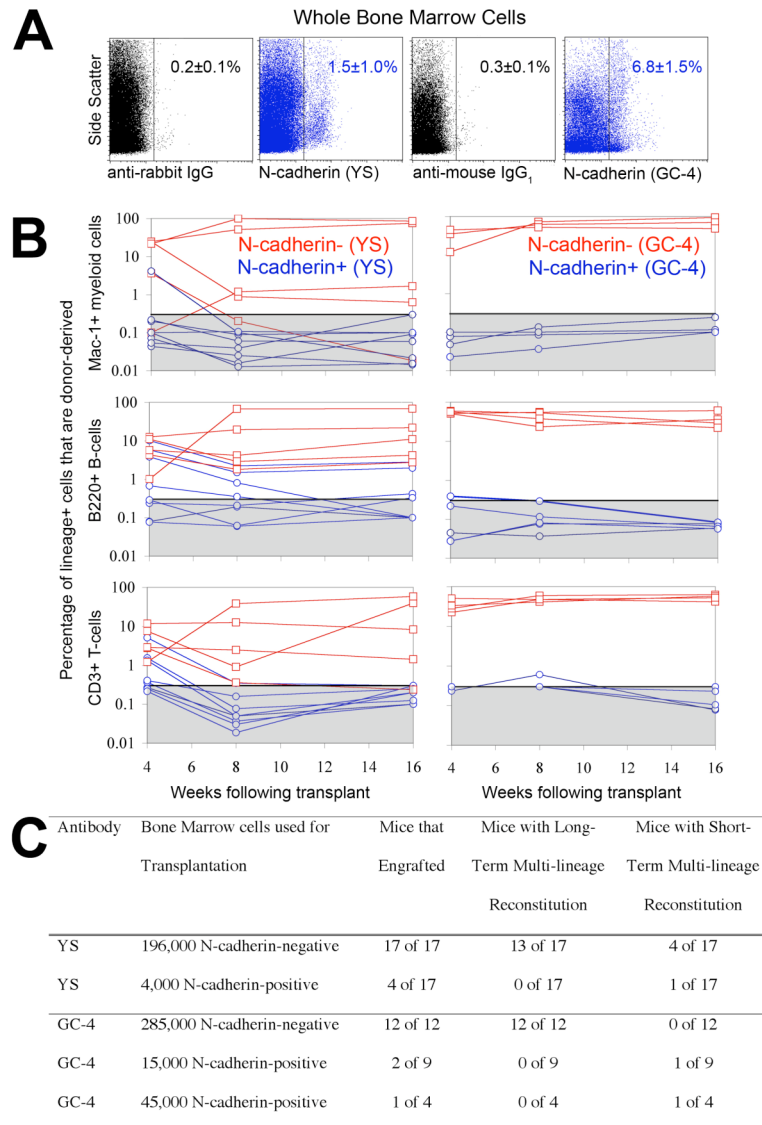
Figure 3.6: All detectable HSC activity is contained within the N-cadherin- fraction of bone marrow cells. (A) 1.5 to 6.8% of bone marrow cells stained positively for N-cadherin, depending on the antibody used (mean±SD from 3-8 independent experiments). B) Bone marrow cells were fractionated into N-cadherin- 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 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 panel 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 3 to 4 independent experiments.

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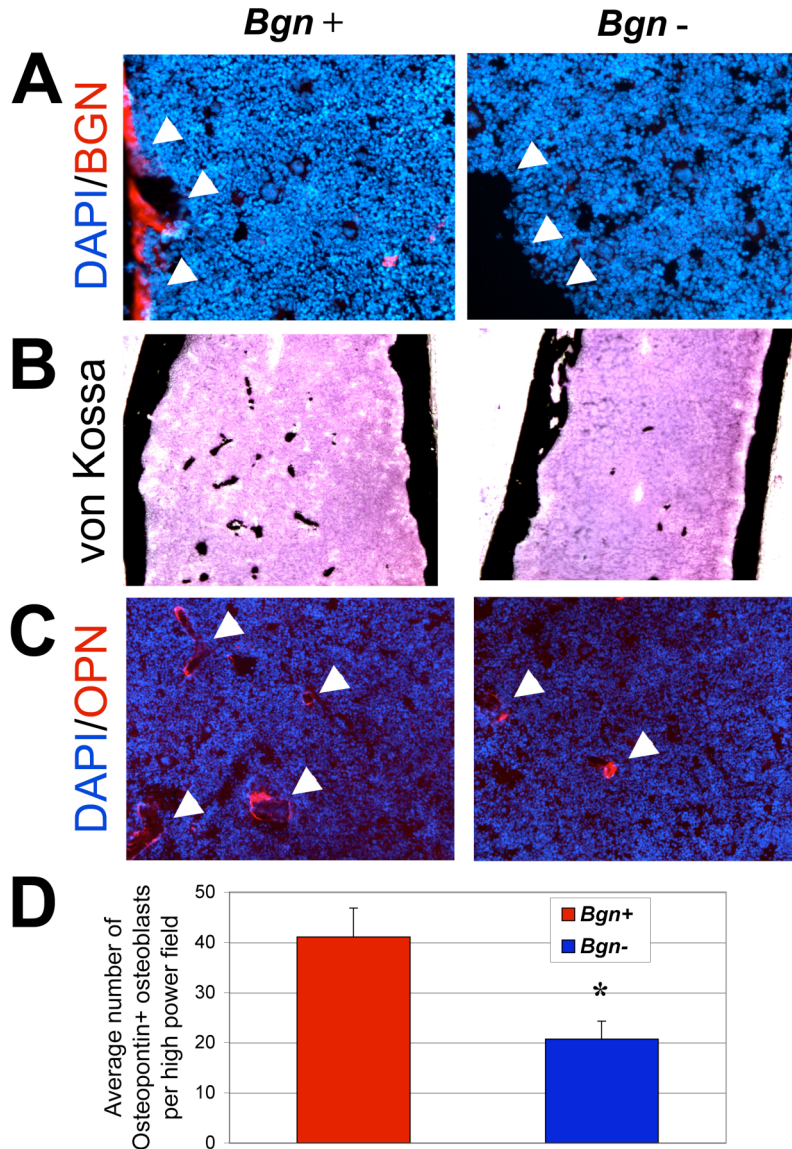
Figure 3.7: *biglycan*-**deficient mice exhibit significant reductions in trabecular bone and osteoblasts relative to littermate controls**. Five micron sections through wild-type (*Bgn*⁺) and *biglycan*-deficient (*Bgn*⁻) femurs were stained with anti-Biglycan antiserum. Biglycan expression (red) was detected at the endosteal surface (arrowheads) and within the bone matrix through the epiphysis and diaphysis as well as in the non-ossified chrondrous regions (not shown) in wild-type tissue (A) but not in *biglycan*-deficient tissue (B). Nuclei (blue) were stained with DAPI. This staining pattern is consistent with prior reports that Biglycan is preferentially expressed by osteoblasts in the bone marrow (Chen et al., 2004; Chen et al., 2002; Xu et al., 1998). 15 month-old *biglycan*-deficient mice exhibited less trabecular bone based on von Kossa staining (B) as well as fewer osteoblasts based on staining for Osteopontin (C). Counting of Osteopontin+ osteoblasts on endosteal surfaces revealed a significant reduction in *biglycan*-deficient mice (D). Note that all mice were male in this analysis.
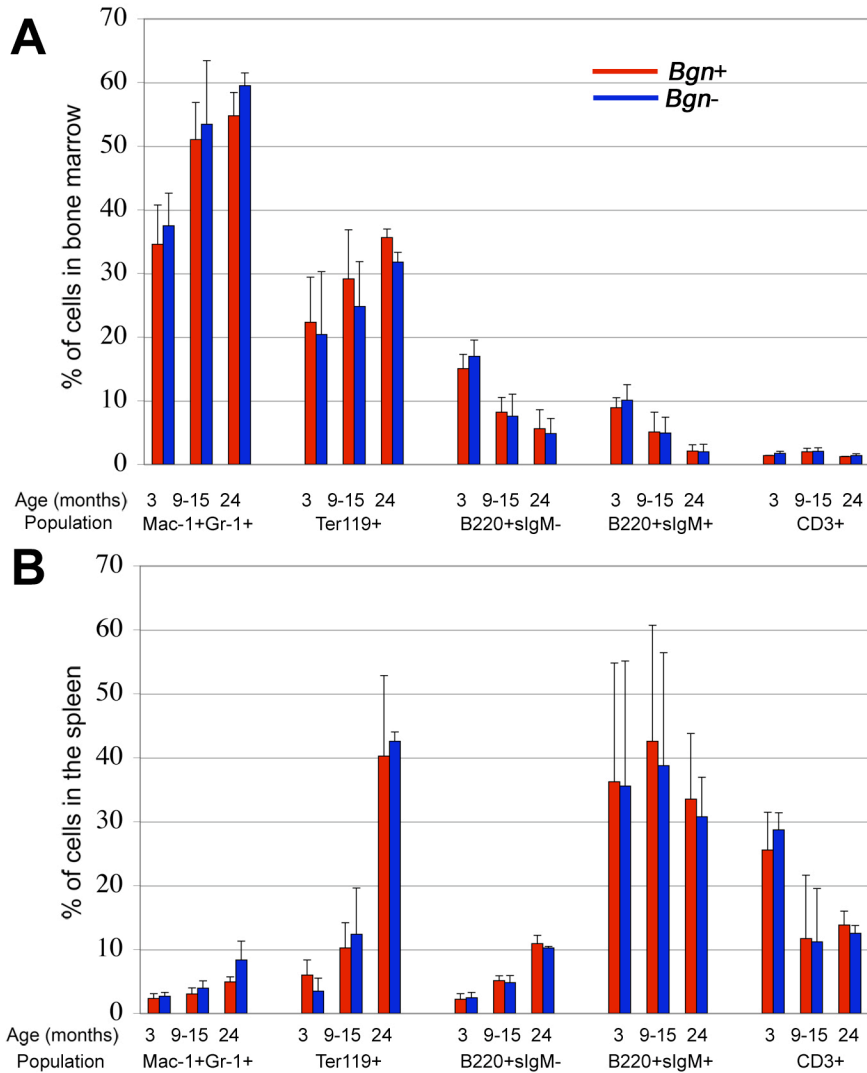
Figure 3.8: *biglycan*-**deficient mice exhibit normal frequencies of hematopoietic cells in bone marrow and spleen throughout life**. No statistically significant differences were observed in the frequencies of Mac-1+Gr-1+ myeloid cells, Ter119+ erythroid cells, B220+surface IgM- B lineage cells, B220+sIgM+ B cells, or CD3+ T-cells from the bone marrow (*A*) or spleen (*B*) of *biglycan*-deficient (*Bgn-*, blue) mice as compared to littermate controls (*Bgn+*, red) at 3, 9 to 12 or 24 months of age. Data represent 2 to 7 mice per genotype per age in 5 independent experiments. All mice were male.
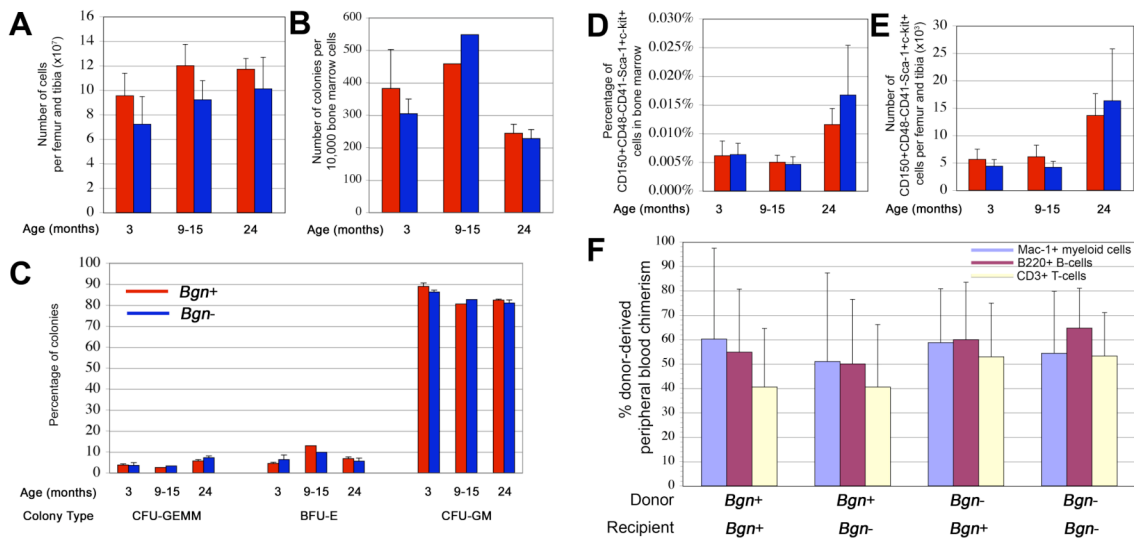
Figure 3.9: Normal hematopoiesis and HSC function despite osteoblast depletion in the absence of *biglycan*. (A) Slightly fewer bone marrow cells were recovered per femur and tibia of *biglycan*-deficient (blue bars) mice as compared to littermate controls (red bars) at 3, 9 to 15 or 24 months of age, though in no case was the difference statistically significant (p=0.09 to 0.31; data from 5 independent experiments with 2 to 5 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-kit+Sca-1+ HSCs in bone marrow from control littermates (*Bgn*+, red bars) and *biglycan*-deficient (*Bgn*-, blue bars) mice at 3, 9 to 15, or 24 months of age (p=0.46-0.89). Data represent mean±SD from 2-5 animals of each genotype in 1-3 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. 300,000 bone marrow cells 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 2 independent experiments. A total of 12 to 17 recipients were analyzed for each treatment with 2-3 independent donors per genotype.

A DAPI/CD150   CD41 CD48 lineage   MECA-32/CD150 CD41 CD48 lineage
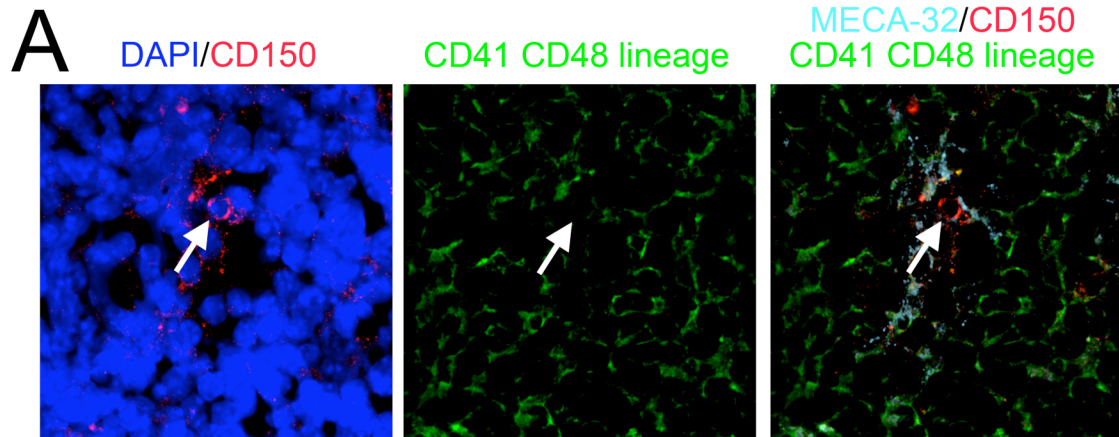
B All bone marrow cells

	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 3.10: 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 pan-endothelial 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 2-3 mice per genotype.

BIBLIOGRAPHY

Adams, G.B., Chabner, K.T., Alley, I.R., Olson, D.P., Szczepiorkowski, Z.M., Poznansky, M.C., Kos, C.H., Pollak, M.R., Brown, E.M., and Scadden, D.T. (2006). Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. Nature *439*, 599-603.

Adams, G.B., and Scadden, D.T. (2006). The hematopoietic stem cell in its place. Nature Immunology *7*, 333-337.

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell *118*, 149-161.

Bianco, P., Fisher, L.W., Young, M.F., Termine, J.D., and Robey, P.G. (1990). Expression and localization of the two small proteoglycans biglycan and decorin in developing human skeletal and non-skeletal tissues. J Histochem Cytochem *38*, 1549-1563.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R.*, et al.* (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. Nature *425*, 841-846.

Chen, X.D., Fisher, L.W., Robey, P.G., and Young, M.F. (2004). The small leucine-rich proteoglycan biglycan modulates BMP-4-induced osteoblast differentiation. Faseb J *18*, 948-958.

Chen, X.D., Shi, S., Xu, T., Robey, P.G., and Young, M.F. (2002). Age-related osteoporosis in biglycan-deficient mice is related to defects in bone marrow stromal cells. J Bone Miner Res *17*, 331-340.

Cheshier, S., Morrison, S.J., Liao, X., and Weissman, I.L. (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proceedings of the National Academy of Sciences USA *96*, 3120-3125.

Christensen, J.L., and Weissman, I.L. (2001). Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proc Natl Acad Sci U S A *98*, 14541-14546.

Hayashi, R., Yamato, M., Sugiyama, H., Sumide, T., Yang, J., Okano, T., Tano, Y., and Nishida, K. (2006). N-cadherin is expressed by putative stem/progenitor cells and melanocytes in the human limbal epithelial stem cell niche. Stem Cells.

Hooper, A.T., Butler, J., Petit, I., and Rafii, S. (2007). Does N-cadherin regulate interaction of hematopoietic stem cells with their niches? Cell Stem Cell *1*, 127-129.

Ivanova, N.B., Dimos, J.T., Schaniel, C., Hackney, J.A., Moore, K.A., and Lemischka, I.R. (2002). A stem cell molecular signature. Science *298*, 601-604.

Katayama, Y., Battista, M., Kao, W.M., Hidalgo, A., Peired, A.J., Thomas, S.A., and Frenette, P.S. (2006). Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell *124*, 407-421.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells. Cell *121*, 1109-1121.

Kim, I., He, S., Yilmaz, O.H., Kiel, M.J., and Morrison, S.J. (2006). Enhanced purification of fetal liver hematopoietic stem cells using SLAM family receptors. Blood *108*, 737-744.

Kollet, O., Dar, A., Shivtiel, S., Kalinkovich, A., Lapid, K., Sztainberg, Y., Tesio, M., Samstein, R.M., Goichberg, P., Spiegel, A.*, et al.* (2006). Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nat Med *12*, 657-664.

Kopp, H.G., Avecilla, S.T., Hooper, A.T., and Rafii, S. (2005). The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiology (Bethesda) *20*, 349-356.

Kostetskii, I., Li, J., Xiong, Y., Zhou, R., Ferrari, V.A., Patel, V.V., Molkentin, J.D., and Radice, G.L. (2005). Induced deletion of the N-cadherin gene in the heart leads to dissolution of the intercalated disc structure. Circ Res *96*, 346-354.

Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. Science *269*, 1427-1429.

Li, W., Johnson, S.A., Shelley, W.C., and Yoder, M.C. (2004). Hematopoietic stem cell repopulating ability can be maintained in vitro by some primary endothelial cells. Exp Hematol *32*, 1226-1237.

Luo, Y., Kostetskii, I., and Radice, G.L. (2005). N-cadherin is not essential for limb mesenchymal chondrogenesis. Dev Dyn *232*, 336-344.

Mancini, S.J., Mantei, N., Dumortier, A., Suter, U., Macdonald, H.R., and Radtke, F. (2005). Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. Blood *105*, 2340-2342.

Molofsky, A.V., Pardal, R., Iwashita, T., Park, I.K., Clarke, M.F., and Morrison, S.J. (2003). Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature *425*, 962-967.

Moore, K.A., and Lemischka, I.R. (2006). Stem cells and their niches. Science *311*, 1880-1885.

Morrison, S.J., Wright, D., and Weissman, I.L. (1997). Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. Proc Natl Acad Sci USA *94*, 1908-1913.

Nilsson, S.K., Johnston, H.M., and Coverdale, J.A. (2001). Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. Blood *97*, 2293-2299.

Nilsson, S.K., Johnston, H.M., Whitty, G.A., Williams, B., Webb, R.J., Denhardt, D.T., Bertoncello, I., Bendall, L.J., Simmons, P.J., and Haylock, D.N. (2005). Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood *106*, 1232-1239.

Ohneda, O., Fennie, C., Zheng, Z., Donahue, C., La, H., Villacorta, R., Cairns, B., and Lasky, L.A. (1998). Hematopoietic stem cell maintenance and differentiation are supported by embryonic aorta-gonad-mesonephros region-derived endothelium. Blood *92*, 908-919.

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R., and Down, J.D. (2007). Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc Natl Acad Sci U S A *104*, 5431-5436.

Radice, G.L., Rayburn, H., Matsunami, H., Knudsen, K.A., Takeichi, M., and Hynes, R.O. (1997). Developmental defects in mouse embryos lacking N-cadherin. Dev Biol *181*, 64-78.

Redies, C., and Takeichi, M. (1993). Expression of N-cadherin mRNA during development of the mouse brain. Dev Dyn *197*, 26-39.

Stier, S., Ko, Y., Forkert, R., Lutz, C., Neuhaus, T., Grunewald, E., Cheng, T., Dombkowski, D., Calvi, L.M., Rittling, S.R., *et al*. (2005). Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J Exp Med *201*, 1781-1791.

Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 signaling in bone marrow stromal cell niches. Immunity *In Press*.

Suzuki, N., Ohneda, O., Minegishi, N., Nishikawa, M., Ohta, T., Takahashi, S., Engel, J.D., and Yamamoto, M. (2006). Combinatorial Gata2 and Sca1 expression defines hematopoietic stem cells in the bone marrow niche. Proc Natl Acad Sci U S A *103*, 2202-2207.

Taichman, R.S., Reilly, M.J., and Emerson, S.G. (1996). Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures. Blood *87*, 518-524.

Tombran-Tink, J., and Barnstable, C.J. (2004). Osteoblasts and osteoclasts express PEDF, VEGF-A isoforms, and VEGF receptors: possible mediators of angiogenesis and matrix remodeling in the bone. Biochem Biophys Res Commun *316*, 573-579.

Visnjic, D., Kalajzic, Z., Rowe, D.W., Katavic, V., Lorenzo, J., and Aguila, H.L. (2004). Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. Blood *103*, 3258-3264.

Wilson, A., Murphy, M.J., Oskarsson, T., Kaloulis, K., Bettess, M.D., Oser, G.M., Pasche, A.C., Knabenhans, C., Macdonald, H.R., and Trumpp, A. (2004). c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev *18*, 2747-2763.

Wilson, A., and Trumpp, A. (2006). Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol *6*, 93-106.

Wright, D.E., Wagers, A.J., Gulati, A.P., Johnson, F.L., and Weissman, I.L. (2001). Physiological migration of hematopoietic stem and progenitor cells. Science *294*, 1933-1936.

Xu, T., Bianco, P., Fisher, L.W., Longenecker, G., Smith, E., Goldstein, S., Bonadio, J., Boskey, A., Heegaard, A.M., Sommer, B., *et al*. (1998). Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice. Nat Genet *20*, 78-82.

Yao, L., Yokota, T., Xia, L., Kincade, P.W., and McEver, R.P. (2005). Bone marrow dysfunction in mice lacking the cytokine receptor gp130 in endothelial cells. Blood *106*, 4093-4101.

Yilmaz, O.H., Kiel, M.J., and Morrison, S.J. (2006a). SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. Blood *107*, 924-930.

Yilmaz, O.H., Valdez, R., Theisen, B.K., Guo, W., Ferguson, D.O., Wu, H., and Morrison, S.J. (2006b). Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature *441*, 475-482.

Zhang, C.C., and Lodish, H.F. (2005). Murine hematopoietic stem cells change their surface phenotype during ex vivo expansion. Blood *105*, 4314-4320.

Zhang, J., and Li, L. (2008). Stem cell niche - Microenvironment and beyond. J Biol Chem.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q*., et al.* (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.

Zhu, J., Garrett, R., Jung, Y., Zhang, Y., Kim, N., Wang, J., Joe, G.J., Hexner, E., Choi, Y., Taichman, R*., et al.* (2007). Osteoblasts support B lymphocyte commitment and differentiation from hematopoietic stem cells. Blood *109*, 3706-3712.

CHAPTER 4

HEMATOPOIETIC STEM CELLS DO NOT DEPEND ON N-CADHERIN EXPRESSION FOR THEIR MAINTENANCE[1]

SUMMARY

According to the "osteoblastic niche" model, hematopoietic stem cells (HSCs) are maintained by N-cadherin-mediated homophilic adhesion to osteoblasts at the bone marrow endosteum. In contrast to this model we have been unable to detect N-cadherin expression by HSCs using several different techniques. It has nonetheless been suggested that HSCs express low levels of N-cadherin that are difficult to detect, but functionally important for HSC maintenance. To test this we conditionally deleted N-cadherin from HSCs and other hematopoietic cells in adult *Mx-1-Cre$^+$N-cadherin$^{fl/-}$* mice. N-cadherin deficiency had no detectable effect on HSC maintenance or hematopoiesis. N-cadherin deficiency did not affect bone marrow cellularity or lineage composition, the numbers of colony-forming progenitors, the frequency of HSCs, the ability of HSCs to sustain hematopoiesis over time, or their ability to reconstitute irradiated mice. These results

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demonstrate that N-cadherin expression by HSCs is not necessary for the function of HSC niches, an observation inconsistent with "osteoblastic niche" models.

INTRODUCTION

HSCs persist throughout adult life in the bone marrow where they continuously produce new blood cells to maintain the hematopoietic system. To understand the mechanisms that sustain HSCs it is necessary to identify the niche – the specialized microenvironment in which HSCs are thought to reside (Adams and Scadden, 2006; Kiel and Morrison, 2008).

Osteoblasts have been widely suggested to contribute to the creation of HSC niches. Genetic manipulations that increase osteoblast numbers in mice also increase the number of HSCs (Calvi et al., 2003; Zhang et al., 2003). Osteoblasts have also been proposed to secrete factors that are necessary for HSC maintenance, including angiopoietin, thrombopoietin, and CXCL12 (Arai et al., 2004; Yoshihara et al., 2007), though none of these factors have yet been conditionally deleted from osteoblasts and each is also secreted by other cell types. Calcium ions from bone resorption (due to osteoblast and osteoclast activity) also regulate HSC localization and maintenance (Adams et al., 2006). These observations raise two general possibilities for how osteoblasts could contribute to HSC maintenance. One possibility is that osteoblasts produce extracellular factors that diffuse into the marrow, directly or indirectly regulating HSC niches that are near, but not at, the endosteum. A second possibility is that osteoblasts directly promote HSC maintenance by binding HSCs, creating "osteoblastic niches" at the endosteal surface.

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The possibility of bone marrow HSC niches near, but not at, the endosteum has been raised by a number of recent observations (Kiel and Morrison, 2008). We have localized HSCs within the bone marrow using SLAM family markers that give high levels of stem cell purity and found that few HSCs localized to the endosteal surface (Kiel et al., 2007b; Kiel et al., 2005). Instead, most HSCs were present around sinusoids, some of which were close to the endosteum while others were more distant. It remains uncertain whether perivascular cells directly promote HSC maintenance; however, the observation that HSCs are considerably more likely than other hematopoietic cells to be adjacent to sinusoids (Kiel et al., 2007b) raises the possibility that there are perivascular niches. Consistent with this possibility, recent studies have suggested that perivascular cells, such as reticular cells and mesenchymal progenitors, express more CXCL12 and angiopoietin than osteoblasts (Sacchetti et al., 2007; Sugiyama et al., 2006). This raises the question of whether HSCs are maintained in direct contact with osteoblasts, or whether they are maintained in other microenvironments that are directly or indirectly influenced by factors secreted by endosteal cells.

The widely discussed "osteoblastic niche" model has favored the idea that HSCs are maintained in direct contact with osteoblasts (Suda et al., 2005; Wilson and Trumpp, 2006; Zhang and Li, 2008; Zhang et al., 2003). This model proposes that HSCs adhere to the surface of osteoblasts via N-cadherin mediated homophilic adhesion and that osteoblasts directly promote the maintenance of HSCs by mechanisms that involve cell-cell contact, including Notch and N-cadherin activation (Haug et al., 2008; Wilson et al., 2004; Zhang et al., 2003). However, genetic evidence supporting such mechanisms is lacking. Conditional deletion of Jagged1 and/or Notch1 (the ligand/receptor combination

proposed to regulate Notch signaling between osteoblasts and HSCs) does not affect HSC maintenance or function (Mancini et al., 2005). Moreover, conditional inactivation of all canonical Notch signaling by disruption of the CSL/Rbp-J transcriptional complex also does not affect the maintenance or function of adult HSCs (Maillard et al., 2008). It has not been tested whether *N-cadherin* deficiency affects HSC maintenance.

Given the central role proposed for N-cadherin in the creation of osteoblastic niches, we recently tested whether HSCs express N-cadherin. We were unable to detect N-cadherin expression among highly purified HSCs by quantitative PCR, by staining with commercially available anti-N-cadherin antibodies, or in N-cadherin:LacZ genetrap mice (Kiel et al., 2007b). Published microarray analyses of HSCs from multiple laboratories also failed to detect N-cadherin expression (Ivanova et al., 2002; Kiel et al., 2005). Only N-cadherin negative bone marrow cells had the capacity to give long-term multilineage reconstitution of irradiated mice (Kiel et al., 2007b), even when we used the commercially available anti-N-cadherin antibody that had been used to identify putative osteoblastic niches in bone marrow sections (Wilson et al., 2004; Zhang et al., 2003). This suggested that HSCs could not adhere to osteoblasts via N-cadherin and that the N-cadherin$^+$ cells imaged at the endosteum in prior studies could not have been HSCs. However, it was subsequently suggested that N-cadherin is expressed at very low levels on HSCs and that it remains functionally important for their localization within a quiescent osteoblastic niche despite being difficult to detect (Haug et al., 2008; Zhang and Li, 2008).

To resolve this controversy it was suggested that *N-cadherin* should be genetically deleted from adult hematopoietic cells (Hooper et al., 2007). If N-cadherin is

not expressed by HSCs, then *N-cadherin* deficiency should not affect HSC maintenance. In contrast, if HSC niches are regulated by low levels of N-cadherin expression within HSCs then *N-cadherin* deletion should lead to the depletion of HSCs and to deficits in hematopoiesis. We have now addressed this by conditionally deleting *N-cadherin* from HSCs and other hematopoietic cells in adult *Mx-1-Cre$^+$N-cadherin$^{fl/-}$* mice. Despite efficiently deleting *N-cadherin* from HSCs, these mice exhibited no detectable effects of *N-cadherin* deficiency on HSC maintenance or hematopoiesis, either acutely after *N-cadherin* deletion or months later. Bone marrow cells from these mice exhibited normal HSC frequency and a normal capacity to reconstitute irradiated mice. *N-cadherin* is therefore not required cell-autonomously to regulate HSC maintenance or function, casting doubt on widely published models of the osteoblastic niche.

RESULTS

***N-cadherin* is efficiently deleted after pIpC treatment**

Mice with germline *N-cadherin* deficiency (*N-cadherin$^{-/-}$*) die by E11 due to severe developmental defects including cardiovascular failure (Radice et al., 1997). Therefore, to study the effects of *N-cadherin* deficiency on adult HSCs, we mated previously described *N-cadherin^{fl}* mice (Kostetskii et al., 2005) with *Mx-1-Cre* mice (Kuhn et al., 1995) to conditionally delete *N-cadherin* from adult HSCs. Each of these strains were backcrossed for at least 6 generations onto a C57BL background. *Mx-1-Cre* expression is activated in HSCs, other hematopoietic cells, and some other tissues by administration of polyinosine-polycytidine (pIpC) (Hock et al., 2004; Kuhn et al., 1995; Yilmaz et al., 2006b).

To test the efficiency of *N-cadherin* excision within HSCs, we cultured individual

CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs (1 cell/well) isolated from *Mx-1-Cre$^+$N-cadherin$^{fl/-}$* mice after they had been treated with 7 doses of pIpC over 14 days.

These cells include more than 95% of all long-term multilineage reconstituting cells in

the bone marrow, and more than 40% of single cells within this population give long-

term multilineage reconstitution after transplantation into irradiated mice (Kiel et al.,

2005; Kiel et al., 2008; Yilmaz et al., 2006a). Genomic DNA was extracted from

individual myeloerythroid colonies formed by these HSCs and analyzed by PCR. Of

colonies cultured immediately after pIpC treatment, 53 of 54 showed complete

recombination of *N-cadherin^{fl}* (Figure 4.1A,B). Of colonies that arose from HSCs

cultured 1 to 2 months following pIpC treatment, 72 of 73 showed complete

recombination of *N-cadherin^{fl}* (Figure 4.1A,B). This indicates that at least 98% of HSCs

deleted *N-cadherin* upon pIpC treatment. The rare non-recombined cells did not appear to

have a competitive advantage over *N-cadherin* deficient HSCs, as their frequency did not

increase with time after pIpC treatment.

N-cadherin deficiency does not alter hematopoiesis

If *N-cadherin* is critical for HSC maintenance then *N-cadherin* deficiency should

lead to defects in hematopoiesis. We therefore tested whether acute or chronic loss of *N-cadherin* affected hematopoiesis in *Mx-1-Cre$^+$N-cadherin$^{fl/-}$* mice. To test this we

harvested the peripheral blood and bone marrow from *Mx-1-Cre$^+$N-cadherin$^{fl/-}$* mice or

littermate controls (mice lacking *Cre* or bearing at least one wild-type allele of *N-cadherin*) either immediately following pIpC treatment (2 weeks pIpC; Figure 4.1C-G) or

1-2 months following 2 weeks of pIpC administration (Figure 4.1H-L). We did not

detect any effect of *N-cadherin* deletion on white blood cell (WBC), erythrocyte (RBC)

or platelet (Plts) concentration in the blood of *Mx-1-Cre⁺N-cadherin^{fl/-}* mice as compared

to littermate controls at either time point (Figure 4.1C, H). We also did not detect any

effect of *N-cadherin* deficiency on bone marrow cellularity (Figure 4.1D, I) or

composition with respect to the erythroid (Ter119+ cells), myeloid (Mac-1+Gr-1+), B

(B220+surfaceIgM±) or T cell (CD3+) lineages (Figure 4.1E, J). Finally, we did not

detect any effect of *N-cadherin* deficiency on the frequency (Figure 4.1F, K) or types

(Figure 4.1G, L) of colony-forming progenitors in the bone marrow. These results

indicate that *N-cadherin* is not required within HSCs for hematopoiesis.

N-cadherin deficiency does not alter HSC frequency or function in vivo

To directly test whether *N-cadherin* deficiency affects HSC maintenance we

examined the HSC content of bone marrow isolated from *Mx-1-Cre⁺N-cadherin^{fl/-}* mice

or littermate controls either immediately following pIpC treatment (Figure 4.2A-C) or 1-2

months later (Figure 4.2D-F). As previously reported for wild-type mice (Kiel et al.,

2007a; Kiel et al., 2005; Kiel et al., 2008), the vast majority of CD150+CD48-CD41-

lineage- cells from the bone marrow of *Mx-1-Cre⁺N-cadherin^{fl/-}* mice or littermate

controls were positive for the HSC markers Sca-1 and c-kit (Figure 4.2A, D). We did not

detect any difference in the frequency or absolute number of CD150+CD48-CD41-

lineage-Sca-1+c-kit+ HSCs in the hindlimb bone marrow of *Mx-1-Cre⁺N-cadherin^{fl/-}*

mice as compared to littermate controls (Figure 4.2B, E). We were also unable to detect

any difference in the frequency or absolute number of Flk2-lineage-Sca-1+c-kit+ HSCs

in *Mx-1-Cre⁺N-cadherin*$^{fl/-}$ mice as compared to littermate controls (Figure 4.3). Finally, when CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs were individually cultured in methylcellulose, 83-89% of cells formed large myeloerythroid colonies irrespective of whether they were from *Mx-1-Cre⁺N-cadherin*$^{fl/-}$ mice or littermate controls (Figure 4.2C, F). We therefore could not detect any effect of *N-cadherin* deficiency on the frequency, absolute number, or colony-forming capacity of HSCs either immediately after *N-cadherin* deletion or 1-2 months later.

To test whether *N-cadherin* deficiency affects HSC function in vivo, we transplanted 1x10^6 bone marrow cells from non-pIpC-treated CD45.2⁺ *Mx-1-Cre⁺N-cadherin*$^{fl/-}$ mice or CD45.2⁺ littermate controls into lethally irradiated CD45.1+ recipients along with 3x10^5 wild-type CD45.1+ bone marrow cells. Four weeks following transplantation, we analyzed the blood of these recipients for engraftment by CD45.2+ donor cells and found that both *Mx-1-Cre⁺N-cadherin*$^{fl/-}$ cells and control cells had engrafted at similar levels, as expected (Figure 4.4A-C; 4 week time point). Starting 6 weeks after transplantation, we administered pIpC for 2 weeks to all mice to delete *N-cadherin*. If N-cadherin is required for HSC maintenance or function in vivo then we should have observed a loss of donor cells over time in the mice transplanted with *Mx-1-Cre⁺N-cadherin*$^{fl/-}$ cells. We monitored the levels of donor cell reconstitution in all recipients for a total of 20 weeks after pIpC treatment but never observed a decline in donor myeloid (Mac-1+; Figure 4.4A), B (B220+; Figure 4.4B) or T cells (CD3+; Figure 4.4C) in recipients of *Mx-1-Cre⁺N-cadherin*$^{fl/-}$ cells. To ensure that *N-cadherin* had been efficiently deleted in this experiment, we performed PCR on genomic DNA from donor-derived myeloid cells isolated from the peripheral blood of these recipients. We observed

complete excision of *N-cadherin* from the myeloid cells collected from recipients of *Mx-1-Cre⁺N-cadherin^{fl/-}* cells but not recipients of control cells (Figure 4.4D). These results indicate that *N-cadherin* is not required for the maintenance of HSC function in vivo and that *N-cadherin*-deficient HSCs are not at a competitive disadvantage relative to wild-type cells.

N-cadherin is not required for HSC reconstitution of irradiated recipients

If N-cadherin is required for HSC homing or engraftment in the niche, then deletion of *N-cadherin* from HSCs prior to transplantation should cause an engraftment defect. To test this we treated *Mx-1-Cre⁺N-cadherin^{fl/-}* mice or littermate controls with pIpC, then transplanted $1x10^6$ bone marrow cells 2 months later along with $3x10^5$ recipient bone marrow cells into CD45.1+ recipient mice. All of the recipients of both *Mx-1-Cre⁺N-cadherin^{fl/-}* cells (n=12) and control cells (n=11) became long-term multilineage reconstituted by donor myeloid, B, and T cells (Figure 4.5A). The level of reconstitution by donor cells did not differ between recipients of *Mx-1-Cre⁺N-cadherin^{fl/-}* cells and control cells in terms of all donor blood cells (CD45.2+, Figure 4.5B), donor myeloid cells (Mac-1+, Figure 4.5C), donor B cells (B220+, Figure 4.5D), or donor T cells (CD3+, Figure 4.5E). *N-cadherin* is therefore not required for the homing or engraftment of HSCs in vivo.

DISCUSSION

The "osteoblastic niche" model proposes that HSCs are maintained in direct contact with osteoblasts via N-cadherin homophilic adhesion (Arai et al., 2004; Haug et

al., 2008; Suda et al., 2005; Wilson et al., 2004; Wilson and Trumpp, 2006; Zhang and Li, 2008; Zhang et al., 2003). According to this model, N-cadherin is postulated to be required within HSCs to maintain adhesion with the niche, to regulate quiescence, to regulate ß-catenin signaling, and to maintain HSCs in an undifferentiated state (Arai et al., 2004; Haug et al., 2008; Suda et al., 2005; Wilson et al., 2004; Wilson and Trumpp, 2006; Zhang and Li, 2008; Zhang et al., 2003). Yet none of these predictions have been tested in *N-cadherin* deficient mice. Our data demonstrate that *N-cadherin* deletion in vivo from HSCs and other hematopoietic cells has no effect on hematopoiesis in the bone marrow (Figure 4.1), HSC frequency (Figure 4.2), HSC maintenance or function over time (Figure 4.4), or on the ability of HSCs to engraft and reconstitute irradiated mice (Figure 4.5). In no assay did *N-cadherin* deficient HSCs show a competitive disadvantage relative to wild-type HSCs. Our data thus demonstrate that *N-cadherin* is not required autonomously within HSCs to regulate their maintenance or function.

Other data have also been inconsistent with this model. HSCs were originally localized to the surface of osteoblasts based on staining for N-cadherin[+] bromo-deoxyuridine (BrdU) label-retaining cells (Zhang et al., 2003). However, in addition to being unable to detect N-cadherin expression within HSCs by several different techniques (Kiel et al., 2007b), we have also found that BrdU label-retention has very poor specificity and very poor sensitivity as an HSC marker: the vast majority of HSCs fail to retain BrdU for long periods of time and the vast majority of cells that do retain BrdU are not HSCs (Kiel et al., 2007a). When we localize highly purified HSCs within bone marrow sections using SLAM family markers, we find a minority of HSCs that localize to the endosteum (<20%) and a majority of HSCs (>60%) that localize to

sinusoids (Kiel et al., 2007b; Kiel et al., 2005). It thus remains possible that a subset of HSCs is maintained in niches that are at, or near, the endosteum by N-cadherin-independent mechanisms but it seems unlikely that all HSCs depend on proximity to endosteal cells for their maintenance. Rather, our data (Kiel et al., 2007b; Kiel et al., 2005) and the data of others (Sacchetti et al., 2007; Sugiyama et al., 2006) raise the possibility of perivascular niches for HSCs, though direct evidence that perivascular cells promote HSC maintenance is lacking.

A recent study from Li and colleagues (Haug et al., 2008) used an anti-N-cadherin antibody (MNCD2; (Matsunami and Takeichi, 1995)) that was not used in prior studies of HSCs to suggest that HSCs do express low levels of N-cadherin and that this can be used to resolve HSCs into two populations: an N-cadherin^{low} population that contains long-term multilineage reconstituting activity and an N-cadherin^{intermediate} "reserve" population that resides within the osteoblastic niche. However, the N-cadherin^{low} staining could not clearly be distinguished from background fluorescence and the N-cadherin^{intermediate} population had little or no HSC activity in reconstitution assays. In our view, if cells fail to exhibit HSC activity in transplantation assays the simplest interpretation is that they are not HSCs. We have also independently tested the MNCD2 antibody and fail to detect staining of HSCs (Figure 4.6). We do observe staining of bone marrow cells with this antibody, but the staining was not affected by *N-cadherin* deletion, suggesting that bone marrow staining is non-specific (Figure 4.6). We thus stand by our conclusion that HSCs do not express N-cadherin.

It remains possible that *N-cadherin* deficiency in osteoblasts could affect HSC maintenance or hematopoiesis, at least indirectly. Expression of dominant negative *N-*

cadherin in osteoblasts (that may affect other cadherins as well) leads to a reduction in trabecular bone and defects in osteoblast maturation (Cheng et al., 2000). Effects on hematopoiesis have not yet been tested in these mice, but it is possible that defects in osteogenesis could lead to changes in HSC frequency or hematopoiesis. Nonetheless, many direct and indirect mechanisms could potentially account for such effects so this would not provide evidence of an osteoblastic niche.

Many potential models of the HSC niche remain compatible with existing data and there is little experimental basis on which to favor any specific model (Kiel and Morrison, 2008; Morrison and Spradling, 2008). One possibility is that HSCs reside in niches near the endosteum that are created through the combined action of factors secreted by endosteal, perivascular, and potentially other cells. Another possibility is that HSCs reside primarily in perivascular niches, at least some of which are close to the endosteum, and potentially influenced by factors secreted by endosteal cells. A third possibility is that there are spatially distinct niches in the bone marrow, some of which are close to the endosteum while others are not. These possibilities are not mutually exclusive and are not the only possibilities. Although some have recently proposed that it may be possible to distinguish between cells that reside in perivascular versus endosteal niches, there is little direct experimental support for this idea. Moreover, this model is conceptually problematic given that the endosteum is among the most highly vascularized sites in the bone marrow: HSCs cannot localize to the endosteum without being perivascular. To more clearly define the niche it will be necessary to genetically determine the physiologically important sources for factors that are required for HSC maintenance.

MATERIALS AND METHODS

Mice

C57BL/Ka-Thy-1.1 (CD45.2) and C57BL/Ka-Thy-1.2 (CD45.1) mice were housed in the Unit for Laboratory Animal Medicine at the University of Michigan. Both *Mx-1-Cre* mice and *N-cadherin*^{fl/-} mice were backcrossed for at least six generations onto a C57BL background. In every experiment, littermate control mice were gender-matched and either lacked *Mx-1-Cre* or had at least one wild-type allele of *N-cadherin*.

pIpC administration

pIpC was administered to mice as previously described (Yilmaz et al., 2006b). Briefly, pIpC was resuspended in Dulbecco's phosphate buffered saline (D-PBS) at 2 mg/ml and mice were injected with 25ug per gram of body weight every other day for 14 days.

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 um, Sefar America, Kansas City, MO) to obtain a single cell suspension.

For isolation of Flk2-c-kit+Sca-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). An antibody against Flk-2 (A2F10.1) was used to isolate Flk-2⁻ progenitors. c-kit staining was visualized using streptavidin APC-Cy7.

For isolation of CD150+CD48-CD41-c-kit+Sca-1+lineage- HSCs, bone marrow cells were incubated with PE-conjugated anti-CD150 (TC15-12F12.2; BioLegend, San Diego, California), FITC-conjugated anti-CD48 (HM48-1; BioLegend), FITC-conjugated anti-CD41 (MWReg30; BD Pharmingen, San Diego, California), 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). Sca-1 was visualized using streptavidin-conjugated APC-Cy7. HSCs were sometimes pre-enriched by selecting Sca-1+ or c-kit+ cells using paramagnetic microbeads and autoMACS (Miltenyi Biotec, Auburn, CA). MNCD2 anti-N-cadherin antibody was obtained from Linheng Li. Non-viable cells were excluded from sorts and analyses using the viability dye DAPI (1ug/ml).

Long-term competitive repopulation assay

Adult recipient mice (CD45.1) were irradiated with an Orthovoltage X-ray source delivering approximately 300 rad/min in two equal doses of 570 rad, delivered at least 2 hr apart. Cells were injected into the retro-orbital venus sinus of anesthetized recipients. Each recipient mouse received 3×10^5 CD45.1 marrow cells for radioprotection. Beginning 4 weeks after transplantation and continuing for at least 16 weeks, blood was obtained from the tail veins of recipient mice, subjected to ammonium-chloride

potassium red cell lysis, and stained with directly conjugated antibodies to CD45.2 (104, FITC), B220 (6B2), Mac-1 (M1/70), CD3 (KT31.1), and Gr-1 (8C5) to monitor engraftment.

N-cadherin excision analysis

Genomic DNA from individual CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSC colonies was isolated by phenol-chloroform extraction. Glycogen (Roche, Indianapolis IN) was used to enhance the recovery of the DNA. PCR products were separated using 2% agarose gels to confirm the presence or absence of bands corresponding to wild-type, floxed or deleted alleles. Primer sequences for amplification of *N-cadherin* alleles were as described previously (Kostetskii et al., 2005): *N-cadherin* NC23 5'-GTATGGCCAAGTAATGGGGAC, *N-cadherin* L07 5'-TGCTGGTAGCATTCCTATGG and *N-cadherin* L08 5'-TACAAGTTTGGGTGACAAGC.

Methylcellulose culture

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

ACKNOWLEDGEMENTS

Figure 4.1: pIpC treatment of adult *Mx-1-Cre⁺N-cadherinᶠˡ⁻* mice leads to efficient *N-cadherin* deletion from HSCs without altering hematopoiesis. A) CD150+CD48-CD41-lineage-Sca-1+c-kit+ bone marrow HSCs from pIpC-treated *Mx-1-Cre⁺N-cadherinᶠˡ⁻* mice or littermate controls were cultured in methylcellulose for 14 days, then genomic DNA was extracted from the colonies and examined for *N-cadherin* deletion. B) Virtually all of the colonies examined in 6 independent experiments from 15 *Mx-1-Cre⁺N-cadherinᶠˡ⁻* mice showed complete deletion. Hematopoiesis was examined in *Mx-1-Cre⁺N-cadherinᶠˡ⁻* mice (black bars) or littermate controls (white bars) either immediately following 2 weeks of pIpC treatment (C-G) or 1-2 months following 2 weeks of pIpC administration (H-L). No statistically significant differences were observed in the concentration of white blood cells (WBC), erythrocytes (RBC) or platelets (Plts; C, H) from the blood of *Mx-1-Cre⁺N-cadherinᶠˡ⁻* mice as compared to littermate controls. We also did not observe any differences in bone marrow cellularity (D, I), composition (E, J), frequency (F, K), or types of colony-forming progenitors (G, L) in *Mx-1-Cre⁺N-cadherinᶠˡ⁻* mice as compared to littermate controls. Data are from 2 to 6 mice per treatment in 2-3 independent experiments. All error bars represent SD.
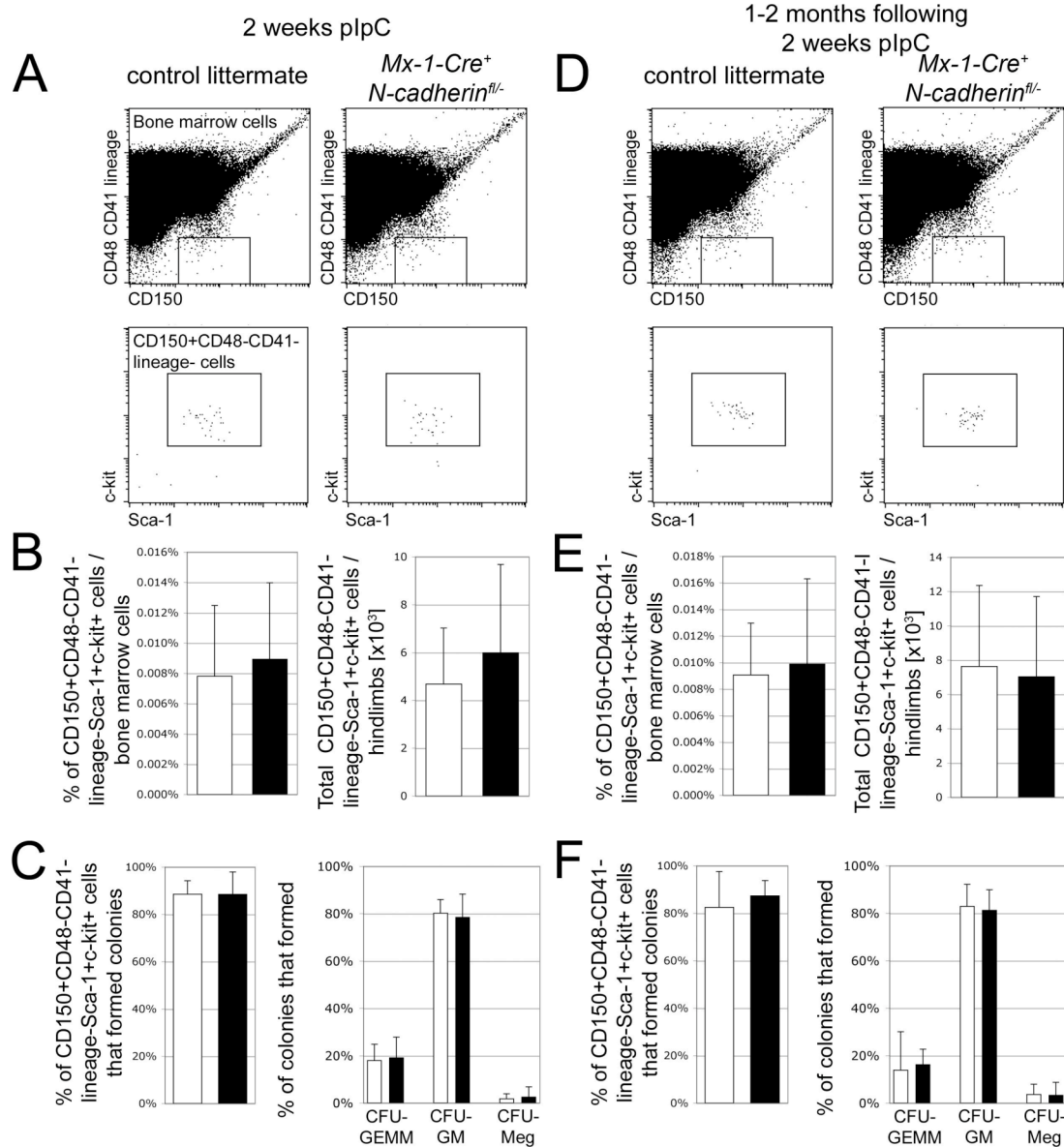
Figure 4.2: *N-cadherin* **deletion does not affect HSC frequency or the ability of HSCs to form colonies in culture.** The frequency of CD150+CD48-CD41-c-kit+Sca-1+lineage- cells was examined by flow-cytometry in *Mx-1-Cre⁺N-cadherin^{fl/-}* mice (black bars) or littermate controls (white bars) either immediately following pIpC administration (A-C) or 1-2 months later (D-F). No significant differences were observed in the frequency or absolute number of CD150+CD48-CD41-c-kit+Sca-1+lineage- cells contained within the bone marrow of *Mx-1-Cre⁺N-cadherin^{fl/-}* mice as compared to littermate controls (B and E). We also did not detect any effect of *N-cadherin* deficiency on the frequency of Flk2-c-kit+Sca-1+lineage- cells (Figure 4.3). When individual CD150+CD48-CD41-c-kit+Sca-1+lineage- cells were cultured in methylcellulose, no significant differences were observed in the percentage of these cells that formed colonies or in the types of colonies they formed (C and F). The data are from 6 independent experiments with 1-2 mice per treatment per experiment. All error bars represent SD.

2 weeks pIpC

control littermate

Mx-1-Cre⁺ N-cadherinᶠˡ/⁻

A

B
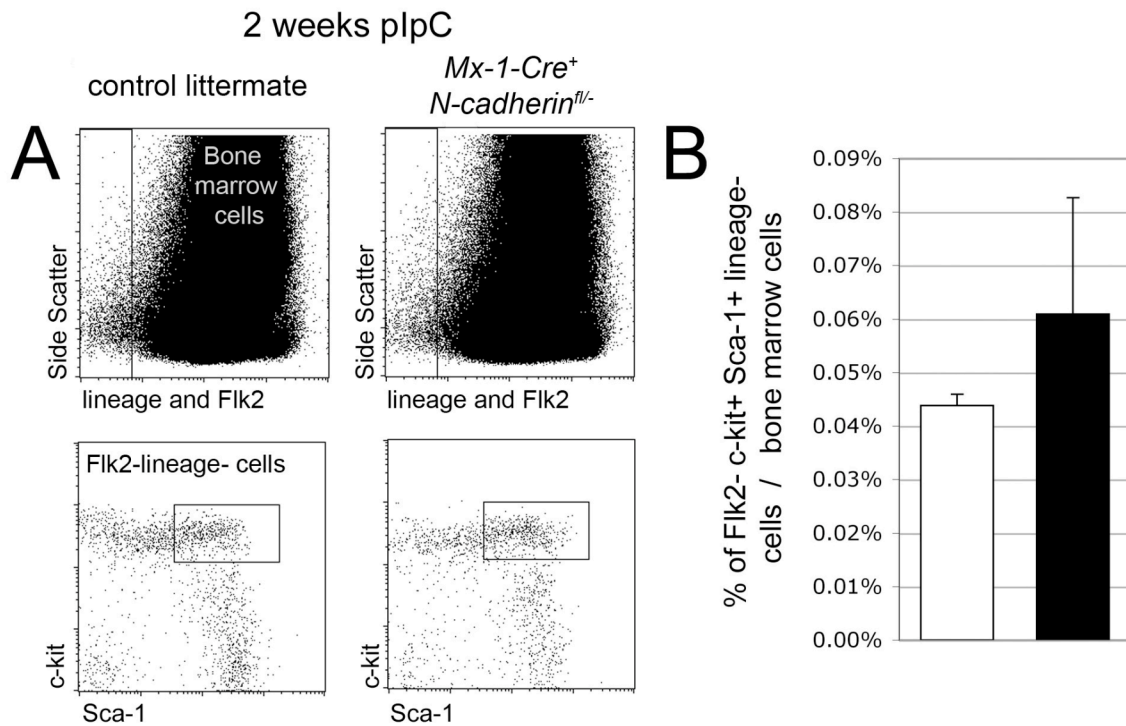
Figure 4.3: *N-cadherin* **deficiency does not affect the frequency of Flk2-c-kit+Sca-1+lineage- cells in the bone marrow.** Representative flow-cytometry plots of bone marrow cells from *Mx-1-Cre⁺N-cadherinᶠˡ/⁻* mice (right) or littermate controls (left) after 2 weeks of pIpC administration (A). No significant difference was observed in the frequency of Flk2-c-kit+Sca-1+lineage- cells within the bone marrow of *Mx-1-Cre⁺N-cadherinᶠˡ/⁻* mice as compared to littermate controls (B). These data are from 2 independent experiments with 1-2 mice of each genotype per experiment.
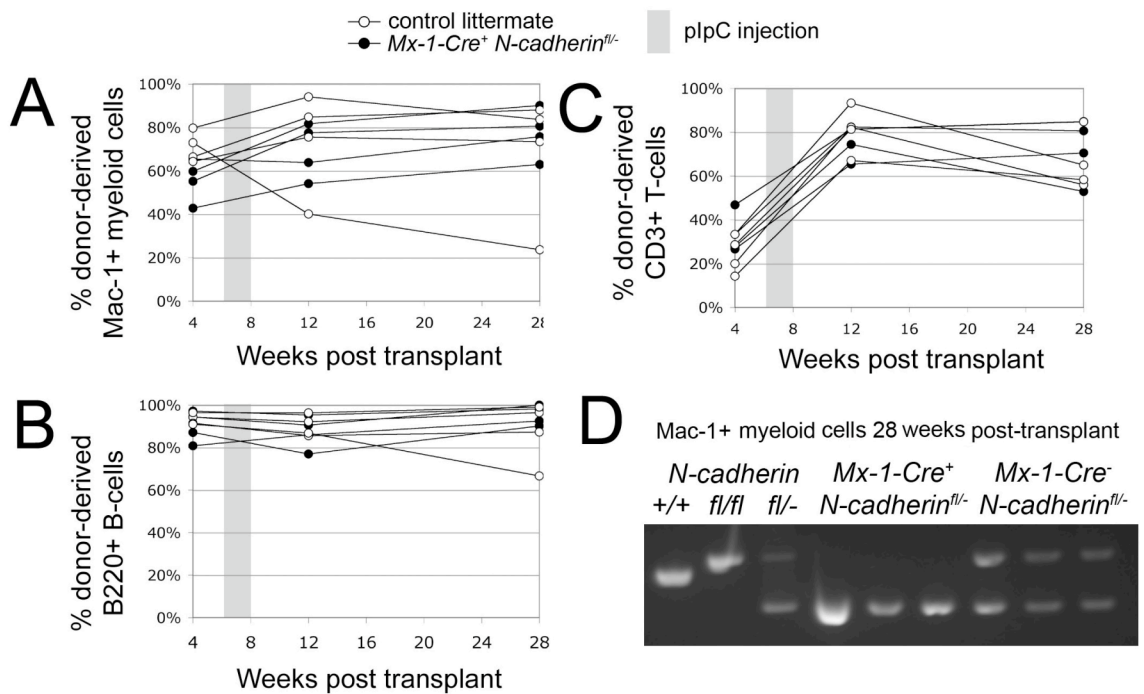
Figure 4.4: *N-cadherin* deficiency does not affect HSC maintenance or function in vivo. 1x10^6 bone marrow cells from CD45.2+ *Mx-1-Cre$^+$N-cadherin$^{fl/-}$* mice (black dots) or CD45.2+ littermate controls (white dots) were injected into lethally irradiated CD45.1$^+$ wild-type recipients. pIpC was administered to all recipients for 2 weeks (shaded area) beginning at week 6. Reconstitution by donor cells was then monitored for an additional 20 weeks. We observed no decline in reconstitution by donor myeloid (Mac-1+, A), B (B220+, B), or T (CD3+, C) cells, indicating that *N-cadherin* deletion did not affect HSC maintenance, function, or the ability to compete with wild-type HSCs. Each line represents a single recipient mouse. Data from one representative experiment are shown. Two independent experiments with a total of 9 recipients per genotype gave similar results. D) After 28 weeks, donor myeloid cells were isolated from the blood of representative recipients and analyzed for *N-cadherin* deletion: recipients of *Mx-1-Cre$^+$N-cadherin$^{fl/-}$* cells were reconstituted by *N-cadherin* deficient cells.
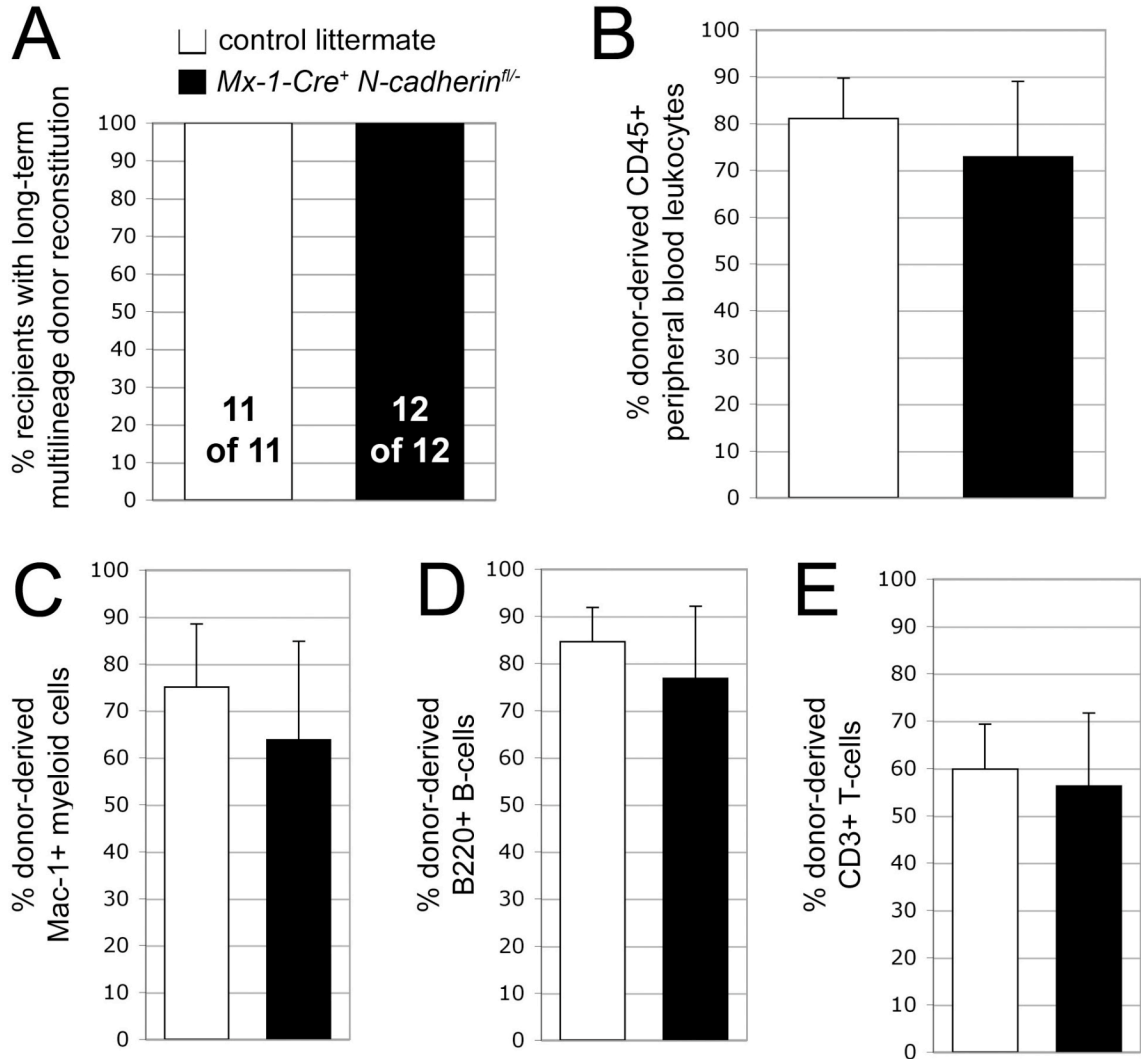
Figure 4.5: *N-cadherin* **deficiency does not affect the ability of HSCs to engraft or reconstitute irradiated recipients.** pIpC was administered to *Mx-1-Cre$^+$N-cadherin$^{fl/-}$* mice or littermate controls. Two months later $1x10^6$ bone marrow cells from CD45.2+ *Mx-1-Cre$^+$N-cadherin$^{fl/-}$* mice (black bars) or littermate controls (white bars) were injected into lethally irradiated CD45.1+ wild-type recipients along with $3x10^5$ CD45.1$^+$ bone marrow cells. All recipients became long-term multilineage reconstituted by donor cells (A, n=11-12 recipients per genotype). The levels of reconstitution 12 weeks after transplantation are shown. *N-cadherin* deficiency did not affect the levels of reconstitution by all donor cells (CD45+, B) or by donor cells in the myeloid (Mac-1+, A), B (B220+, B) or T (CD3+, C) lineages. Four different control littermates and four different *Mx-1-Cre$^+$N-cadherin$^{fl/-}$* donors were separately transplanted into a total of 11-12 recipients per genotype. Error bars represent SD.
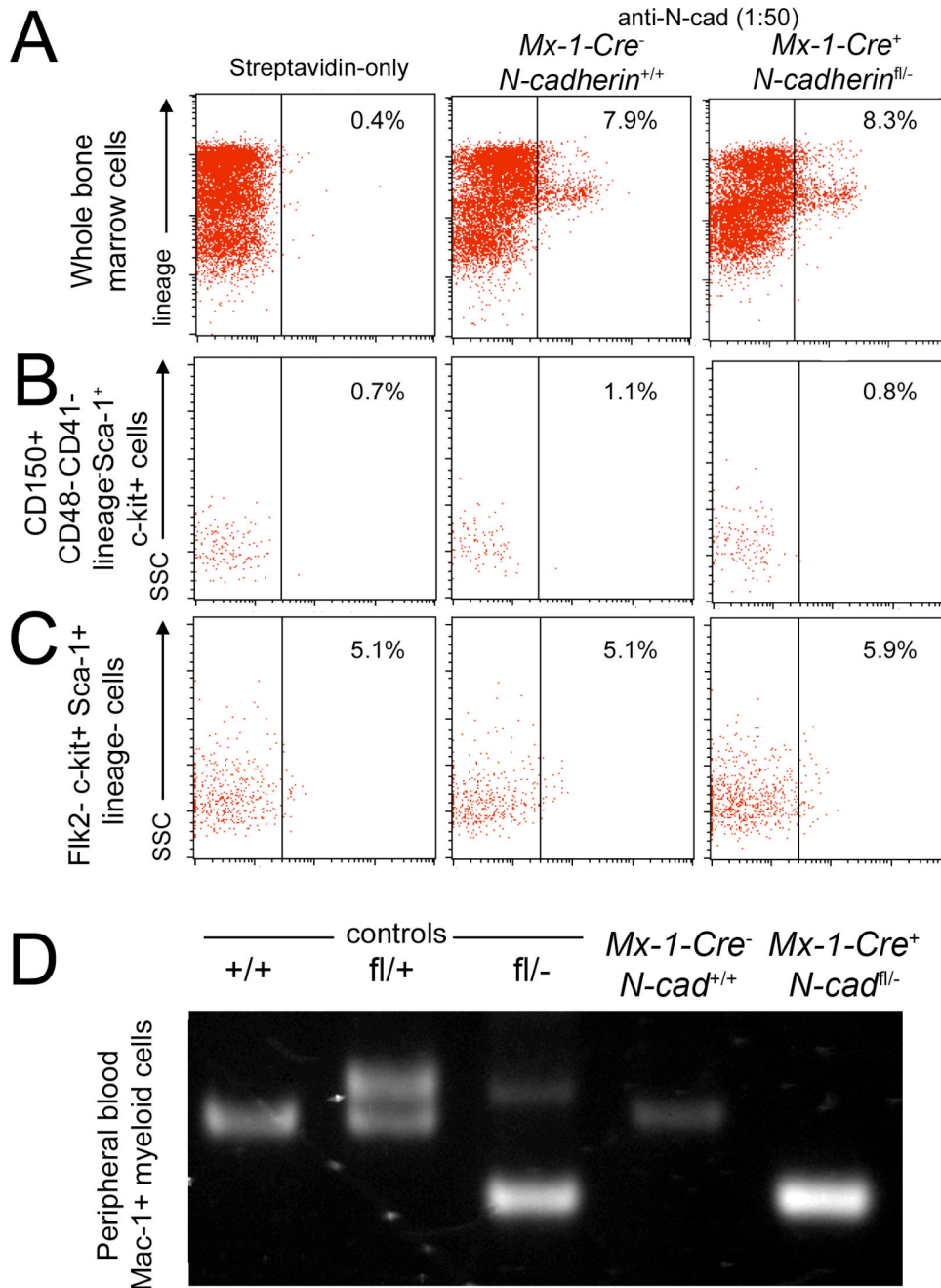
Figure 4.6: MNCD2 anti-N-cadherin antibody shows no staining of HSCs and the staining that is observed in whole bone marrow is unaffected by *N-cadherin* deletion. MNCD2 staining was compared to streptavidin-only negative control (first column) in whole bone marrow cells (A), CD150+CD48-CD41-Sca-1+c-kit+lineage-HSCs (B), and Flk2-lineage-Sca-1+c-kit+ HSCs (C). Staining above background was only observed in whole bone marrow (A). In all cases, staining was unaffected by *N-cadherin* deficiency (third column shows *Mx-1-Cre⁺N-cadherin^fl/-* mice after pIpC treatment). *N-cadherin* deficiency was confirmed by PCR in pIpC-treated mice (D).

BIBLIOGRAPHY

Adams, G.B., Chabner, K.T., Alley, I.R., Olson, D.P., Szczepiorkowski, Z.M., Poznansky, M.C., Kos, C.H., Pollak, M.R., Brown, E.M., and Scadden, D.T. (2006). Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. Nature *439*, 599-603.

Adams, G.B., and Scadden, D.T. (2006). The hematopoietic stem cell in its place. Nature Immunology *7*, 333-337.

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell *118*, 149-161.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R.*, et al.* (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. Nature *425*, 841-846.

Cheng, S.L., Shin, C.S., Towler, D.A., and Civitelli, R. (2000). A dominant negative cadherin inhibits osteoblast differentiation. J Bone Miner Res *15*, 2362-2370.

Haug, J.S., He, X.C., Grindley, J.C., Wunderlich, J.P., Gaudenz, K., Ross, J.T., Paulson, A., Wagner, K.P., Xie, Y., Zhu, R.*, et al.* (2008). N-cadherin expression level distinguishes reserved versus primed states of hematopoietic stem cells. Cell stem cell *2*, 367-379.

Hock, H., Meade, E., Medeiros, S., Schindler, J.W., Valk, P.J., Fujiwara, Y., and Orkin, S.H. (2004). Tel/Etv6 is an essential and selective regulator of adult hematopoietic stem cell survival. Genes Dev *18*, 2336-2341.

Hooper, A.T., Butler, J., Petit, I., and Rafii, S. (2007). Does N-cadherin regulate interaction of hematopoietic stem cells with their niches? Cell stem cell *1*, 127-129.

Ivanova, N.B., Dimos, J.T., Schaniel, C., Hackney, J.A., Moore, K.A., and Lemischka, I.R. (2002). A stem cell molecular signature. Science *298*, 601-604.

Kiel, M.J., He, S., Ashkenazi, R., Gentry, S.N., Teta, M., Kushner, J.A., Jackson, T.L., and Morrison, S.J. (2007a). Haematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU. Nature *449*, 238-242.

Kiel, M.J., and Morrison, S.J. (2008). Uncertainty in the niches that maintain haematopoietic stem cells. Nat Rev Immunol *8*, 290-301.

Kiel, M.J., Radice, G.L., and Morrison, S.J. (2007b). Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. Cell stem cell *1*, 204-217.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells. Cell *121*, 1109-1121.

Kiel, M.J., Yilmaz, O.H., and Morrison, S.J. (2008). CD150- cells are transiently reconstituting multipotent progenitors with little or no stem cell activity. Blood *111*, 4413-4414.

Kostetskii, I., Li, J., Xiong, Y., Zhou, R., Ferrari, V.A., Patel, V.V., Molkentin, J.D., and Radice, G.L. (2005). Induced deletion of the N-cadherin gene in the heart leads to dissolution of the intercalated disc structure. Circ Res *96*, 346-354.

Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. Science *269*, 1427-1429.

Maillard, I., Koch, U., Dumortier, A., Shestova, O., Xu, L., Sai, H., Pross, S.E., Aster, J.C., Bhandoola, A., Radtke, F.*, et al.* (2008). Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. Cell stem cell *2*, 356-366.

Mancini, S.J., Mantei, N., Dumortier, A., Suter, U., Macdonald, H.R., and Radtke, F. (2005). Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. Blood *105*, 2340-2342.

Matsunami, H., and Takeichi, M. (1995). Fetal brain subdivisions defined by R- and E-cadherin expressions: evidence for the role of cadherin activity in region-specific, cell-cell adhesion. Developmental biology *172*, 466-478.

Morrison, S.J., and Spradling, A.C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell *132*, 598-611.

Radice, G.L., Rayburn, H., Matsunami, H., Knudsen, K.A., Takeichi, M., and Hynes, R.O. (1997). Developmental defects in mouse embryos lacking N-cadherin. Developmental biology *181*, 64-78.

Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P.G., Riminucci, M.*, et al.* (2007). Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell *131*, 324-336.

Suda, T., Arai, F., and Hirao, A. (2005). Hematopoietic stem cells and their niche. Trends Immunol *26*, 426-433.

Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity *25*, 977-988.

Wilson, A., Murphy, M.J., Oskarsson, T., Kaloulis, K., Bettess, M.D., Oser, G.M., Pasche, A.C., Knabenhans, C., Macdonald, H.R., and Trumpp, A. (2004). c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev *18*, 2747-2763.

Wilson, A., and Trumpp, A. (2006). Bone-marrow haematopoietic-stem-cell niches. Nature reviews *6*, 93-106.

Yilmaz, O.H., Kiel, M.J., and Morrison, S.J. (2006a). SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. Blood *107*, 924-930.

Yilmaz, O.H., Valdez, R., Theisen, B.K., Guo, W., Ferguson, D.O., Wu, H., and Morrison, S.J. (2006b). Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature *441*, 475-482.

Yoshihara, H., Arai, F., Hosokawa, K., Hagiwara, T., Takubo, K., Nakamura, Y., Gomei, Y., Iwasaki, H., Matsuoka, S., Miyamoto, K.*, et al.* (2007). Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with in the osteoblastic niche. Cell stem cell *doi:10.1016/j.stem.2007.10.020.*

Zhang, J., and Li, L. (2008). Stem cell niche - Microenvironment and beyond. The Journal of biological chemistry.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q.*, et al.* (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.

CHAPTER 5

HEMATOPOIETIC STEM CELLS DO NOT ASYMMETRICALLY SEGREGATE CHROMOSOMES OR RETAIN BROMO-DEOXYURIDINE[1]

SUMMARY

Stem cells are proposed to asymmetrically segregate chromosomes during self-renewing divisions such that older ('immortal') DNA strands are retained in daughter stem cells while newly-synthesized strands segregate to differentiating cells (Cairns, 1975; Conboy et al., 2007; Karpowicz et al., 2005; Potten et al., 1978; Shinin et al., 2006; Smith, 2005). Stem cells are also proposed to retain DNA labels, like bromo-deoxyuridine (BrdU), either because they segregate chromosomes asymmetrically or because they divide slowly (Cotsarelis et al., 1990; Shinin et al., 2006; Tumbar et al., 2004; Zhang et al., 2003). However, the purity of stem cells among BrdU label-retaining cells has not been documented in any tissue and the 'immortal strand hypothesis' has not been tested in a system with definitive stem cell markers. We tested these hypotheses in hematopoietic stem cells (HSCs), which can be highly purified using well-characterized markers. We administered BrdU to newborn, cyclophosphamide/G-CSF mobilized, and normal adult mice for 4 to 10 days, followed by 70-days without BrdU. In each case, less than 6% of HSCs retained BrdU and less than 0.5% of all BrdU-retaining hematopoietic

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cells were HSCs, revealing poor specificity and poor sensitivity as an HSC marker. Sequential administration of chloro-deoxyuridine (CldU) and iodo-deoxyuridine (IdU) suggested that all HSCs segregate their chromosomes randomly. Division of individual HSCs in culture revealed no asymmetric segregation of label. HSCs therefore cannot be identified based on BrdU label-retention and do not retain older DNA strands during division, indicating these are not general properties of stem cells.

INTRODUCTION

The 'immortal strand hypothesis' was proposed as a mechanism by which stem cells could avoid accumulating mutations that arise during DNA replication (Cairns, 1975). While most cells segregate their chromosomes randomly (Armakolas and Klar, 2006; Potten et al., 1978), it was argued that adult stem cells in steady-state tissues might retain older strands during asymmetric self-renewing divisions, segregating newly-synthesized strands to daughters fated to differentiate (Figure 5.1a). Evidence has supported this model in some epithelial stem cells (Potten et al., 1978), neural stem cells (Karpowicz et al., 2005), mammary epithelial progenitors (Smith, 2005), and muscle satellite cells (Conboy et al., 2007; Shinin et al., 2006). A related idea is that adult stem cells in steady-state tissues might consistently retain DNA labels, either because chromosomes segregate randomly but stem cells divide more infrequently than other cells (Figure 5.1b) or because the older DNA strand is labeled and segregated asymmetrically (Figure 5.1c). Tritiated thymidine (Cotsarelis et al., 1990) or histone (Tumbar et al., 2004) label-retaining cells from the hair follicle are enriched for epithelial stem cells, though purity remains uncertain. Label-retaining cells have also been identified in the

hematopoietic system (Arai et al., 2004; Zhang et al., 2003), in mammary epithelium (Welm et al., 2002), in intestinal epithelium (Potten et al., 1978; Potten et al., 2002), and in the heart (Urbanek et al., 2006), but the purity of stem cells among these label-retaining cells has not been tested. As a result, it remains uncertain whether label-retention can identify stem cells with specificity or sensitivity.

Under steady-state conditions in adult bone marrow, all HSCs divide regularly but infrequently (Cheshier et al., 1999) to sustain hematopoiesis as well as to maintain nearly constant numbers of HSCs. As a result of this observation, as well as the observation that HSC divisions yield asymmetric outcomes in culture (Takano et al., 2004), it has been proposed that adult HSCs divide asymmetrically (Takano et al., 2004), though the rarity of HSCs in vivo and their relative quiescence has made it impossible to confirm this directly. Nonetheless, if BrdU label-retention and/or asymmetric chromosome segregation are general properties of adult stem cells, then either or both of these characteristics should be evident in HSCs, depending on experimental conditions.

RESULTS AND DISCUSSION

To test the rate at which HSCs go into cycle we administered BrdU to mice for 1, 4, or 10 days, then sorted HSCs onto microscope slides and stained with an anti-BrdU antibody. HSCs were sorted as CD150+CD48-CD41-lineage-Sca-1+c-kit+ cells (Figure 5.2a). This population contains all of the detectable HSC activity in bone marrow and 47% of single cells from this population give long-term multilineage reconstitution in irradiated mice (Kiel et al., 2005). After 1 to 10 days of BrdU, 51 to 94% of whole bone marrow cells and 6.5 to 46% of CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs

became BrdU$^+$ (Figure 5.2c,d). We calculated the rate at which HSCs went into cycle (Cheshier et al., 1999) as 6.0% per day (Figure 5.2e). Consistent with this, only 3.2% of CD150+CD48-CD41-lineage-Sca-1+c-kit+ cells were in S/G2/M phase of the cell cycle at any one time (Figure 5.3). These results are similar to a prior study that identified HSCs with somewhat different markers (Cheshier et al., 1999).

The linearity of BrdU incorporation over time (Figure 5.2e) suggests that most HSCs divide at a similar rate. If a minority of HSCs divided more rapidly, significantly more than 6.0% of HSCs should have incorporated BrdU after one day; however, we did not observe this (Figure 5.2d). If a minority of HSCs were more deeply quiescent than most other HSCs, these HSCs should remain BrdU negative even after long periods of BrdU. This also has not been observed as more than 99% of HSCs are labeled after six months of BrdU (Cheshier et al., 1999). Therefore, there is no evidence for more rapidly dividing or more slowly dividing subsets of long-term self-renewing HSCs under steady-state conditions, though we cannot exclude the possibility that a minority of HSCs divide somewhat more slowly than 6% per day.

To evaluate BrdU label-retention, we administered BrdU for 10 days, followed by a 70 day chase without BrdU, like prior studies in the hematopoietic system (Arai et al., 2004; Zhang et al., 2003). Given that 6.0% of HSCs entered cycle each day and 46% of HSCs were labeled after 10 days of BrdU (Figure 5.2d), we modeled the fraction of HSCs that would be expected to retain BrdU over time (Figure 5.4a; see Materials and Methods section for explanation).

If HSCs follow the immortal strand model they should lose their BrdU only one division after BrdU is discontinued as the labeled chromosomes are segregated to

differentiating daughter cells (Figure 5.1a), and only 0.6% of HSCs would be expected to retain BrdU after 70 days without BrdU (Figure 5.4a). If HSCs segregate chromosomes randomly, then BrdU would be lost stochastically over time and the fraction of BrdU$^+$ HSCs after a 70 day chase would depend upon the threshold of BrdU required for detection. If the threshold is equivalent to 0.5N labeled chromosomes (one quarter of the genome), this level of BrdU dilution could be achieved in cells that had divided 1 or 2 times after BrdU was discontinued (depending on whether they had divided once or twice in the presence of BrdU), and only 1.4% of HSCs would be expected to retain BrdU after the 70 day chase (Figure 5.3a). In contrast, if the threshold of detection is equivalent to 0.0625N labeled chromosomes, this could be achieved on average in cells that had divided 4 or 5 times after BrdU was discontinued, and 19.8% of HSCs would be expected to retain BrdU after the 70 day chase (Figure 5.4a). These calculations thus predict that few (<20%) HSCs should retain BrdU after a 70-day chase, irrespective of how chromosomes are segregated.

To test this we administered BrdU for 10 days then stained whole bone marrow cells and CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs after 40, 70, and 120 days of chase. After 70 days of chase, 0.4±0.1% of bone marrow cells and 4.6±0.9% of HSCs were BrdU+ (Figure 5.4c). This demonstrates, as predicted (Figure 5.4a), that few HSCs retain BrdU. Moreover, only 2.0±1.0% of HSCs were BrdU+ after 120 days of chase, demonstrating that the frequency of BrdU-retaining HSCs continues to decline over time rather than identifying a deeply quiescent subset of HSCs that retains BrdU indefinitely. Although BrdU label-retaining cells were 10-fold enriched for HSCs, the rarity of HSCs means that only 0.08% of BrdU$^+$ bone marrow cells were HSCs (0.0066% x 4.6%/0.4%).

BrdU label-retention is therefore a very insensitive and non-specific marker of HSCs as the vast majority of HSCs did not retain detectable BrdU, and only rare BrdU label-retaining cells were HSCs. Very similar results were obtained when we used flow-cytometry to detect BrdU incorporation (Figure 5.5) or when HSCs were isolated using different surface markers (c-kit+Flk-2-lineage-Sca-1+ cells; Figure 5.7). These data are most consistent with random chromosome segregation by HSCs and the failure to detect BrdU after approximately 3 divisions in the absence of BrdU (Figure 5.4a).

According to the immortal strand model, stem cells can incorporate BrdU into DNA strands that become the 'older' strands during symmetric cell divisions and these labeled DNA strands would be retained indefinitely by stem cells that resume asymmetric divisions (Figure 5.1c). To test this, we administered BrdU to newborn mice for 10 days or to cyclophosphamide/G-CSF treated mice for 4 days. The absolute number of HSCs expands dramatically in both newborn (Bowie et al., 2006) and cyclophosphamide/G-CSF mobilized mice (Morrison et al., 1997) (indicating symmetric divisions), before stabilizing to steady-state levels as mice enter adulthood or G-CSF is discontinued. After 10 days of BrdU in neonatal mice, 93±3.7% of bone marrow cells and 80±11% of CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs were BrdU+. Seventy days later, 0.1% of bone marrow cells and 6.4±2.5% of CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs were BrdU+ (Figure 5.4d). After 4 days of BrdU in mobilized mice, 94±3% of CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs were BrdU$^+$. Seventy days later, 0.1% of bone marrow cells and 2.1±1.1% of CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs were BrdU$^+$ (Figure 5.4d). Thus even when BrdU was administered to symmetrically dividing HSCs, only 2 to 6% of HSCs retained the label and only 0.2% to 0.4% of BrdU-

retaining bone marrow cells were HSCs. We were unable to identify any context in which BrdU label-retention identified HSCs with sensitivity or specificity, and none of these results were consistent with the immortal strand hypothesis.

To address the possibility that the HSCs in the above experiments might have continued to divide symmetrically after BrdU was discontinued we also administered BrdU to mice from 20 to 29 days postnatally. HSCs are thought to transition from rapidly dividing cells with a fetal phenotype to relatively quiescent cells with an adult phenotype between 21 and 28 days postnatally (Bowie et al., 2006). We obtained similar results with only 6.5±1.1% of HSCs retaining BrdU after a 70-day chase. There was therefore no period during neonatal development when BrdU could be administered in a way that resulted in retention within significant numbers of HSCs.

To test the immortal strand model directly, we treated mice with 10 days of CldU then 10 days of IdU. If HSCs segregate older and younger DNA strands asymmetrically, then HSCs should rarely incorporate both CldU and IdU under steady-state conditions because newly synthesized (labeled) DNA strands should be segregated to differentiating daughter cells after each division (Figure 5.6a). In contrast, if HSCs segregate older and younger DNA strands randomly then CldU-labeled HSCs should have the same chance of incorporating IdU as unlabeled cells and approximately 25% (50% x 50%) of HSCs should be double labeled (Figure 5.6b).

After 10 days of CldU followed by 10 to 11 days of IdU we observed that 14% of HSCs incorporated only CldU, 32% of HSCs incorporated only IdU, and 27% of HSCs incorporated both CldU and IdU (Figure 5.6c; Figure 5.8). The frequency of CldU+IdU+ cells (27%) was therefore similar to the product of the frequencies of total CldU+ cells

and total IdU+ cells (41% x 59%=24%) indicating that CldU+ cells had a similar probability of incorporating IdU as other cells. We repeated this experiment by administering CldU to mice for 60 days followed by 15 days of IdU, and found the frequency of CldU+IdU+ cells (63%) was again similar to the product of the frequencies of total CldU+ cells and total IdU+ cells (73% x 84%=61%). These results were not significantly affected by a slow clearance of CldU from mice, as CldU was cleared in less than 1 day after being discontinued from the drinking water (Figure 5.9). These observations directly contradict a key prediction made by the immortal strand hypothesis but are as would be expected by random chromosome segregation.

The foregoing experiments left the formal possibility that if HSCs divide by a combination of symmetric and asymmetric divisions in vivo we might underestimate the frequency of HSCs that retain older DNA strands. To address this we examined the division of individual HSCs in culture that were isolated from mice treated for 10 days with BrdU. Single CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs were sorted into cultures under conditions in which half of HSC divisions give asymmetric outcomes (daughter cells with different developmental potentials) (Takano et al., 2004). After 2 to 3 days of culture we observed a total of 346 colonies in which HSCs had divided once (2 daughter cells) or twice (3 or 4 daughter cells). Either all daughter cells were BrdU+ (162 colonies; 46%) or all daughter cells were BrdU- (184 colonies) (Figure 5.4f) as would be expected by random chromosome segregation given that 46% of HSCs incorporate BrdU in vivo after 10 days (Figure 5.2d). We observed no colonies after 1 or 2 rounds of division that contained a mixture of BrdU+ and BrdU- cells. These in vitro experiments

on individual HSCs thus failed to detect any asymmetric segregation of labeled chromosomes.

Our results were not confounded by effects of BrdU/CldU/IdU on HSC proliferation, survival, or DNA repair. The cell cycle status (Figure 5.3) and frequency (data not shown) of HSCs was not affected by these treatments. BrdU/CldU/IdU incorporation from DNA repair was not detectable (Figure 5.3).

Our results indicate that BrdU label-retention is neither a sensitive nor a specific marker of HSCs. Nonetheless BrdU label-retention could be a better marker of stem cells in other tissues. Moreover, histone-GFP (Tumbar et al., 2004) may do a better job of marking stem cells, including HSCs, because it can be selectively expressed in subsets of progenitors and may be retained with different kinetics than BrdU. Our data demonstrate the need to test the sensitivity and specificity of BrdU and other label-retention markers before assuming they mark stem cells with fidelity.

Our data also demonstrate that the immortal strand model (Cairns, 1975) does not apply to HSCs and cannot be considered a general model of stem cell division. Our data do not address whether stem cells from other tissues asymmetrically segregate chromosomes or whether HSCs segregate a limited number of chromosomes asymmetrically (Armakolas and Klar, 2006). Nonetheless, asymmetric chromosome segregation cannot be a mechanism by which HSCs avoid accumulating mutations over time.

MATERIALS AND METHODS

BrdU/CldU/IdU administration

All experiments employed C57BL/Ka-CD45.2:Thy-1.1 mice. For experiments in adult mice, BrdU (Sigma, St. Louis, MO) was administered when the mice were 8 to 10 weeks of age. Mice were given an intraperitoneal injection of 100 mg of BrdU/kg of body mass in Dulbecco's phosphate buffered saline (DPBS; Gibco, Carlsbad, CA) and maintained on 1 mg/ml of BrdU in the drinking water for 1 to 10 days. Amber bottles containing BrdU water were changed every 1 to 3 days. For retention studies, BrdU water was replaced with regular water and the mice were maintained for 40 to 120 days before analysis.

BrdU injections into neonatal mice were performed as described (Taylor et al., 2000). Beginning within 3 days after birth, neonatal mice were injected subcutaneously with 50 mg BrdU/kg body weight twice daily for 10 days. Mice were weighed every two days and the dose of BrdU was adjusted.

To assess BrdU retention following cytokine mobilization, adult mice were injected with cyclophosphamide (200 mg/kg; Bristol-Myers Squibb, New York) and then on each of 4 subsequent days with 250 μg/kg per day of human G-CSF (Amgen, Thousand Oaks, CA) (Morrison et al., 1997). On the fourth day of G-CSF injection, a single intraperitoneal injection of 100 mg BrdU/kg of body mass was given and the mice were put on 1 mg/ml BrdU water for 4 additional days. The mice were then returned to regular water for 70 days before analysis.

For 5-chloro-2-deoxyuridine (CldU) and 5-iodo-2-deoxyuridine (IdU) experiments, mice were given an intraperitoneal injection of 100 mg of CldU/kg of body mass in DPBS and maintained on 1 mg/ml of CldU (Sigma) in the drinking water for 10

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days. Mice were then given an intraperitoneal injection of 100 mg of IdU/kg of body mass in DPBS, and switched to 1 mg/ml of IdU (Sigma) in the drinking water for 10 to 11 days before being sacrificed.

BrdU segregation in cultured HSCs

Single CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs were sorted from BrdU-treated mice into a V-bottom 96-well plate containing Stempro-34 medium (Invitrogen) supplemented with 2 mM L-glutamine, $50\,\mu$M 2-mercaptoethanol (Sigma), murine IL-3 (10 ng/ml), murine SCF (100 ng/ml) and murine Tpo (100 ng/ml; all cytokines from R&D Systems) with 10% charcoal absorbed fetal bovine serum (Cocalico Biologicals, Inc., Reamstown, PA) and cultured for 2 to 3 days in low oxygen chambers (Morrison et al., 2000). For analysis, plates were centrifuged at 500xg for 10 minutes, then cells from each colony were pipetted onto individual wells of Teflon printed glass slides and allowed to dry overnight prior to staining for BrdU and DAPI.

BrdU staining

Slides were thawed at room temperature for 15 minutes, fixed in 70% ethanol at – 20°C for 30 minutes, rinsed in 0.1M phosphate buffer (PB) twice, incubated in 2N HCl with 0.8% Triton in PB for 30 minutes, incubated in 0.1M Sodium Borate (pH 8.5) for 15 minutes, then rinsed in 0.1M PB at room temperature, at 37ºC, and at room temperature again. The slides were then incubated with 0.3% Triton in 0.1M PB supplemented with 5% Goat Serum for 1 hour at 4°C. Slides were incubated at 4ºC overnight with the primary anti-BrdU antibody that specifically recognizes BrdU and CldU but does not

recognize IdU (clone BU1/75, Accurate Chemical and Scientific Corp.). Slides were

then rinsed in 0.1M PB twice and incubated with Alexa488-conjugated goat anti-rat IgG

(Invitrogen-Molecular Probes, Carlsbad, California) for 2 hours at room temperature.

Slides were rinsed in 0.1M PB twice and incubated with DAPI for 1 hour at room

temperature. Finally, slides were rinsed three times in 0.1M PB, the excess buffer was

shaken off and the slides were mounted in 70% glycerol in PBS. Images were gathered

using an Olympus BX-51 florescence microscope equipped with a Cooke Pixelfly CCD

camera. In some experiments, BrdU incorporation was measured by flow-cytometry

using an antibody directly conjugated to allophycocyanin (APC; APC BrdU flow kit, BD

Pharmingen). Sorted samples were fixed and permeablized according to manufacturer's

instructions, incubated in 2N HCl for 30 minutes at room temperature, neutralized in

Sodium Borate (pH 8.5), washed in 0.5% Triton in DPBS, stained with anti-BrdU APC

and resuspended in DAPI (10 µg/ml) prior to FACS analysis. All flow-cytometry was

performed on a FACSVantage SE-dual laser, three-line flow-cytometer (Becton-

Dickinson).

Analysis of cell cycle distribution and cell death in HSCs

Cell cycle distribution was analyzed by Hoechst 33342 (Invitrogen-Molecular

Probes, Carlsbad, California) staining. CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs

were sorted and resorted into ice-cold 70% ethanol and stored at -20°C overnight. Cells

were resuspended in PBS containing 0.02 mg/ml Hoechst 33342, incubated for 30

minutes and analyzed by flow-cytometry using a UV laser.

For activated caspase-3 staining, frozen slides bearing sorted CD150+CD48-

CD41-lineage-Sca-1+c-kit+ HSCs or sections through E11 mouse forebrain were thawed

at room temperature for 10 minutes, fixed at room temperature in 10% buffered neutral

formalin (VWR, West Chester, Pennsylvania) for 10 minutes, rinsed in 0.1M PB twice,

and blocked with 0.3% Triton in 0.1M PB supplemented with 5% Goat Serum for 1 hour

at 4°C. Slides were then incubated with anti-activated caspase-3 antibody (BD

Pharmingen, San Diego, California) at room temperature for 2 hours. Slides were then

rinsed in 0.1M PB twice and incubated with Alexa488-conjugated goat anti-rabbit IgG

(Invitrogen-Molecular Probes, Carlsbad, California) for 1 hour at room temperature.

Slides were rinsed in 0.1M PB twice and incubated with DAPI for 30 minutes at room

temperature. Finally, slides were rinsed three times in 0.1M PB, the excess buffer was

shaken off and the slides were mounted in 70% glycerol in PBS. To test for the

incorporation of BrdU due to DNA repair, BrdU was administered to adult mice for 12

hours followed by irradiation with 100 rad from a Gammacell40 Extractor (MDS

Nordion, Ottawa, ON, Canada) followed by 48 hours of further BrdU administration.

CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs were then sorted onto slides and

stained for BrdU as described above.

Mathematical models of BrdU uptake and retention

To model the uptake and retention of BrdU in a population of stem cells we

assumed that for days 0 through T the stem cells are exposed to adequate BrdU so that

cells incorporate BrdU when they divide. Based on our data a random 6.0% of HSCs

enter cycle each day. At day T, the BrdU supply is removed and the level of BrdU

incorporated into the chromosomes decreases for every cell division after day T. The rate

at which BrdU is diluted from cells during this chase period depends upon how the cells

segregate their chromosomes. If chromosomes segregate randomly, then irrespective of

whether stem cells divide asymmetrically or symmetrically with respect to daughter cell

fate, BrdU labeled chromosomes will stochastically become diluted over time: on average

the BrdU label will be diluted by half during each round of division and multiple

divisions will be required to dilute the BrdU label to the point that it is no longer

detectable by immunohistochemistry. This is modeled as case 1 below for asymmetrically

dividing cells. In contrast, according to the immortal strand model (Cairns, 1975), stem

cells divide asymmetrically under steady-state conditions and BrdU is preferentially

incorporated into newly synthesized DNA strands that are asymmetrically segregated into

differentiating daughter cells with each round of division. Under these assumptions,

modeled as case 2 below, stem cells retain only the unlabeled older DNA strands once

BrdU is withdrawn.

Case 1: Random segregation of chromosomes (see Figure 5.1b)

BrdU Incorporation: When chromosomes are allowed to segregate randomly, BrdU could

be incorporated into one or two DNA strands within each chromosome, depending on the

number of times a stem cell divides during the period of BrdU incorporation and the way

in which the chromosomes segregate. To model the rate of BrdU incorporation:

y_0 = fraction of cells without BrdU

y_1 = fraction of cells with one strand BrdU+ after only one division

y_2 = fraction of cells with both strands BrdU+ after two or more divisions

α = the proliferation rate of stem cells (6.0% of HSCs enter cycle per day)

Note as well that cell death was not incorporated into this model because we did not observe significant cell death or changes in HSC frequency during the experiments. The equations for uptake:

$$\frac{dy_0}{dt} = -\alpha y_0$$

Equation 1: Cells leave the y_0 population when they divide.

$$\frac{dy_1}{dt} = -\alpha y_1 + \alpha y_0$$

Equation 2: Cells from the y_0 population are added into the y_1 population through the incorporation of BrdU into one of the DNA strands. Cells leave the y_1 population when they divide.

$$\frac{dy_2}{dt} = \alpha y_1$$

Equation 3: Cells from the y_1 population are added into the y_2 population through further incorporation of BrdU through cell division.

To determine the frequency of BrdU+ stem cells at any time after the addition of BrdU we solve the system of ordinary differential equations with all HSCs initially being unlabeled prior to BrdU administration. Note that similar equations have previously been used to model BrdU incorporation and depletion from other cells (Bonhoeffer et al., 2000).

The process by which cells lose the BrdU label: At day T (when BrdU is removed), we determine the total number of BrdU+ stem cells by adding the y_1 and y_2 populations. Cells in the y_1 population have a BrdU level of 1, whereas y_2 cells have a BrdU level of up to 2. To model the rate at which these cells lose BrdU during subsequent divisions after removing BrdU:

y_{10} = fraction of cells, initially with one labeled DNA strand that undergo 0 divisions after day T

y_{11} = fraction of cells, initially with one labeled DNA strand that undergo 1 division after day T

...

y_{1N} = fraction of cells, initially with one labeled DNA strand that undergo N divisions after day T

y_{20} = fraction of cells, initially with two labeled DNA strands that undergo 0 divisions after day T

y_{21} = fraction of cells, initially with two labeled DNA strands that undergo 1 division after day T

…

y_{2N} = fraction of cells, initially with two labeled DNA strands that undergo N divisions after day T

Cells move from $y_{1(k-1)}$ to y_{1k} at the proliferation rate α (cells move in y_{2k} similarly). The equations for dilution of the BrdU label are:

$$\frac{dy_{10}}{dt} = -\alpha y_{10}$$

$$\frac{dy_{11}}{dt} = -\alpha y_{11} + \alpha y_{10}$$

...

$$\frac{dy_{1N}}{dt} = \alpha y_{1(N-1)}$$

$$\frac{dy_{20}}{dt} = -\alpha y_{20}$$

$$\frac{dy_{21}}{dt} = -\alpha y_{21} + \alpha y_{20}$$

...

$$\frac{dy_{2N}}{dt} = \alpha y_{2(N-1)}$$

With each cell division, the cell's BrdU level is decreased by half. For instance, cells in y_{10} have BrdU level 1, cells in y_{11} have half as much BrdU on average, cells in y_{12} have one quarter as much BrdU on average, etc. Similarly, cells in y_{20} have BrdU level 2, cells in y_{21} have BrdU level 1, cells in y_{22} have BrdU level 0.5, etc. We can determine the total fraction of cells that have a BrdU level that is above a minimum detection level (which can be set at any desired level in the simulations) by adding all relevant populations at any time after day T.

This set of ordinary differential equations is solved for initial conditions that are determined by the observed results of the BrdU incorporation at time T. In this way, it is possible to plot the frequency of BrdU+ HSCs over time, depending on whether it takes 1, 2, 3, or 4 rounds of division to dilute BrdU to the point at which it is no longer detectable (Figure 5.4a). Note that 1 round of division corresponds to a 2-fold dilution of BrdU while 4 rounds of division correspond to 16-fold dilution. Our empirical data

suggest that approximately 3 rounds of division are required to dilute BrdU to the point that it is no longer detectable in HSCs.

Case 2: Asymmetric chromosome segregation (immortal strand model; Figure 5.1a)

BrdU Incorporation: Under this model, only one strand of DNA within each chromosome in stem cells can contain BrdU irrespective of how long BrdU is administered (though the proportion of labeled stem cells will increase over time).

The equations for Case 2 are:

y_0 = fraction of cells without BrdU

y_1 = fraction of cells with one strand BrdU+ (after one or more divisions)

α = proliferation rate of stem cells

The equations for uptake:

$$\frac{dy_0}{dt} = -\alpha y_0$$

Equation 4: Cells leave the y_0 population when they divide.

$$\frac{dy_1}{dt} = \alpha y_0$$

Equation 5: Cells from the y_0 population are added into the y_1 population through the incorporation of BrdU into one of the DNA strands.

As before, in order to determine the proportion of cells within each population at the end of day T we solve the system of ordinary differential equations with initial conditions corresponding to the case where initially all HCSs are unlabeled prior to BrdU administration. Cells in population y_0 do not contain any BrdU, while those in y_1 have a BrdU level of 1.

The process by which cells lose BrdU label: We take all cells containing BrdU at day T (all cells in population y_1 and monitor BrdU loss through cell division in the absence of

BrdU). In this case, the asymmetric segregation of chromosomes means that all dividing stem cells will lose all of the BrdU label in a single division in the absence of BrdU. Therefore, the fraction of stem cells that remain BrdU+ at time T, simplifies to the fraction of BrdU$^+$ HSCs that do not divide after removing BrdU:

y_{10} = fraction of cells, initially with one labeled strand that undergo 0 divisions after day T

Cells move from y_{10} (and lose their BrdU label) at the proliferation rate α. The equations for loss of BrdU label:

$$\frac{dy_{10}}{dt} = -\alpha y_{10}$$

This differential equation with initial condition $y_{10}(0) = y_1(T)$ determines the fraction of HSCs that retain BrdU over time, according to the immortal strand hypothesis.

ACKNOWLEDGEMENTS
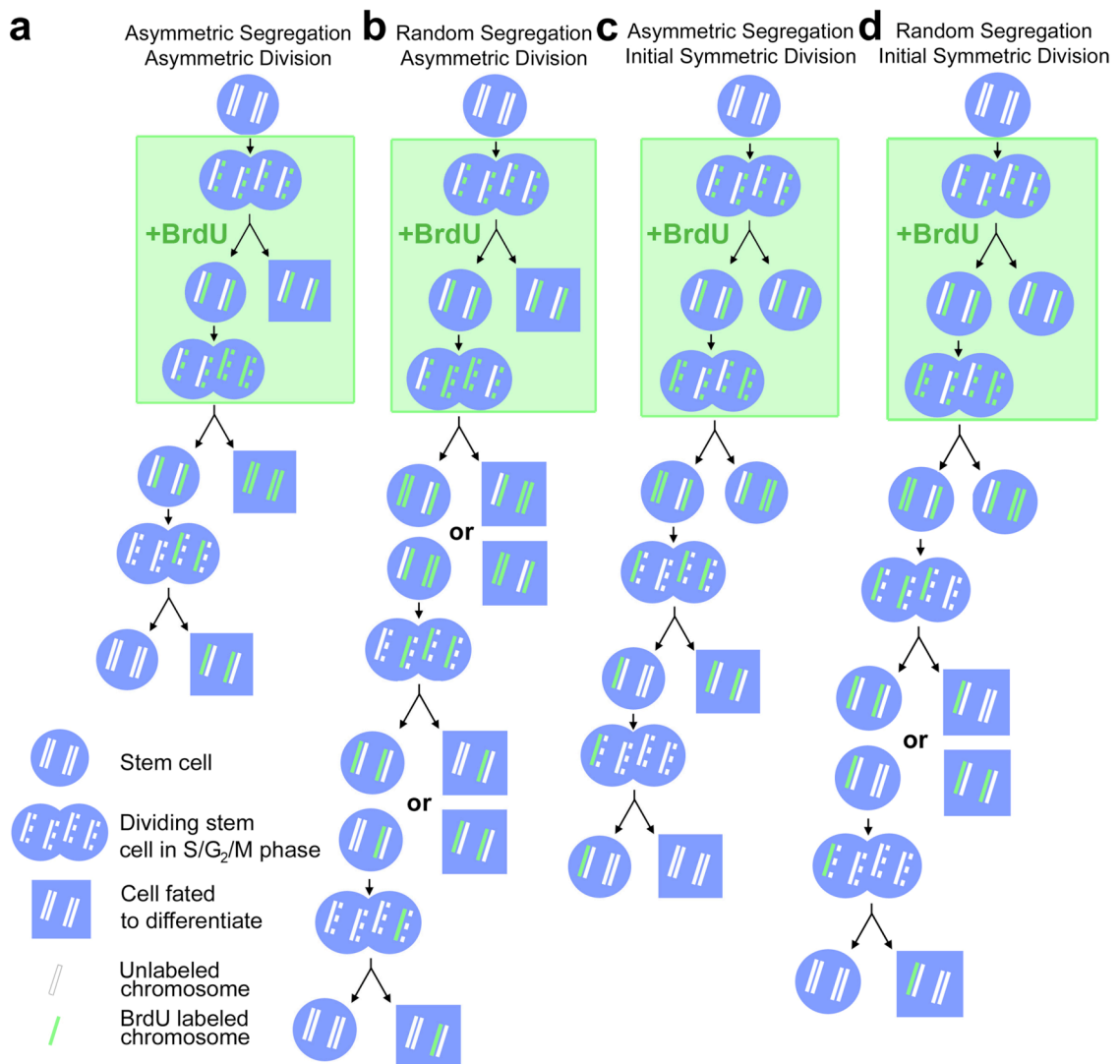
Figure 5.1: Contrasting predictions regarding stem cell labeling based on the immortal strand model versus random chromosome segregation. a) According to the immortal strand model (Cairns, 1975), stem cells divide asymmetrically under steady-state conditions and BrdU is incorporated into newly-synthesized DNA strands that are asymmetrically segregated into differentiating daughter cells with each round of division, such that stem cells retain only the unlabeled older DNA strands. b) In contrast, if chromosomes segregate randomly, then BrdU labeled chromosomes will be stochastically diluted over multiple rounds of divisions. c) By the immortal strand model, if stem cells divide symmetrically then BrdU can be incorporated into DNA strands that become the 'older' strands once stem cells resume asymmetric division. Under these circumstances the BrdU+ older strands would be retained indefinitely in stem cells. d) In contrast, if chromosome segregation is random then BrdU+ chromosomes are stochastically diluted over time after BrdU is discontinued.

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a bone marrow cells — CD48 CD41 lineage vs CD150; 0.007±0.001%

CD150+CD48-CD41-lineage- cells — c-kit vs Sca-1; 94±4.7%

b no BrdU / D10 BrdU — DAPI, BrdU

c % BrdU+ bone marrow cells

	Day 0	Day 1	Day 4	Day 10
1	0.0	49.6	78.3	92.0
2	0.0	49.3	91.0	96.5
3	0.0	53.6		93.3
Mean±SD	0.0±0.0	51±2.4	85±9.0	94±2.3

d % BrdU+ HSCs

	Day 0	Day 1	Day 4	Day 10
1	0.0	4.5	25.0	44.6
2	0.0	5.6	23.6	43.2
3	0.0	5.0		50.0
4	0.0	10.9		
Mean±SD	0.0±0.0	6.5±3.0	24±1.0	46±3.6

e 6.0% of HSCs enter cycle/day
r^2=0.9819

-log (% BrdU negative HSCs) vs Days of BrdU administration

Figure 5.2: 6.0% of HSCs stochastically enter cell cycle each day. a) HSCs can be isolated by flow-cytometry as CD150+CD48-CD41-lineage-Sca-1+c-kit+ cells that represent only 0.0066±0.0003% (0.007% x 94%) of bone marrow cells but which contain all detectable HSC activity and which are very highly enriched for HSCs (nearly 50% of single cells give long-term multilineage reconstitution in irradiated mice(Kiel et al., 2005)). b) BrdU incorporation into HSCs evaluated by immunofluorescence after sorting HSCs onto microscope slides (DAPI is a nuclear stain). The percentage of BrdU+ bone marrow cells (c) and CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs (d) after various periods of BrdU administration (3 independent experiments with 3 to 4 mice per experiment and 200 to 400 bone marrow cells or 100 to 400 HSCs counted per mouse). e) The percentage of HSCs that enter cycle each day (6.0%) can be derived by plotting the negative logarithm of the percentage of HSCs that were BrdU⁻ over time (Cheshier et al., 1999).

a

no CldU or IdU

4 days CldU+4 days IdU

DNA content- Hoescht H33342

S/G2/M
3.2±1.1%

S/G2/M
3.3±0.7%

b

no CldU or IdU   10 days CldU+10 days IdU   E11 forebrain

DAPI

activated caspase-3

0±0%   0±0%   ~1%

c

no BrdU   60hr. BrdU   100rad+60hr. BrdU

DAPI

BrdU

0±0%   17.4±1.6%   18.6±4.5%
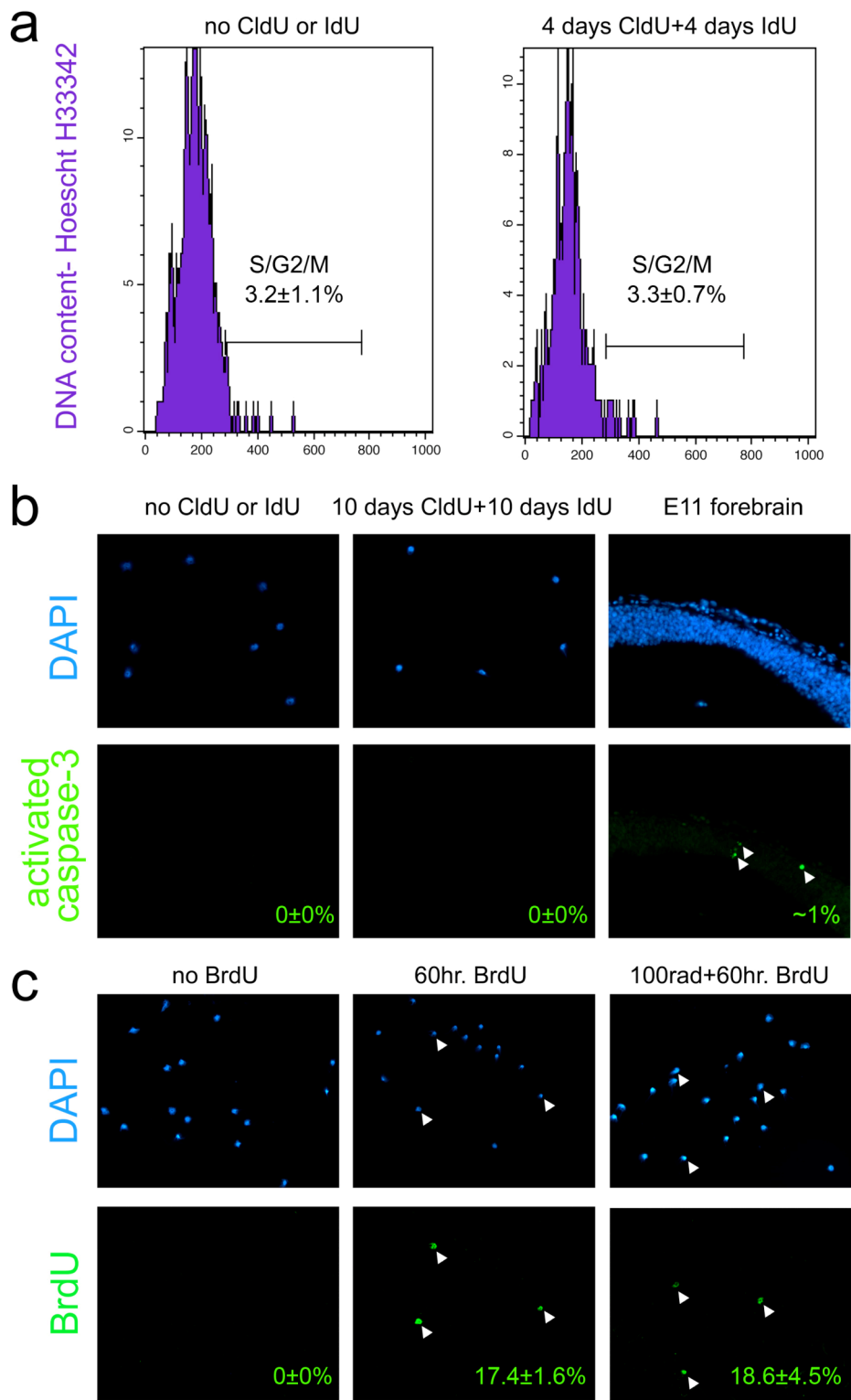
Figure 5.3

Figure 5.3: Administration of CldU and IdU does not affect HSC proliferation or cell death and BrdU incorporation during DNA repair is negligible. a) Cell cycle distribution (based on DNA content) of CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs isolated from a mouse that did not receive CldU or IdU (left) or from a mouse that received 4 days of CldU followed by 4 days of IdU (right) (two independent experiments). In separate experiments, the frequency of HSCs was not affected by these treatments as CD150+CD48-CD41-lineage-Sca-1+c-kit+ cells represented 0.0070±0.0011% of bone marrow cells in mice treated with BrdU or CldU or IdU for 10 days and 0.0066±0.0014% of bone marrow cells in controls. b) Activated caspase-3 staining of CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs sorted onto a microscope slide following 10 days of CldU administration and 10 days of IdU administration. Arrowheads identify activated caspase-3+ cells in a section through embryonic day 11 mouse forebrain (positive control), but no activated caspase-3+ HSCs were detected in either CldU/IdU-treated or control mice (200 to 300 HSCs examined per treatment; two independent experiments). c) BrdU was administered for 12 hours followed by gamma irradiation with 100 rad and then 48 hours of further BrdU administration. No BrdU+ HSCs were detected in negative control mice (no BrdU). Among mice treated with BrdU for 60 hours, 17.4±1.6% of HSCs from non-irradiated mice were BrdU+ and 18.6±4.5% of HSCs from irradiated mice were BrdU+ (250 to 600 HSCs analyzed per treatment; 3 independent experiments). Since DNA damage would be expected in all cells that receive this dose of irradiation(Taniguchi et al., 1993), this demonstrates that the amount of BrdU that is incorporated as a result of DNA damage repair is negligible and cannot be detected by immunofluorescence in these assays. Our failure to detect BrdU incorporation from DNA repair is consistent with prior studies that found that nucleotide incorporation as a result of DNA repair is negligible relative to DNA replication, even over many years (Spalding et al., 2005).

a +BrdU / no BrdU

BrdU Incorporation
Asymmetric segregation- immortal strand
Random segregation
BrdU lost at 0.5N labeled DNA, 1-2 div.
BrdU lost at 0.25N labeled DNA, 2-3 div.
BrdU lost at 0.125N labeled DNA, 3-4 div.
BrdU lost at 0.0625N labeled DNA, 4-5 div.

19.8%
11.4%
4.9%

b D70 of chase without BrdU

DAPI / BrdU

c % BrdU+ cells from adult mice

bone marrow cells	chase D40	chase D70	chase D120
1	0.3	0.4	0.3
2	0.6	0.3	0.4
3		0.5	0.1
Mean±SD	0.4±0.2	0.4±0.1	0.3±0.1

HSCs	chase D40	chase D70	chase D120
1	7.4	4.0	1.5
2	14.5	5.7	1.4
3	8.3	3.4	3.1
4	6.9	5.0	
5		4.8	
Mean±SD	9.3±3.5	4.6±0.9	2.0±1.0

d % BrdU+ cells from neonatal or mobilized mice

bone marrow cells	chase D70 neonate	chase D120 neonate	chase D70 mobilized
1	0.0	0.1	0.0
2	0.2	0.1	0.0
3	0.2	0.0	0.2
Mean±SD	0.1±0.1	0.1±0.1	0.1±0.1

HSCs	chase D70 neonate	chase D120 neonate	chase D70 mobilized
1	6.1	2.5	2.8
2	5.6	0.8	2.6
3	4.3	2.1	0.9
4	10.8		
5	5.3		
Mean±SD	6.4±2.5	1.8±0.9	2.1±1.1

Figure 5.4: Few HSCs retain BrdU and the vast majority of BrdU-retaining bone marrow cells are not HSCs. a) Model predicting the fraction of HSCs that retain BrdU over time after administering BrdU 10 days, depending on whether chromosomes segregate asymmetrically or randomly and on the threshold of BrdU that can be detected by immunofluorescence (0.5N, 0.25N, 0.125N, or 0.0625N labeled DNA). b) CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs sorted onto a microscope slide following 10 days BrdU administration and 70 days chase (without BrdU). Arrowheads identify BrdU⁺ cells. c) The frequency of BrdU⁺ bone marrow cells and HSCs after 10 days BrdU administration and 40, 70, or 120 days chase. d) The frequency of BrdU⁺ bone marrow cells and HSCs after 10 days BrdU administration to neonatal mice followed by 70 or 120 days chase; or after 4 days BrdU administration to cyclophosphamide/G-CSF treated mice followed by 70 days chase. All data are based on 3 to 5 independent experiments with 3 mice per experiment and 400 to 700 bone marrow cells or 300 to 400 HSCs counted per mouse.
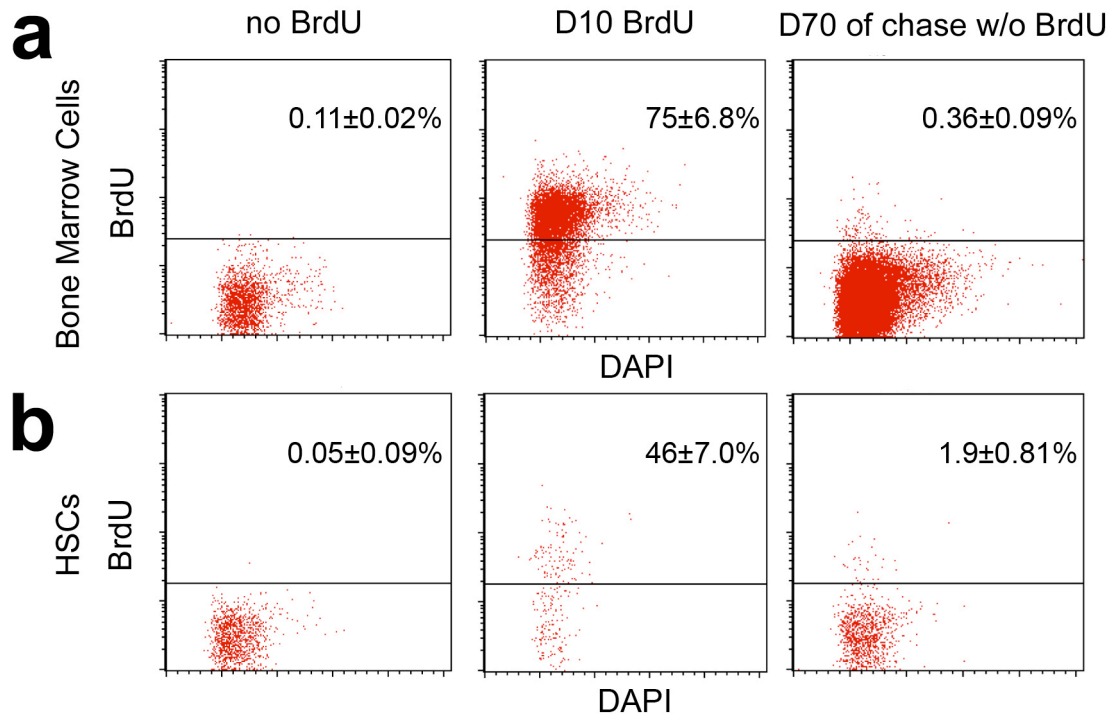
Figure 5.5: Few HSCs retain BrdU, and the vast majority of BrdU label-retaining bone marrow cells are not HSCs even when BrdU incorporation is measured by flow-cytometry. In case flow-cytometric detection of BrdU was more sensitive than immunofluorescence microscopy, we repeated all of the experiments using this approach. The percentage of BrdU+ whole bone marrow cells (a) or CD150+CD48-CD41-lineage-HSCs (b) in negative control mice (no BrdU), or after 10 days of BrdU administration, or after an additional 70 days of chase (without BrdU). Note that fewer HSC markers were used to free up a fluorescence channel to detect BrdU by flow-cytometry; however, this should not introduce significant impurities as 45% of CD150+CD48-CD41- cells give long-term multilineage reconstitution in irradiated mice (Kiel et al., 2005). Only 1.9% of HSCs detectably retained BrdU after 70 days chase and less than 0.04% of BrdU+ bone marrow cells were HSCs (0.0066% x 1.9%/0.36%). The data are based on 3 independent experiments in which 6,000 to 60,000 bone marrow cells or 400 to 1200 HSCs were analyzed per experiment.
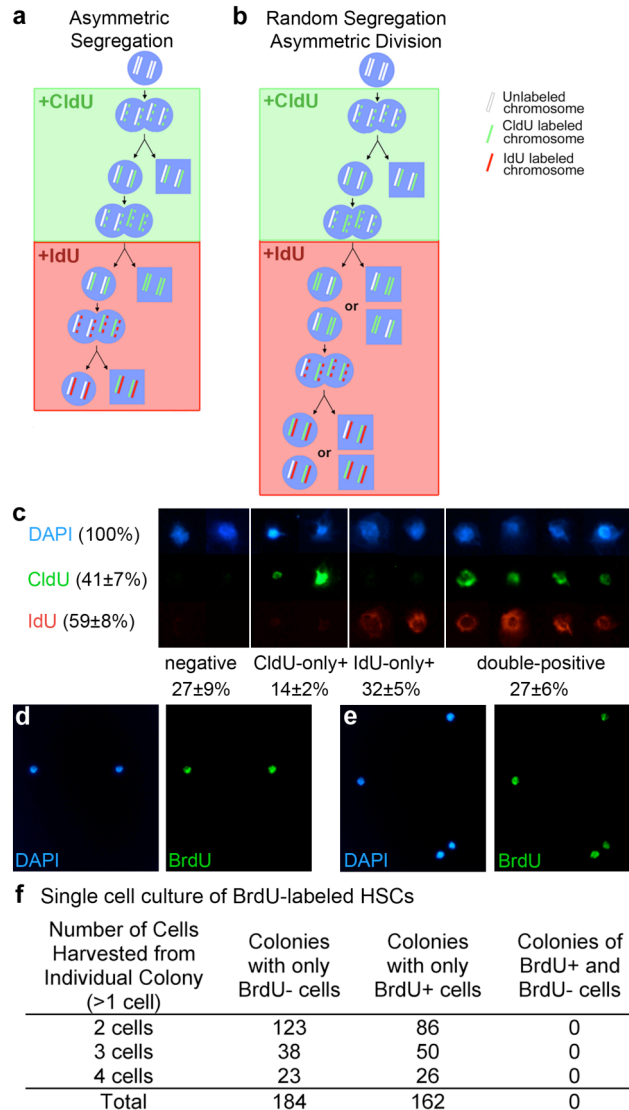
Figure 5.6: HSCs segregate chromosomes randomly in vivo and in vitro. a) By the immortal strand model, stem cells sequentially exposed to CldU (10 days) then IdU (10 days) would not incorporate both labels, with the exception of rare cells in S/G2/M phase of their first division after switching from CldU into IdU (expected frequency <<3%). b) In contrast, if chromosome segregation is random then CldU+ stem cells would have the same probability of incorporating IdU as unlabeled cells (expected frequency of CldU+IdU+ HSCs approximately 25%). c) CldU was administered to mice for 10 days followed by IdU for 10 to 11 days and CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs were stained. d) Examples of HSCs that incorporated neither label, only CldU, only IdU, or both labels (data are based on two independent experiments with 2 or 3 mice per experiment and 100 to 250 HSCs per mouse). HSCs from BrdU-treated mice divided once (d) or twice (e) in culture to form daughter cells. f) The progeny of these HSCs were either all BrdU+ or all BrdU-. We detected no clones in which label was asymmetrically segregated to a subset of daughter cells.

a

bone marrow cells

lineage | CD135 | 2.0±1.1%

lineage-CD135- cells

c-kit | Sca-1 | 1.0±0.6%

b

	no BrdU	D10 BrdU	D70 of chase w/o BrdU
DAPI			
BrdU			

c   % BrdU+ HSCs

	D0 of BrdU adult	D10 of BrdU adult	D70 of chase adult	D70 of chase neonate	D70 of chase mobilized
1	0.3	53.3	3.9	8.2	4.4
2	0.0	31.5	4.6	4.6	2.4
3	0.0	46.4			
Mean±SD	0.1±0.2	44±11	4.3±0.5	6.3±2.7	3.4±1.4

Figure 5.7: HSCs identified as c-kit+Flk-2-lineage-Sca-1+ cells also cannot be reliably identified based on BrdU label-retention.   a) In case the isolation of HSCs using different markers would identify a population that is more consistently marked by BrdU label-retention, we isolated c-kit+Flk-2-lineage-Sca-1+ cells, which represent around 0.02% of bone marrow cells (2.0% x 1.0%; a), and which are highly enriched for HSCs (Christensen and Weissman, 2001). b) c-kit+Flk-2-lineage-Sca-1+ cells were isolated from mice that had been administered BrdU for 10 days followed by a 70 day chase without BrdU. Cells were sorted onto microscope slides and stained with DAPI (to identify nuclei) and BrdU. c) The frequency of BrdU+ c-kit+Flk-2-lineage-Sca-1+ cells after 10 days of BrdU administration or after an additional 70 days of chase in normal young adult mice or neonatal mice was very similar to what we observed when HSCs were isolated as CD150+CD48-CD41-lineage-Sca-1+c-kit+ cells (Figure 5.4c, d). The frequency of BrdU+ c-kit+Flk-2-lineage-Sca-1+ cells after 4 days of BrdU administration followed by 70 days of chase in cyclophosphamide/G-CSF treated mice was also similar to what we observed in CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs (Figure 5.4d). These results were based on two independent experiments in which 6 mice and 200 to 300 cells per mouse were analyzed per experiment.

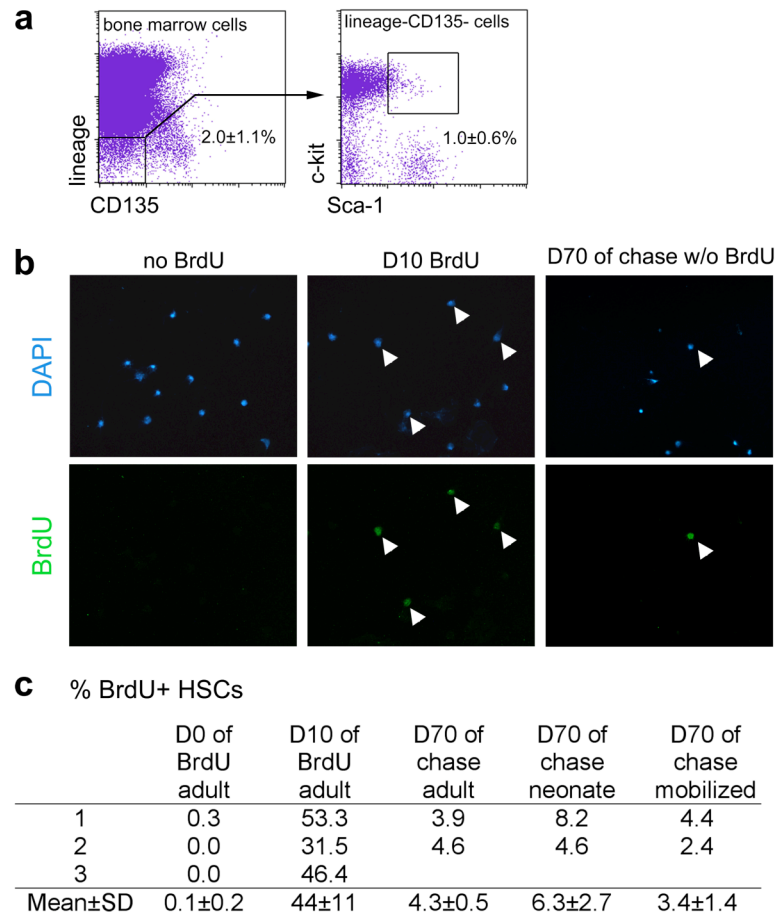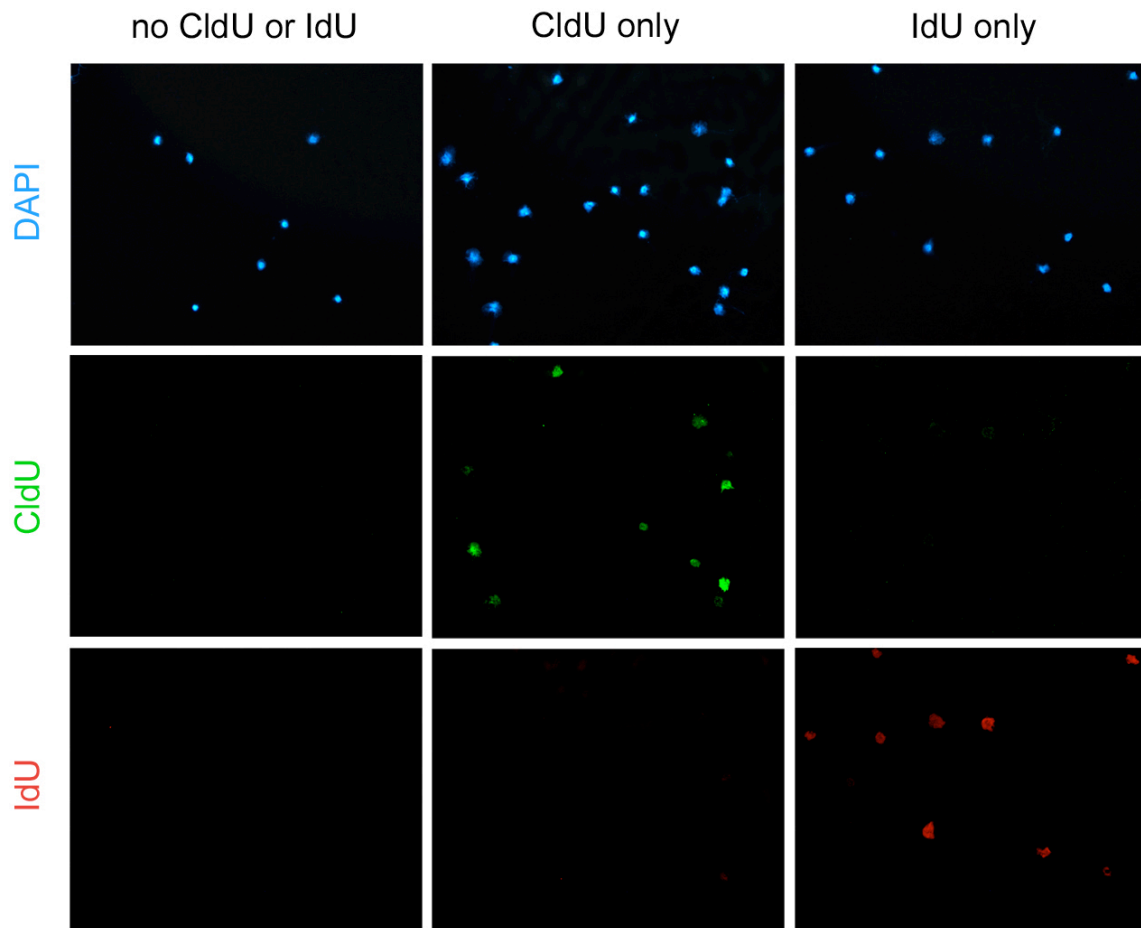**Figure 5.8: CldU+ and IdU+ cells can be distinguished by antibody staining.** CldU and IdU staining of CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs from control mice that were administered neither label (left column), only CldU (middle column), or only IdU (right column). These controls were performed side-by-side with all CldU/IdU samples using the same staining conditions. IdU staining was only observed in samples from mice administered IdU and CldU staining was only observed in samples from mice administered CldU.

a

	D0	D1	D2

CldU   IdU

b % HSCs positive for CldU and/or IdU

	CldU-only+	IdU-only+	Total CldU+	Total IdU+	CldU+IdU +
1	11.7	6.2	16.0	10.5	4.3
2	10.2	5.4	13.6	8.8	3.4
3	13.4	6.0	14.8	7.4	1.4
Mean±SD	12±2	6±0.4	15±1	9±2	3±2

Figure 5.9: CldU persists in mice for less than a day after administration is discontinued. To test whether the CldU/IdU double labeling of HSCs might simply have reflected a slow clearance of CldU from mice after its administration was discontinued (such that dividing HSCs were inadvertently exposed to both CldU and IdU) we sequentially administered CldU and IdU to mice for only 1 day each (a). If significant residual CldU remained in the mice for at least a day during IdU administration, all IdU+ HSCs should also be CldU+ in this experiment. In contrast, if CldU is cleared from mice within hours of being discontinued, then only a minority of IdU+ HSCs should also be CldU+. b) The frequency of CD150+CD48-CD41-lineage-Sca-1+c-kit+ HSCs that stained only with CldU, or only with IdU, the total frequencies of CldU+ or IdU+ cells, and the frequency of CldU+IdU+ HSCs. Nearly two-thirds of IdU+ HSCs (6% IdU-only+ versus 9% total IdU+) were able to incorporate IdU without incorporating CldU over this brief period of administration, indicating that CldU does not persist in mice for longer than 8 hours after administration. The data are based on 3 separate mice analyzed in two independent experiments with 162 to 238 HSCs analyzed per mouse. The fact that some residual CldU remains in these mice during the first several hours of IdU administration can account for the consistent 2 to 3% increase in the frequency of CldU$^+$IdU$^+$ HSCs above that predicted by the product of single positive HSCs (27% observed versus 24% predicted with 10 day pulses (Figure 5.6c); 3% observed versus 1.3% predicted based on 1 day pulses (panel b above)).

BIBLIOGRAPHY

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell *118*, 149-161.

Armakolas, A., and Klar, A.J. (2006). Cell type regulates selective segregation of mouse chromosome 7 DNA strands in mitosis. Science *311*, 1146-1149.

Bonhoeffer, S., Mohri, H., Ho, D., and Perelson, A.S. (2000). Quantification of cell turnover kinetics using 5-bromo-2'-deoxyuridine. J Immunol *164*, 5049-5054.

Bowie, M.B., McKnight, K.D., Kent, D.G., McCaffrey, L., Hoodless, P.A., and Eaves, C.J. (2006). Hematopoietic stem cells proliferate until after birth and show a reversible phase-specific engraftment defect. J Clin Invest *116*, 2808-2816.

Cairns, J. (1975). Mutation selection and the natural history of cancer. Nature *255*, 197-200.

Cheshier, S., Morrison, S.J., Liao, X., and Weissman, I.L. (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proceedings of the National Academy of Sciences USA *96*, 3120-3125.

Christensen, J.L., and Weissman, I.L. (2001). Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proc Natl Acad Sci U S A *98*, 14541-14546.

Conboy, M.J., Karasov, A.O., and Rando, T.A. (2007). High incidence of non-random template strand segregation and asymmetric fate determination in dividing stem cells and their progeny. PLoS Biol *5*, e102.

Cotsarelis, G., Sun, T.T., and Lavker, R.M. (1990). Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell *61*, 1329-1337.

Karpowicz, P., Morshead, C., Kam, A., Jervis, E., Ramunas, J., Cheng, V., and van der Kooy, D. (2005). Support for the immortal strand hypothesis: neural stem cells partition DNA asymmetrically in vitro. J Cell Biol *170*, 721-732.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells. Cell *121*, 1109-1121.

Morrison, S.J., Csete, M., Groves, A.K., Melega, W., Wold, B., and Anderson, D.J. (2000). Culture in reduced levels of oxygen promotes clonogenic sympathoadrenal differentiation by isolated neural crest stem cells. J Neurosci *20*, 7370-7376.

Morrison, S.J., Wright, D., and Weissman, I.L. (1997). Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. Proc Natl Acad Sci USA *94*, 1908-1913.

Potten, C.S., Hume, W.J., Reid, P., and Cairns, J. (1978). The segregation of DNA in epithelial stem cells. Cell *15*, 899-906.

Potten, C.S., Owen, G., and Booth, D. (2002). Intestinal stem cells protect their genome by selective segregation of template DNA strands. J Cell Sci *115*, 2381-2388.

Shinin, V., Gayraud-Morel, B., Gomes, D., and Tajbakhsh, S. (2006). Asymmetric division and cosegregation of template DNA strands in adult muscle satellite cells. Nat Cell Biol *8*, 677-687.

Smith, G.H. (2005). Label-retaining epithelial cells in mouse mammary gland divide asymmetrically and retain their template DNA strands. Development *132*, 681-687.

Spalding, K.L., Bhardwaj, R.D., Buchholz, B.A., Druid, H., and Frisen, J. (2005). Retrospective birth dating of cells in humans. Cell *122*, 133-143.

Takano, H., Ema, H., Sudo, K., and Nakauchi, H. (2004). Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med *199*, 295-302.

Taniguchi, S., Hirabayashi, Y., Inoue, T., Kanisawa, M., Sasaki, H., Komatsu, K., and Mori, K.J. (1993). Hemopoietic stem-cell compartment of the SCID mouse: double-exponential survival curve after gamma irradiation. Proc Natl Acad Sci U S A *90*, 4354-4358.

Taylor, G., Lehrer, M.S., Jensen, P.J., Sun, T.T., and Lavker, R.M. (2000). Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell *102*, 451-461.

Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W.E., Rendl, M., and Fuchs, E. (2004). Defining the epithelial stem cell niche in skin. Science *303*, 359-363.

Urbanek, K., Cesselli, D., Rota, M., Nascimbene, A., De Angelis, A., Hosoda, T., Bearzi, C., Boni, A., Bolli, R., Kajstura, J.*, et al.* (2006). Stem cell niches in the adult mouse heart. Proc Natl Acad Sci U S A *103*, 9226-9231.

Welm, B.E., Tepera, S.B., Venezia, T., Graubert, T.A., Rosen, J.M., and Goodell, M.A. (2002). Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev Biol *245*, 42-56.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q.*, et al.* (2003). Identification of the hematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.

CHAPTER 6

CONCLUSIONS[1]

In this dissertation, I have presented evidence in favor of the existence of a perivascular niche for HSCs identified using markers of the SLAM-family of surface antigens. Moreover, I have questioned the validity of some of the most widely cited evidence in favor of the existence of an osteoblastic HSC niche.

Overall, many questions still remain relating to the role of the endosteum environment in the localization and maintenance of HSCs in the bone marrow. Some of the factors implicated in HSC maintenance by endosteal cells have not yet been tested for necessity *in vivo*. Evidence supporting a role for these factors in HSC maintenance is based upon experiments conducted in culture or on over-expression experiments *in vivo*. It will be important to test whether these factors are really required for HSC maintenance *in vivo*. Similarly, a fundamental question is whether there are redundant sources for critical growth factors in the bone marrow. It will ultimately be necessary to conditionally delete critical growth factors from specific stromal cell types to confirm that they are the physiologically important source for these factors. It is clear that endosteal cells have important roles in the regulation of HSC maintenance and function. However, answers

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to these questions would provide insight into precisely how endosteal cells regulate HSCs and what fraction of the HSC pool depends on such mechanisms.

With respect to whether there is a perivascular niche, multiple cell types in addition to endothelial cells themselves are located perivascularly and may contribute to the cell-extrinsic regulation of HSCs. Mesenchymal progenitors, that localize perivascularly in the bone marrow (Shi and Gronthos, 2003), also appear capable of regulating HSC maintenance (Sacchetti et al., 2007). Cultured bone marrow stromal cells can form bony ossicles that become hematopoietic when transplanted subcutaneously in mice (Kaigler et al., 2005). These ectopic bone marrow compartments have cellular compositions similar to endogenous bone marrow. In contrast, transplantation of osteoblasts can form bone, but not hematopoietic bone. Rather, a recent study found that CD146-expressing mesenchymal progenitors, that reside perivascularly in the bone marrow, are uniquely capable of forming hematopoietic bone (Sacchetti et al., 2007). This suggests that self-renewing, perivascular mesenchymal progenitors can form HSC niches. Additionally, these perivascular mesenchymal progenitors express factors that regulate HSC maintenance, including Angiopoietin and CXCL12. An interesting question concerns the relationship between these mesenchymal progenitors and the CXCL12-expressing perivascular reticular cells (Sugiyama et al., 2006) – could these be the same cells?

Additional cells that might have a role in the maintenance of HSCs also localize to the perivascular environment, including megakaryocytes. Megakaryocytes localize preferentially to sinusoids (Avecilla et al., 2004), and are often found adjacent to HSCs (Kiel et al., 2005). Mice with defects in megakaryocyte and platelet development also

have abnormalities in bone-marrow hematopoiesis (Shivdasani and Orkin, 1995; Vannucchi et al., 2002). Finally, there is considerable evidence of cross-regulation among megakaryocytes, endothelial cells, and osteoblasts (Avecilla et al., 2004; Kacena et al., 2006; Kaigler et al., 2005), raising the possibility that all of these cells might work together to create HSC niches using a combination of direct and indirect mechanisms (see Figure 1.3).

Does the perivascular environment actually promote the maintenance of HSCs, or are the HSCs around sinusoids in the process of migrating elsewhere? Significant numbers of HSCs enter and exit the circulation every day (Wright et al., 2001) and HSCs that are in the process of entering or exiting the bone marrow might be expected to be present around sinusoids. Additional studies will be required to genetically test whether perivascular cells maintain bone marrow HSCs using specific mechanisms. Moreover, it would be helpful to be able to image HSCs within the bone marrow to determine how long they spend in perivascular environments and whether they undergo self-renewing divisions in these locations. It is not clear whether this will be technically possible, but the ability to dynamically image the homing of hematopoietic progenitors to specialized regions of endothelium within the bone marrow of living mice is encouraging (Sipkins et al., 2005). The ability to perform live imaging of HSCs within the bone marrow (Suzuki et al., 2006) could also help to resolve the question of whether HSCs behave differently in different locations.

If vascular niches have an important role in the maintenance and expansion of HSCs, an interesting question is whether all sinusoids can participate in the formation of vascular niches or whether only specialized domains within certain sinusoids are

competent to do so. If vascular structures can acquire competence to support HSCs and hematopoiesis in response to hematopoietic stress, it would suggest that the hematopoietic system has considerable plasticity with respect to the numbers and locations of stem cell niches.

Whereas most evidence suggests important contributions by endosteal and perivascular cells to the maintenance of HSCs, it remains possible that there are crucial, but as yet undiscovered, roles for other cell types or other microenvironments in the maintenance of HSCs. Using SLAM family markers, 38% of HSCs in extramedullary tissues and 26% of HSCs in bone marrow were not detectably in contact with either sinusoids or endosteum (Kiel et al., 2005). It remains unclear whether these cells were migrating or whether they were present in distinct niches that we do not yet know how to identify. For example, it is possible that adipocytes secrete factors that regulate HSCs or hematopoiesis in the bone marrow (DiMascio et al., 2007). We remain at an early stage of elucidating the contributions of cells within the bone marrow and other tissues to the regulation of HSCs and hematopoiesis.

A recent study suggests that HSCs often occupy hypoxic microenvironments in the bone marrow. The degree of blood perfusion in different regions of tissues can be tested by infusing brief pulses of Hoechst dye intravenously and then comparing the degree of Hoechst uptake by cells in different locations (Parmar et al., 2007). Cells in microenvironments that are well perfused by blood should be exposed to the highest concentrations of Hoechst dye, whereas cells in microenvironments that are poorly perfused should be exposed to much lower concentrations. Cells that stain with the lowest levels of Hoechst dye are therefore inferred to be in relatively hypoxic environments.

After intravenous infusion of Hoechst dye, HSCs were enriched among the bone-marrow cells that stained with the lowest levels of Hoechst by flow-cytometry (Durand et al., 1990). At least certain regions of the endosteum appear to be hypoxic, though other regions of the bone marrow also contain hypoxic areas (Parmar et al., 2007). It remains unclear whether some perisinusoidal regions are hypoxic, a possibility given that sinusoids carry slow venous circulation that is not designed to transport oxygen so much as to allow cells to move in and out of circulation. Additional studies will be required to fully characterize the hypoxic regions within bone marrow and the manner in which they regulate HSCs.

Recent evidence also indicates that HSC mobilization may be influenced by the sympathetic nervous system. Mice deficient in UDP-galactose ceramide galactosyltransferase, an enzyme that is required for normal peripheral nerve myelination and conduction, fail to mobilize hematopoietic progenitors in response to granulocyte colony stimulating factor (G-CSF) (Levesque et al., 2007). Pharmacological ablation of noradrenergic neurons also reduced the mobilization of hematopoietic progenitors, whereas ß2 adrenergic agonists enhanced mobilization (Katayama et al., 2006). These data indicate that the sympathetic nervous system regulates the localization of hematopoietic progenitors in the bone marrow. Since all of these treatments also had effects on osteoblast morphology, the sympathetic nervous system was interpreted to regulate an endosteal niche for HSCs. The sympathetic nervous system also regulates the vasculature (Katayama et al., 2006) and it was not tested whether there were effects on bone marrow vasculature in these mice. Additional work will be required to determine

which bone marrow cells mediate the effects of the sympathetic nervous system on hematopoietic progenitor mobilization.

Drosophila spermatogenesis provides one paradigm for what a stem cell niche is, and how it works (Julien et al., 1995) (Figure 6.1). An average of 9 spermatogonial stem cells (blue) are arrayed around the hub, a cluster of 12 somatic cells (green) that produce a factor called Unpaired (Upd). Stimulation by Unpaired is required for the maintenance of spermatogonial stem cells, and Unpaired is concentrated in the extracellular matrix surrounding hub cells, so the stem cells can only be maintained immediately adjacent to the hub (Fuller and Spradling, 2007; Tulina and Matunis, 2001). The stem cells divide asymmetrically such that one daughter cell remains adjacent to the hub and retains stem cell identity, whereas the other daughter cell is displaced from the niche and is fated to differentiate (purple) by virtue of losing access to Unpaired (Kiger et al., 2001). The differentiating progeny continue to proliferate and differentiate into sperm. In this way, spermatogonial stem cells both sustain the stem cell pool and generate differentiated progeny. Since the niche is constrained by the need for contact with the hub, this physically limits the number of stem cells that can reside within the tissue and ensures that half of the progeny of stem cells are displaced from the niche and fated to differentiate. Nonetheless, if stem cells are ablated, the daughter cell that is normally destined to differentiate can reoccupy the open space in the niche to maintain homeostasis (Morrison and Kimble, 2006).

The Drosophila germline is attractive in the simplicity of the mechanisms by which it maintains limited numbers of stem cells while also sustaining spermatogenesis.

Does the hematopoietic system employ analogous mechanisms to maintain proscribed numbers of HSCs while sustaining hematopoiesis? We might imagine that this is the case, though it need not be. As more and more niches are characterized in vertebrates and invertebrates we are sure to uncover new mechanisms and perhaps even new paradigms. For now, much less is known about HSC niches in two crucial respects. First, whereas we have single-cell resolution in the Drosophila germline, with the ability to reliably identify each stem cell and each supporting (hub) cell, HSCs are so rare, and hematopoietic tissues so vast, that it can be much more difficult to locate the actual stem cells. Second, the mechanisms that have been implicated in the regulation of the Drosophila germline are supported by genetic evidence showing necessity under physiological conditions *in vivo* (Brawley and Matunis, 2004). In the hematopoietic system, we draw more complex models but many elements of these models remain genetically untested.

Despite the limitations in existing knowledge, both endosteal and perivascular cells appear to contribute to the maintenance, expansion, and localization of HSCs. One possibility is that perivascular and endosteal cells contribute to the formation of a common niche that is influenced by both cell types (Figure 6.2C). Alternatively, they may form spatially distinct niches (Figure 6.2A). If they do form distinct niches, are these niches functionally distinct in terms of their effect on HSCs (Figure 6.2B)? Finally, it is possible that there is less microenvironmental specialization than we imagine in the bone marrow, and that HSCs in a variety of locations have access to factors required for HSC maintenance (Figure 6.2D). For now, we can only speculate about the answers to these questions.

Another fundamental question is whether the mechanisms that are responsible for the maintenance of HSCs change after injury. It is striking that increased Jagged1–Notch1 signalling (Calvi et al., 2003; Fuller and Spradling, 2007; Varnum-Finney et al., 1998) and increased canonical WNT signalling (Reya et al., 2003; Stier et al., 2002) are sufficient to promote HSC expansion, though not under all circumstances (Scheller et al., 2006; Willert et al., 2003). Yet the genes encoding Jagged1, Notch1 and ß-catenin are not necessary for the maintenance of adult HSCs *in vivo* (Kirstetter et al., 2006; Mancini et al., 2005) (Table 6.1). One possibility is that there are redundant signals that can promote HSC maintenance or expansion under physiological conditions, any of which is sufficient, but none of which is necessary. It is also possible that WNT signaling or Notch signaling is necessary for the response of HSCs to certain stresses but not under steady-state conditions. The idea that different signals may regulate HSCs under different circumstances is also consistent with the observation that the cellular composition of the bone marrow, and the potential for extramedullary hematopoiesis change in response to stress. Going forward it will be important to distinguish between environmental mechanisms that maintain HSCs under steady-state conditions versus mechanisms that regulate HSC survival or expansion after injury.

These questions also pose some conceptual issues. Are HSC niches set in number such that they specify the numbers of HSCs that can be present in tissues, or are many cells facultatively capable of creating HSC niches? Are niches transiently or permanently competent to support HSCs, and are they transiently or permanently occupied? What changes allow HSCs to expand in number during development (Cobas et al., 2004)? What changes allow HSCs to expand in number prior to mobilization (Bodine et al.,

1993; Morrison et al., 1995)? What changes allow extramedullary tissues to maintain large numbers of HSCs and high levels of hematopoiesis after hematopoietic stresses? It seems as though there are cells in hematopoietic tissues that do not form HSC niches under steady-state conditions but that can acquire this potential in the context of hematopoietic stress. How is this regulated to prevent hematopoietic tissues from accumulating inappropriate numbers of niches and HSCs? Do these environmental mechanisms go awry in hematopoietic malignancies?

Are all sinusoidal vascular environments and all endosteal environments equally capable of promoting the maintenance of HSCs (directly or indirectly), or are there specialized domains that are uniquely capable of regulating HSCs? If so, are these domains invariant, or constantly shifting under physiological circumstances? Could this have something to do with the frequent migration of HSCs under steady state conditions (Morrison et al., 1997)? Or could the frequent migration of HSCs mean that they require only periodic contact with supportive cells? If so, is it really meaningful to think in terms of specialized niches that maintain HSCs?

At this point, there are more questions than answers. Nonetheless, progress is rapid and the answers that arise over the next several years will provide fundamental insights into the mechanisms used by mammalian tissues to regulate development, homeostasis, and regeneration after injury.

Table 6.1: Factors proposed to be important in extrinsic control of HSCs but for which genetic evidence in vivo raises uncertainty.

Factor	Proposed action in HSC niche	Genetic evidence against role in vivo
Wnts	Wnt signaling in HSCs was believed to promote symmetric HSC division and expansion (Reya et al. 2003, Willert et al. 2003)	beta-catenin, the downstream target of Wnts, is dispensable for proper HSC function and maintenance (Cobas et al. 2004, Koch et al. 2007).
Jagged-1, Notch ligands	Interaction of Jagged-1 expressed on osteoblasts with its receptor Notch-1 on the surface of HSCs was believed to regulate HSC number (Calvi et al. 2003, Stier et al. 2002)	Conditional deletion of Jagged-1 or Notch-1 in either hematopoietic cells or in hematopoietic stromal cells does not impair HSC activity or hematopoiesis (Mancini et al. 2005)
N-cadherin (Cdh2)	Contact of HSCs with osteoblasts mediated via homophilic cell-cell interactions was proposed to retain HSCs at the endosteal surface and maintain their quiescence (Arai et al. 2004, Wilson et al. 2004, Wilson et al. 2006, Zhang et al. 2003)	Lack of expression of N-cadherin on the surface of in vivo reconstituting HSCs (Kiel et al. 2007). N-cadherin conditional deletion from HSCs has no effect on HSC maintenance or function (Chapter 4).

Several reasons may reconcile these apparent discrepancies.   First, it is formally possible that these factors are sufficient but not necessary to produce the proposed effects on HSCs.   Second, it is possible that there are molecules with redundant function that mask the effect of loss of these factors in vivo.   Finally, it is possible that the effects of these factors are mediated indirectly or are context dependent and require, for example, stresses to the hematopoietic system before effects of loss of function can be seen.
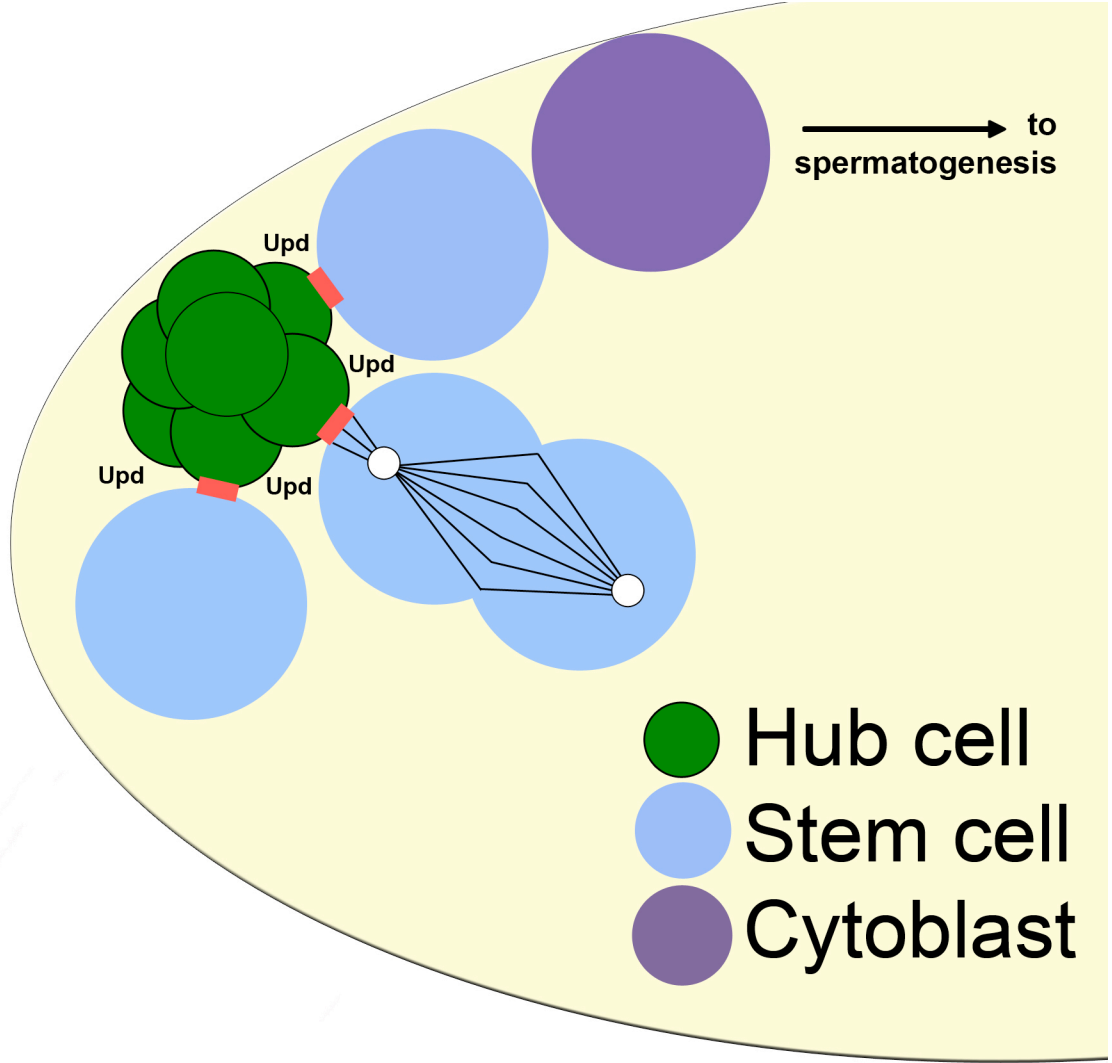
Figure 6.1: Spermatogenesis in *Drosophila melanogaster* provides a paradigm for how a stem cell niche works.
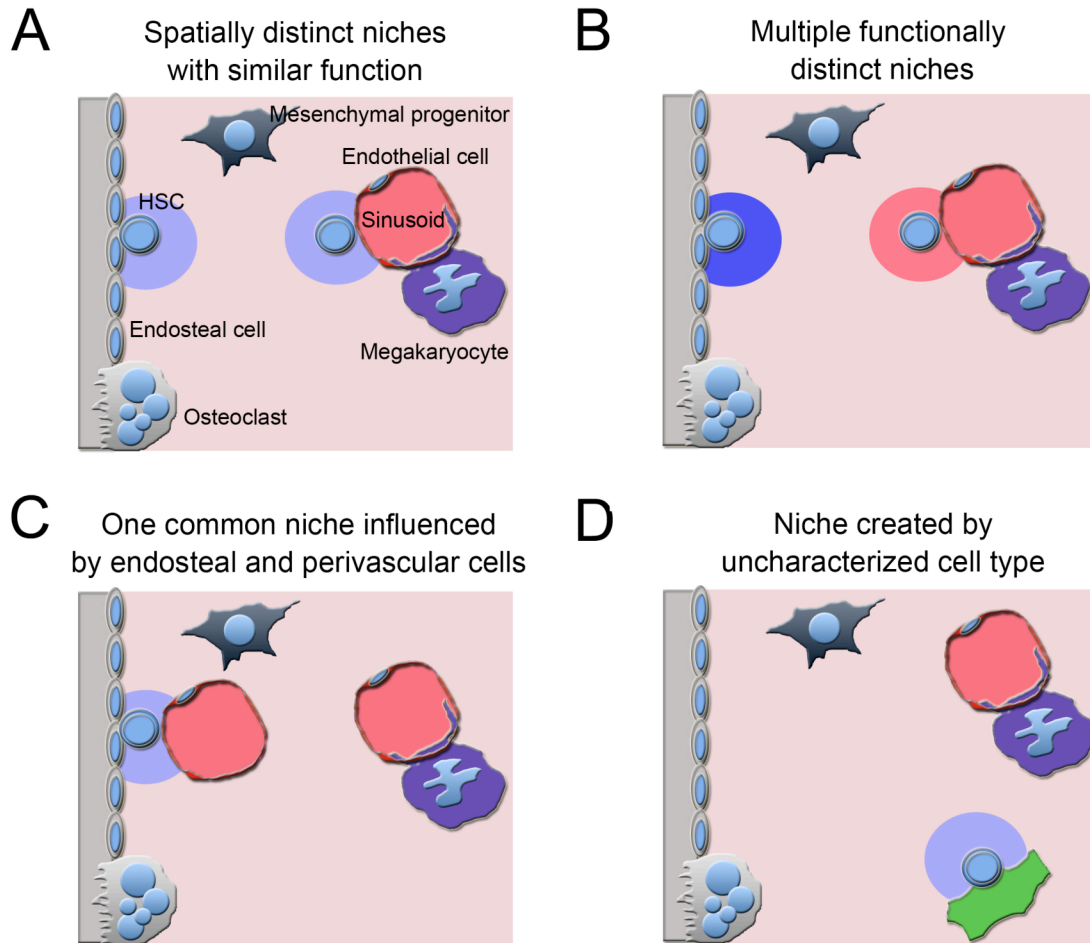
Figure 6.2: Possible relationships between endosteal and perivascular niches. Both endosteal and perivascular cells have been hypothesized to contribute to the formation of hematopoietic stem cell (HSC) niches (Adams and Scadden, 2006; Kopp et al., 2005; Li and Xie, 2005; Suda et al., 2005; Wright et al., 2001). A) One possibility is that there are spatially distinct endosteal and perivascular niches that have functionally redundant roles in the maintenance of HSCs. B) Another possibility is that these niches are spatially and functionally distinct, regulating HSCs in different ways. Although it has been hypothesized that 'activated' HSCs localize to vascular niches there is no evidence to support this possibility at present as the HSCs identified using Signalling lymphocyte activation molecule (SLAM) markers (that are frequently observed adjacent to sinusoids) include few dividing cells (Kiel and Morrison, 2006). C) Rather than multiple spatially distinct niches, it is possible that there is a single niche near the endosteum that is regulated by factors secreted by both endosteal and perivascular cells. D) Finally, it is formally possible that there is little spatial specialization within the bone marrow in terms of the capacity to maintain HSCs and that much of the bone marrow is close enough to endosteal, perivascular, and other cell types to support HSC maintenance. However, the observation that HSCs are more likely than other bone marrow cells to localize near sinusoids and endosteum argues against this possibility (Kiel et al., 2007; Kiel et al., 2005).

BIBLIOGRAPHY

Adams, G.B., and Scadden, D.T. (2006). The hematopoietic stem cell in its place. Nature Immunology *7*, 333-337.

Avecilla, S.T., Hattori, K., Heissig, B., Tejada, R., Liao, F., Shido, K., Jin, D.K., Dias, S., Zhang, F., Hartman, T.E.*, et al.* (2004). Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. Nat Med *10*, 64-71.

Bodine, D.M., Seidel, N.E., Zsebo, K.M., and Orlic, D. (1993). In vivo administration of stem cell factor to mice increases the absolute number of pluripotent hematopoietic stem cells. Blood *82*, 445-455.

Brawley, C., and Matunis, E. (2004). Regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo. Science *304*, 1331-1334.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R.*, et al.* (2003). Osteoblastic cells regulate the hematopoietic stem cell niche. Nature *425*, 841-846.

Cobas, M., Wilson, A., Ernst, B., Mancini, S.J., MacDonald, H.R., Kemler, R., and Radtke, F. (2004). {beta}-Catenin Is Dispensable for Hematopoiesis and Lymphopoiesis. J Exp Med *199*, 221-229.

DiMascio, L., Voermans, C., Uqoezwa, M., Duncan, A., Lu, D., Wu, J., Sankar, U., and Reya, T. (2007). Identification of adiponectin as a novel hemopoietic stem cell growth factor. J Immunol *178*, 3511-3520.

Durand, R.E., Chaplin, D.J., and Olive, P.L. (1990). Cell sorting with Hoechst or carbocyanine dyes as perfusion probes in spheroids and tumors. Methods Cell Biol *33*, 509-518.

Fuller, M.T., and Spradling, A.C. (2007). Male and female Drosophila germline stem cells: two versions of immortality. Science *316*, 402-404.

Julien, C., Zhang, Z.Q., and Barres, C. (1995). How sympathetic tone maintains or alters arterial pressure. Fundam Clin Pharmacol *9*, 343-349.

Kacena, M.A., Gundberg, C.M., and Horowitz, M.C. (2006). A reciprocal regulatory interaction between megakaryocytes, bone cells, and hematopoietic stem cells. Bone *39*, 978-984.

Kaigler, D., Krebsbach, P.H., West, E.R., Horger, K., Huang, Y.C., and Mooney, D.J. (2005). Endothelial cell modulation of bone marrow stromal cell osteogenic potential. Faseb J *19*, 665-667.

Katayama, Y., Battista, M., Kao, W.M., Hidalgo, A., Peired, A.J., Thomas, S.A., and Frenette, P.S. (2006). Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell *124*, 407-421.

Kiel, M.J., and Morrison, S.J. (2006). Maintaining hematopoietic stem cells in the vascular niche. Immunity *25*, 862-864.

Kiel, M.J., Radice, G.L., and Morrison, S.J. (2007). Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. Cell Stem Cell *1*, 204-217.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells. Cell *121*, 1109-1121.

Kiger, A.A., Jones, D.L., Schulz, C., Rogers, M.B., and Fuller, M.T. (2001). Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. Science *294*, 2542-2545.

Kirstetter, P., Anderson, K., Porse, B.T., Jacobsen, S.E., and Nerlov, C. (2006). Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. Nat Immunol *7*, 1048-1056.

Kopp, H.G., Avecilla, S.T., Hooper, A.T., and Rafii, S. (2005). The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiology (Bethesda) *20*, 349-356.

Levesque, J.P., Winkler, I.G., Hendy, J., Williams, B., Helwani, F., Barbier, V., Nowlan, B., and Nilsson, S.K. (2007). Hematopoietic progenitor cell mobilization results in hypoxia with increased hypoxia-inducible transcription factor-1 alpha and vascular endothelial growth factor A in bone marrow. Stem Cells *25*, 1954-1965.

Li, L., and Xie, T. (2005). Stem cell niche: structure and function. Annu Rev Cell Dev Biol *21*, 605-631.

Mancini, S.J., Mantei, N., Dumortier, A., Suter, U., Macdonald, H.R., and Radtke, F. (2005). Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. Blood *105*, 2340-2342.

Morrison, S.J., Hemmati, H.D., Wandycz, A.M., and Weissman, I.L. (1995). The purification and characterization of fetal liver hematopoietic stem cells. Proc Natl Acad Sci USA *92*, 10302-10306.

Morrison, S.J., and Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. Nature *441*, 1068-1074.

Morrison, S.J., Wright, D., and Weissman, I.L. (1997). Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. Proc Natl Acad Sci USA *94*, 1908-1913.

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R., and Down, J.D. (2007). Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc Natl Acad Sci U S A *104*, 5431-5436.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., and Weissman, I.L. (2003). A role for Wnt signalling in self-renewal of hematopoietic stem cells. Nature *423*, 409-414.

Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P.G., Riminucci, M., *et al*. (2007). Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell *131*, 324-336.

Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M.M., Birchmeier, W., Tenen, D.G., and Leutz, A. (2006). Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. Nat Immunol *7*, 1037-1047.

Shi, S., and Gronthos, S. (2003). Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res *18*, 696-704.

Shivdasani, R.A., and Orkin, S.H. (1995). Erythropoiesis and globin gene expression in mice lacking the transcription factor NF-E2. Proc Natl Acad Sci U S A *92*, 8690-8694.

Sipkins, D.A., Wei, X., Wu, J.W., Runnels, J.M., Cote, D., Means, T.K., Luster, A.D., Scadden, D.T., and Lin, C.P. (2005). In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature *435*, 969-973.

Stier, S., Cheng, T., Dombkowski, D., Carlesso, N., and Scadden, D.T. (2002). Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. Blood *99*, 2369-2378.

Suda, T., Arai, F., and Hirao, A. (2005). Hematopoietic stem cells and their niche. Trends Immunol *26*, 426-433.

Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity *25*, 977-988.

Suzuki, N., Ohneda, O., Minegishi, N., Nishikawa, M., Ohta, T., Takahashi, S., Engel, J.D., and Yamamoto, M. (2006). Combinatorial Gata2 and Sca1 expression defines hematopoietic stem cells in the bone marrow niche. Proc Natl Acad Sci U S A *103*, 2202-2207.

Tulina, N., and Matunis, E. (2001). Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. Science *294*, 2546-2549.

Vannucchi, A.M., Bianchi, L., Cellai, C., Paoletti, F., Rana, R.A., Lorenzini, R., Migliaccio, G., and Migliaccio, A.R. (2002). Development of myelofibrosis in mice genetically impaired for GATA-1 expression (GATA-1(low) mice). Blood *100*, 1123-1132.

Varnum-Finney, B., Purton, L.E., Yu, M., Brashem-Stein, C., Flowers, D., Staats, S., Moore, K.A., LeRoux, I., Mann, R., Gray, G.*, et al.* (1998). The notch ligand, jagged-1, influences the development of primitive hematopoietic precursor cells. Blood *91*, 4084-4091.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., 3rd, Nusse, R., Howard Hughes Medical, I., and Department of Developmental Biology, S.U.S.o.M.S.C.U.S.A. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature *423(6938)*, 448-452.

Wright, D.E., Wagers, A.J., Gulati, A.P., Johnson, F.L., and Weissman, I.L. (2001). Physiological migration of hematopoietic stem and progenitor cells. Science *294*, 1933-1936.