DNMT3B'S ROLE IN HEMATOPOIETIC STEM CELLS

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Submitted to Graduate Faculty of

School of Medicine

in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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Hematopoiesis proceeds from a bone marrow resident population of stem cells responsible for generation of all lineages within the blood. Distinct molecular programs within hematopoietic stem cells regulate maintenance of this population under homeostatic conditions, however the coordination of these programs remains largely undefined. DNA methylation is an epigenetic means of gene regulation in a mammals carried out by a family of DNA methyltransferases. Of these, the de novo methyltransferase is highly expressed in hematopoietic stem cells as compared to other members of this family and somatic mutations in DNMT3b lead to a syndrone characterized by immunodeficiency. Therefore we hypothesized that DNMT3b regulates hematopoietic stem cells *via* its ability to methylate DNA. By knock-down of DNMT3b with a retrovirally delivered shRNA or Cre mediated recombination of floxed DNMT3b alleles work presented in this thesis demonstrates a critical role for DNMT3b in hematopoiesis in mice. Loss of DNMT3b leads to limited reconstitution of hematopoiesis in irradiated recipients associated with a proliferative defect *in vitro* and a failure of hematopoietic stem cell self-renewal *in vivo*. Targeted deletion of DNMT3b in hematopoietic stem cells leads to decreased engraftment following transplantation and decreased proliferation *in vitro*. DNMT3b's function in hematopoietic cells requires the methyltransferase activity of the enzyme and the defects in hematopoiesis are associated with loss of DNA methylation and decreased expression of MLL. Therefore DNMT3b is necessary for maintenance of the proliferative ability and engraftment capacity of hematopoietic cells and hematopoiesis is dependent upon appropriate DNA methylation.

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1.0 INTRODUCTION

Somatic stem cells are largely responsible for tissue repair and regeneration over the lifetime of an individual. Hematopoietic stem cell transplantation is currently being used for the treatment of congenital deficiencies and an increasing number of malignant diseases[1]. Hematopoietic stem cells have become increasingly useful with the advent of efficient isolation techniques and with their ability to acquire unique functions through genetic engineering. However, despite the enthusiasm surrounding hematopoietic stem cell biology and therapeutics, the full potential of hematopoietic stem cell manipulation *in vitro* has yet to be fully realized and is highly dependent upon the development of technologies that enhance specific characteristics of these cells. The ultimate success of hematopoietic stem cell expansion *in vitro* and efficient repopulation of the entire hematopoietic and immune system *in vivo* will require a greater understanding of the molecular mechanisms coordinating hematopoietic stem cell self-renewal and differentiation. As hematopoietic stem cell function plays a vital role in hematopoiesis over an individual's lifetime, understanding the biological and functional regulation of these cells is of paramount importance.

Epigenetic regulation of gene expression by DNA methylation has been shown to play a role during the differentiation and self-renewal of a number of pluripotent cell types[2-4]. DNA methylation in mammals is carried out by a family of DNA methyltransferases: DNMT1, DNMT3a, and DNMT3b. Loss of DNMT1 has been shown to limit hematopoietic stem cell function[5] and DNA methylation is necessary for B cell development from hematopoietic progenitor cells[6]. DNMT3b is highly

expressed in hematopoietic stem cells[7, 8] and inherited mutations in this gene in humans are associated with a syndrome characterized by immunodeficiency[9], however a unique role for DNMT3b in hematopoietic stem cells has not been determined. To that end this thesis addresses the role of DNMT3b in hematopoietic stem cells. Knockdown of DNMT3b by a retrovirally-delivered shRNA or by Cre mediated recombination of floxed DNMT3b alleles led to limited hematopoietic stem engraftment in irradiated recipients that was associated with loss of the self-renewal ability of hematopoietic stem cells. DNMT3b function in hematopoietic cells appears to require its methyltransferase activity and improper methylation of the trithorax homolog MLL (mixed lineage leukemia) gene and subsequent down-regulation of its homeobox targets may underlie this defect. Elucidation of the role of DNMT3b in hematopoiesis may allow for future studies addressing the role DNA methylation plays in hematopoietic stem cell regulation as well as potential targets for *ex vivo* manipulation to enhance stem cell engraftment and function following transplantation.

1.1 HEMATOPOIETIC STEM CELL ISOLATION AND NICHE

1.1.1 Hematopoietic Stem Cell Enrichment

Hematopoietic stem cells are a rare population of bone marrow cells responsible for generation of all lineages within the blood over the lifetime of an organism. The availability of monoclonal antibodies and the ability to isolate cells by fluorescence activated cell sorting (FACS) has allowed for the successful enrichment of bone marrow cells containing nearly all the hematopoietic reconstituting ability of the bone marrow[10]. Combinatorial strategies involving selection for the thymocyte marker Thy-1 and depletion of cells expressing differentiated cell markers was shown to enrich for

approximately 0.1% of adult mouse bone marrow that still contained the entire colony forming ability in the spleen[11]. This population could be further enriched by selecting for cells expressing Stem Cell Antigen-1 (Sca-1), a member of the Ly-6 family[12] discovered in a screen of pre-T cell hybridoma cell surface expression molecules[10], and c-kit, the cell surface receptor for stem cell factor[13]. Depletion of bone marrow cells expressing markers of mature hematopoietic lineages ($B220^+$ B cells, $CD3^+$, $CD4^+$, or $CD8^+$ T cells, $Gr\text{-}1^+$ and/or $CD11b^+$ myeloid cells, and $Ter\text{-}119^+$ erythroid precursors) as well as positive selection for Sca-1 and c-kit expression (LKS cells) allows for enrichment of stem cell activity from approximately 1 in 20,000 whole bone marrow cells to greater than 1 in 10 based on competitive long-term repopulation in irradiated recipients[14]. Long term repopulating hematopoietic stem cells in mice can be further enriched based on a low level of CD34 expression, which represents about 1 in 5 LKS cells[15]. When transplanted into irradiated recipients only long term hematopoietic stem cells (LT-HSCs) are capable of engraftment greater than 3 months owing to their sustained self-renewal potential. Conversely, short term hematopoietic stem cells (ST-HSCs), that are CD34+LKS, are only able to maintain their reconstituting ability for approximately two months[15]. Remarkably as few as one murine HSC is able to efficiently reconstitute the hematopoietic system of a lethally-irradiated syngeneic recipient [16-22] although not every transplanted recipient will show engraftment[23].

More recently, based on microarray data, the signaling lymphocyte activation molecule (SLAM) marker CD150 has been shown to be uniquely expressed on hematopoietic stem cells with long-term engraftment ability as compared to multipotent progenitors with limited self-renewal. Combination of CD150 expression with absence of a second SLAM marker CD48 ($CD150^+CD48^-$) can allow for equally efficient enrichment of hematopoietic stem cells from the bone marrow as compared to combinatorial strategies based on LKS and CD34 expression[24]. The SLAM markers have proven useful for immunohistochemical staining of hematopoietic stem cells with the bone marrow and further elucidation of their relationship with the bone marrow niche[24].

Intermediate progenitors with limited self-renewal potential can also be isolated from the bone marrow based on their immunophenotype as summarized in Figure 1-1. Most transiently repopulating progenitors express c-kit, but lack Sca-1 expression, and do not express lineage markers. Multipotent or highly proliferative progenitors, as the name suggests, cycle rapidly, but lack the capacity for extended self-renewal and only transiently contribute to hematopoiesis when transplanted into irradiated recipients[25]. This population expresses the feline sarcoma virus (fms)-like tyrosine kinase receptor Flt-3 and may largely contribute to a transient lymphoid reconstitution following transplantation[25]. All lymphoid cells arise for a common lymphoid progenitor that expresses the receptor for Il-7[26]. Myeloid cells are derived from a group of unique progenitors that can be isolated based on their expression of CD34 and Fc Receptor γ (FcR γ) on the cell surface[27]. The most immature myeloid restricted progenitor is $CD34^{+}FcR\gamma^{lo}$ and is named the common myeloid progenitor. This population of cells gives rise to distinct progenitor populations; granulocyte-monocyte progenitors which are $CD34^{+}FcR\gamma^{hi}$ and megakaryocyte erythrocyte progenitors which are $CD34^{-}FcR\gamma^{lo}$[27]. These lymphoid and myeloid progenitors then proceed to differentiate through a number of pro and blast-like cells based on their cellular immunophenotype and gene expression levels to the mature lineages of the hematopoietic system in the peripheral blood and hematopoietic organs.

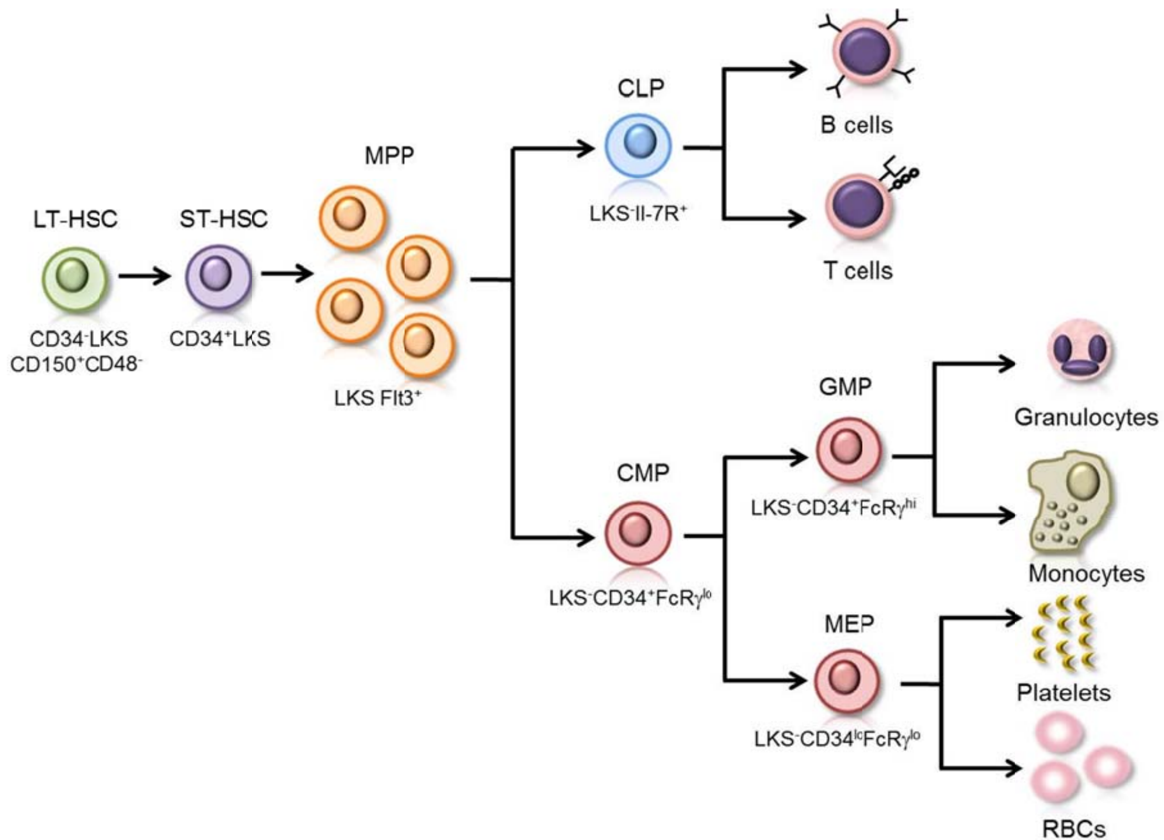


Figure 1-1. The Hematopoietic Tree

Hematopoiesis proceeds from a population of stem cells defined by the length in time in which they engraft in irradiated recipients. Long-term hematopoietic stem cells (LT-HSC) are considered the most immature and can be isolated based on LKS or SLAM markers. LT-HSC give rise to short-term hematopoietic stem cells (ST-HSC) with limited self-renewal ability and subsequently progenitors with increased cycling that largely lack the ability to self-renew. Multipotent progenitors (MPP) can give rise to both myeloid and lymphoid cells, while common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) have lineage restricted progeny. CMP can be further subdivided on into granulocyte-monocyte progenitors (GMP) or megakaryocyte-erythrocyte progenitors (MEP) based on CD34 and FcRγ expression as shown.

1.1.2 Hematopoietic Niche

Hematopoietic stem cells reside in a specialized niche within the bone marrow of adult mammals in close proximity to the endosteal surface (Figure 1-2). A functional relationship between the endosteal surface and cells with hematopoietic reconstituting ability was first indicated by the observation that the cells responsible for colony-forming units in the spleen (discussed in 1.2.1) reside near the endosteum as opposed to the central bone marrow[28] although these cells were later shown to be transiently reconstituting multipotent progenitors[14] that are relatively non-quiescent[28]. CFSE stained immunophenotypically defined hematopoietic stem cells have since been shown to migrate to within 12 cell diameters of the endosteal surface following transplantation[29] as well as in close proximity to perivascular cells within sinusoids[24]. However, immunohistochemical staining of mouse bone marrow with SLAM markers failed to identify co-localization of this relatively pure hematopoietic stem cell population directly on the endosteum[24]. The endosteal surface of bone is lined with osteoblastic progenitors at varying degrees of differentiation which have been shown to play a role in hematopoietic stem cell maintenance. Expansion of osteoblastic cells by overexpression of a constitutively active parathyroid hormone receptor or exogenous administration of parathyroid hormone[30] or inactivation of bone morphogenic protein receptor type IA[31] leads to osteoblastic cell expansion and concomitant increase in hematopoietic stem cell pools. However acute depletion of osteoblastic cells by ganciclovir treatment of mice expressing thymidine kinase under the control of a collagen 1A1 promoter resulted in a more rapid and dramatic decrease in lymphoid progenitors, particularly B-cell progenitors, and a slow decrease in LKS cells [32, 33]. This data suggests that while osteoblasts can regulate hematopoietic stem cell pools this regulation may not require direct cell-to-cell contact between these two cell populations but may act through paracrine signaling or intermediate cell types and other cell types may play overlapping roles in the hematopoietic niche.

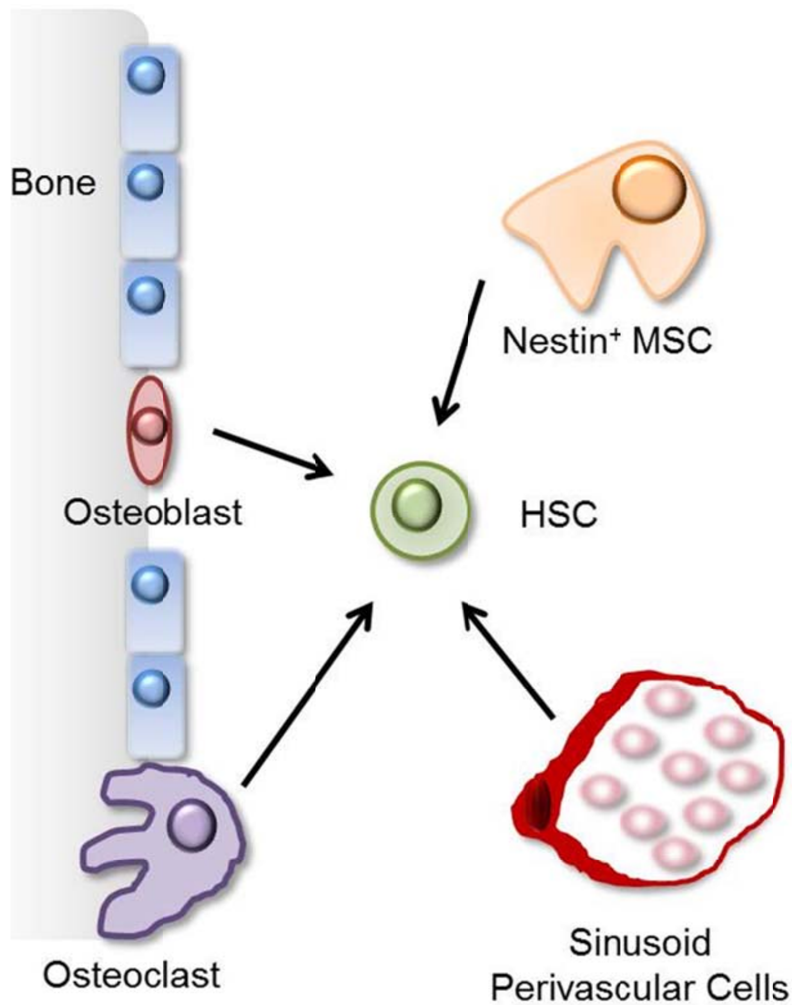


Figure 1-2. The Hematopoietic Stem Cell Niche

Hematopoietic stem cells reside in an endosteal niche in the bone marrow and are maintained or activated by signals from locally residing osteoblasts and osteoclasts as well as mesenchymal stem cells and perivascular cells. In addition long range signaling by cytokines produced outside of the bone marrow contribute to hematopoietic stem cell maintenance within this envrionment.

Osteoblastic cells have been shown to secrete a number of proteins necessary for hematopoietic stem cell maintenance or localization to the bone marrow. Murine hematopoietic stem cells expressing the angiopoietin receptor Tie2 are quiescent and anti-apoptotic[34]. Treatment of bone marrow cells with angiopoietin *in vitro* enhances cobblestone formation on irradiated stromal cells, a surrogate marker of hematopoietic stem cell self-renewal, and maintains hematopoietic stem cells in a quiescent state *in vivo*[34]. Thrombopoietin, which has been shown to be secreted from endosteal osteoblastic cells, also promotes hematopoietic stem cell quiescence; loss of thrombopoietin or its receptor c-Mpl leads to hematopoietic stem cell depletion[35, 36]. In contrast to angiopoietin and thrombopoietin, osteopoietin negatively regulates hematopoietic stem cells; mice lacking osteopoietin have an increased frequency of hematopoietic stem cells in the bone marrow[37]. Although these three factors are produced by osteoblastic cells, multiple other cell types can also produce them, including the liver and kidney in the case of thrombopoietin[38]. Thus in the absence of studies involving targeted depletion of osteoblast-derived growth factors, contribution to hematopoietic stem cell maintenance by osteoblasts *via* these growth factors remains controversial[38].

Osteoclasts, sinusoidal perivascular cells, and bone marrow-resident mesenchymal stem cells may also play a role in the hematopoietic stem cell niche. Bone degradation by osteoclasts may destroy the endosteal niche leading to hematopoietic stem cell mobilization[39], i.e. enhanced hematopoietic stem cell cycling and increased presence of stem and progenitor cell types in the peripheral blood, lymph nodes, and spleen. Mice lacking the calcium receptor have been shown to have reduced bone marrow cellularity concurrent with increased progenitor mobilization suggesting that hematopoietic stem cell lodging at the bone marrow is dependent on calcium ions[40] which are released by the activity of osteoclasts. Hematopoietic stem cell homing near sinusoids may allow for rapid response of these cells to circulating cytokines[38]. Moreover perivascular cells within the sinusoid secrete CXCL12 which, by deletion of its receptor CXCR4, has been shown to maintain hematopoietic stem cells within the niche and their functionality in transplantation assays[41]. Nestin^{+}

mesenchymal stem cells have also been shown to closely associate with hematopoietic stem cells both *in vivo* and *in vitro* and provide a source for osteoblastic cells that further regulate hematopoietic stem cell homing and maintenance[42]. Thus while multiple cell types can regulate the hematopoietic niche, rigorous assessment of the function of the individual cells is lacking and redundancy in signaling to hematopoietic stem cells may underlie this compartment.

1.2 HEMATOPOIETIC STEM CELL FUNCTION

1.2.1 Historical Perspective

The concept of a stem cell is derived from the field of evolutionary biology and the theory developed by Ernest Hackel in the 19th century that multicellular organisms were derived from a unicellular precursor, which was termed the German “Stamzelle”[43]. Hackel also proposed that this term applied to the fertilized embryo due to its ability to give rise to multiple distinct lineages and the term was subsequently extended to include germ line stem cells and hematopoietic precursors, as yet unidentified, in the bone marrow with similar properties[43].

Although the ability of bone marrow transplantation to protect irradiated recipients from hematopoietic failure was known[44], the first identification of cells with this property occurred in 1961 by Till and McCulloch with their observation of splenic colonies 10 days after bone marrow transplantation[45]. Till and McCulloch described hematopoietic precursors in terms of spleen colony forming units (CFU-S), a functional definition of the bone marrow that was shown to correlate with the ability of the marrow to protect transplanted recipients from hematopoietic failure[46]. The colonies were later shown, by chromosomal marking, to be derived from a single cell[47] as well as having the

capacity for self-renewal and multipotent differentiation[48], the two key properties of stem cells. When serially transplanted into irradiated recipients a decrease in the colony forming ability in the spleen was observed greater than that from aged mice suggesting an increased loss of stem cell function with serial transplantation as compared to aging[49]. Thus exhaustion of stem cell functionality is enhanced under the stress of serial transplantation and transplantation of these cells into irradiated recipients can reveal hematopoietic stem cell defects not readily apparent under homeostatic conditions. In addition early studies revealed the stem cell compartment to be relatively quiescent such that cytotoxic agents that require progression through the cell cycle for their activity spare these quiescent hematopoietic stem cells and increase their proportion in the bone marrow[50, 51]. This work completed before prospective isolation of hematopoietic stem cells was achieved, provided a functional definition of hematopoietic stem cells as a relatively quiescent population of bone marrow cells that can be activated to self-renew or differentiate to maintain homeostasis within the hematopoietic system. Despite the inability at the time to prospectively enrich for these cells from the bone marrow the key properties of stem cells had been described for blood-forming bone marrow cells laying a conceptual framework for future studies.

1.2.2 Hematopoietic Stem Cell Assays

The balance between the two key properties of hematopoietic stem cells, self-renewal and the ability to differentiate, can be described in terms of symmetric versus asymmetric division. In a symmetric division both daughter cells have the same phenotype, either that of the parental cell or a more differentiated progenitor. An asymmetric division yields one daughter cell of the same phenotype as the original cell and a more differentiated progenitor cell type. Under physiological conditions, a balance exists between symmetric and asymmetric divisions to maintain both the stem cell pool and homeostasis within the hematopoietic system. A relative increase in symmetric divisions yielding two

progenitor cells will deplete the stem cell pool causing HSC exhaustion. Conversely increased symmetric divisions yielding two stem cells will expand the HSC population.

The gold standard assessment of hematopoietic stem cell functionality is the determination of the ability of these cells to recapitulate hematopoiesis when transplanted into irradiated recipients. Lethal irradiation of mice with a single 9 to 10 Gray dose will lead to hematopoietic failure and ultimately death of the mouse after approximately 3 weeks without bone marrow transplantation[52]. Transplantation experiments often employ bone marrow cells from knock-out mice or cells carrying floxed alleles of the gene of interest that can be deleted by inducing Cre recombinase expression with tamoxifen (ER-Cre)[53] or poly(dI-dC) (Mx1-Cre)[54]. Competitive bone marrow transplantation experiments involve transplantation of two populations of cells into one mouse. These populations can be identified based on the sex of the donor animal[55], retroviral marking of the populations[56], or by employing bone marrow cells from mice expressing different isoforms of the pan-hematopoietic marker CD45[57]. The latter methodology takes advantage of the ability to distinguish the source of the engrafted cells, as well as the recipient cells, by using flow cytometry analysis with antibodies for CD45.1 and CD45.2. The competitor cells allow for normalization of donor cell engraftment within each mouse and can also provide a source of rapidly reconstituting cells to overcome the time necessary for more immature stem cell populations to home and engraft within the bone marrow. Following transplantation, contribution of donor-derived cells to the peripheral blood is monitored monthly for 3 months in order to generate an accurate functional assessment of the most immature hematopoietic stem cells. Altered stem cell self-renewal, i.e. expansion or depletion of this population, following transplantation can be analyzed by direct measurement of hematopoietic stem cell numbers by flow cytometry. However given that the LKS immunopheotype becomes less reliable for hematopoietic stem cell identification after transplantation into irradiated recipients[58] direct assessment of functionality by transplantation into secondary recipients is necessary. Thus the ability to engraft in irradiated recipients is the most accurate depiction of hematopoietic stem cell

functionality, the cellular mechanism for which can be explained by *in vitro* proliferation and colony forming assays.

The proliferative ability of a stem cell enriched population can be assessed *in vitro* by culture in liquid medium with minimal cytokine support, typically with stem cell factor (SCF) and thrombopoietin (TPO)[53]. Although maintenance of stem cell functionality (hematopoietic reconstitution of irradiated hosts) is lost during culture, this assay can provide insights into the mechanism by which an observed *in vivo* engraftment defect occurs. Single cell liquid culture of highly enriched hematopoietic stem cells can also be employed to provide more direct evidence of stem cell phenotype following gene targeting. Owing to the ease by which single cells can be isolated by FACS this assay has largely replaced the long-term culture initiating cell (LTC-IC) assay which relied on the propagation of cobblestone areas on irradiated bone marrow stroma over the course of four to five weeks. The ability of hematopoietic progenitor cells, the direct progeny of hematopoietic stem cells, to differentiate can be elucidated by their ability to form myeloid lineage colonies in methylcellulose which is known as the colony forming cell (CFC) assay. The ability of these hematopoietic progenitors to proliferate can be further assessed by serial CFC assays[59]. An increase in the number of cells in liquid culture in conjunction with maintained colony-forming ability in serial CFC assays can represent stem cell expansion. Alternatively a proliferative defect and decreased number of colonies with serial CFC assays suggests a loss of self-renewal ability. Taken together these *in vitro* assays can provide a cellular mechanism for an observed engraftment phenotype following molecular targeting.

1.3 MOLECULAR REGULATION OF HEMATOPOIETIC STEM CELLS

1.3.1 Cell Cycle Regulation

The observation that hematopoietic stem cells largely reside within G_1/G_0 phases of the cell cycle and cycle with slow kinetics[60] suggests that progression through the cell cycle is an important mechanism regulating hematopoietic stem cell function. Movement through G_1 is governed by the phosphorylation of members of the retinoblastoma family of genes, including Rb, p107, and p130, by cyclin dependent kinases[61]. Loss of Rb alone has a limited cell intrinsic effect on hematopoietic stem cell function[62]; loss of all three retinoblastoma family members in mice, Rb, p107, and p130, leads to excessive proliferation within the hematopoietic stem cell compartment and eventually a myeloproliferative disease arising from hematopoietic progenitors[63]. Mice lacking D type cyclins lose hematopoietic stem cells during development which is associated with decreased hematopoietic progenitor proliferation during *in vitro* culture and increased percentage of cells in G1 in vivo [64]. Similarly cdk4/cdk6 double knockout mice die at late gestation with evidence of limited hematopoiesis owing to decreased proliferation of myeloid progenitors [65]. . This data indicates that proper regulation of the cell cycle in hematopoietic stem cells is necessary not only for their maintenance but also serves to limit possible malignant transformation.

The cyclin dependent kinases are in turn inhibited by two families of cyclin-dependent kinase inhibitors, the CIP-KIP family and the INK4 family. The former is composed of p21, p27, and p57, while the latter is composed of p16, p15, p18 and p19[66]. The CIP/KIP family of cyclin dependent kinase inhibitors block progression into S-phase promoted by cdk2/cyclin E or cyclin A. Mice deficient in p21 show increased cycling within the hematopoietic stem cell compartment and as a result increased incidence of hematopoietic failure following treatment with the S-phase toxin 5-fluorouracil[67]. Moreover serial transplantation of these cells into irradiated recipients, leads to exhaustion of their repopulating ability after 5 rounds of transplantation whereas wild-type cells

maintained fifty percent of their reconstituting ability[67]. In contrast to loss of p21, loss of p27 leads to expansion of hematopoietic progenitors without affecting hematopoietic stem cell number or self-renewal[68]. Owing to this progenitor expansion p27 knock-out cells out-compete wild-type cells in competitive bone marrow transplantation[68]. Thus despite their overlapping roles in regulating cdk2 these molecules have distinct roles in maintaining stem cell quiescence and restricting progenitor cell proliferation.

The INK4 family of cyclin dependent kinase inhibitors restrict the activity of cyclin dependent kinases 4 and 6 and downstream phosphorylation of the retinoblastoma family of proteins. p18 knock-out mice exhibit increased stem cell pools in the bone marrow as compared to wild-type mice and bone marrow from p18 knock-out mice can out-compete freshly isolated wild-type bone marrow in competitive transplantation models[55]. Fetal hematopoietic stem cells, which can outperform hematopoietic stem cells from aged mice, express lower levels of p18 which can compensate for limited hematopoietic stem cell function in the absence of p21[57]. Thus while both p21 and p18 can increase the cycling of hematopoietic stem cells the outcome of these divisions favors self-renewal in the absence of p18 as opposed to loss of the stem cell function in the absence of p21.

p16 has been shown to play a role in stem cell aging. Hematopoietic stem cells from aged mice express higher levels of p16 than young mice. Bone marrow cells from eight to twelve week old p16 knock-out mice are roughly equivalent to their wild-type counterparts in their ability to repopulate irradiated recipients. However bone marrow cells in mice older than fifty-six weeks that lack p16 can outcompete aged match controls most likely due to an increased number of cycling hematopoietic stem cells as measured by BrdU uptake as well as decreased levels of apoptosis[69]. A similar phenotype was observed in neural precursor cells lacking p16[70]. These studies serve to underscore the necessity of proper regulation of the cell cycle within the hematopoietic stem cell compartment to maintain a reserve of these cells over the lifetime of the individual. Moreover genes with overlapping functions at the molecular level may have distinct roles in the regulation of the functionality of hematopoietic stem cells.

1.3.2 Homeobox Proteins

The mammalian homeobox family is composed of 39 proteins sharing a common helix-turn-helix DNA binding motif clustered in 4 groups named A through D[71]. Of these groups the 3' end of homeobox clusters A and B have been shown to be uniquely over-expressed in both mouse[72] and human[73, 74] hematopoietic stem and progenitor cells. Expansion of hematopoietic stem cells with maintenance of their differentiation and hematopoietic reconstitution ability was achieved by retroviral transduction of HoxB4 into mouse[75] and human[76] bone marrow cells. This expansion was enhanced in a p21-null background[77], further validating p21's role in suppression of stem cell self-renewal. HoxB4 has proven to be unique in that overexpression of this molecule within the hematopoietic compartment can generate a cell intrinsic expansion of functional hematopoietic stem cells.

The HoxA cluster of proteins has been implicated in leukemia development, particularly in the setting of Nup98-HoxA9 translocation[78] or overexpression of multiple homeobox proteins in leukemias harboring an MLL translocation[79, 80]. Overexpression of HoxA9 in hematopoietic progenitor populations ultimately leads to acute myeloid leukemia with a latency of up to ten months[81]. Prior to leukemogenesis HoxA9 induces hematopoietic stem cell expansion and leukemogenesis was not observed up to 8 months following transplantation of lymphoid restricted cells over-expressing HoxA9[82]. Transduction with HoxA10 led to disruption of both myelo- and lymphopoiesis and induction of an acute myeloid leukemia in mice with a latency similar to that observed for hematopoietic progenitor cells transduced with HoxA9[83]. Similar to their role in tissue patterning in the developing embryo, hematopoiesis requires cell-type specific expression of the HoxA cluster in that overexpression of HoxA9 and HoxA10 can lead to physiologic expansion of hematopoietic stem cells but their overexpression in hematopoietic progenitors can lead to leukemogenesis.

The HoxA cluster is in turn regulated by the mixed lineage leukemia (MLL) gene, a histone methyltransferase, in hematopoietic stem cells. Embryonic mice heterozygous for MLL have hematopoietic and skeletal defects associated with caudally shifted expression of HoxA7 and HoxC9[84]. Conditional deletion of MLL from adult hematopoietic cells has been shown to lead to loss of self-renewal in irradiated recipients most likely due to inappropriate cell cycle entry within the hematopoietic stem cell compartment[85, 86]. However a role for MLL under homeostatic conditions remains controversial[85, 86]. These defects following MLL deletion occur in the setting of down-regulation of expression of the HoxA cluster within hematopoietic stem and progenitor cells[85] and MLL translocations lead to overexpression of HoxA genes[87]. The observation of the histone modifying enzyme MLL's regulation of HoxA cluster expression underscores the potential role for epigenetic regulation of gene expression within hematopoietic stem cells.

1.3.3 Developmental Regulators in Hematopoietic Stem Cells

A role for a number of other molecules in hematopoietic stem cell function has been elucidated by conditional deletion or overexpression within this population as summarized in Figure 1-3. In general genetic perturbations that increase the rate of hematopoietic stem cell cycling due so at the expense of an increased number of symmetric divisions yielding two progenitor, i.e. more differentiated, daughter cells, and subsequent stem cell exhaustion. Hematopoietic stem cells lacking the gene Growth factor independent 1 (Gfi-1) show increased rates of BrdU uptake *in vivo* and failure to sustain long-term hematopoietic reconstitution in irradiated recipients[88]. Loss of the tumor suppressor PTEN (Phosphatase and tensin homolog) leads to a myeloproliferative disease and rapid

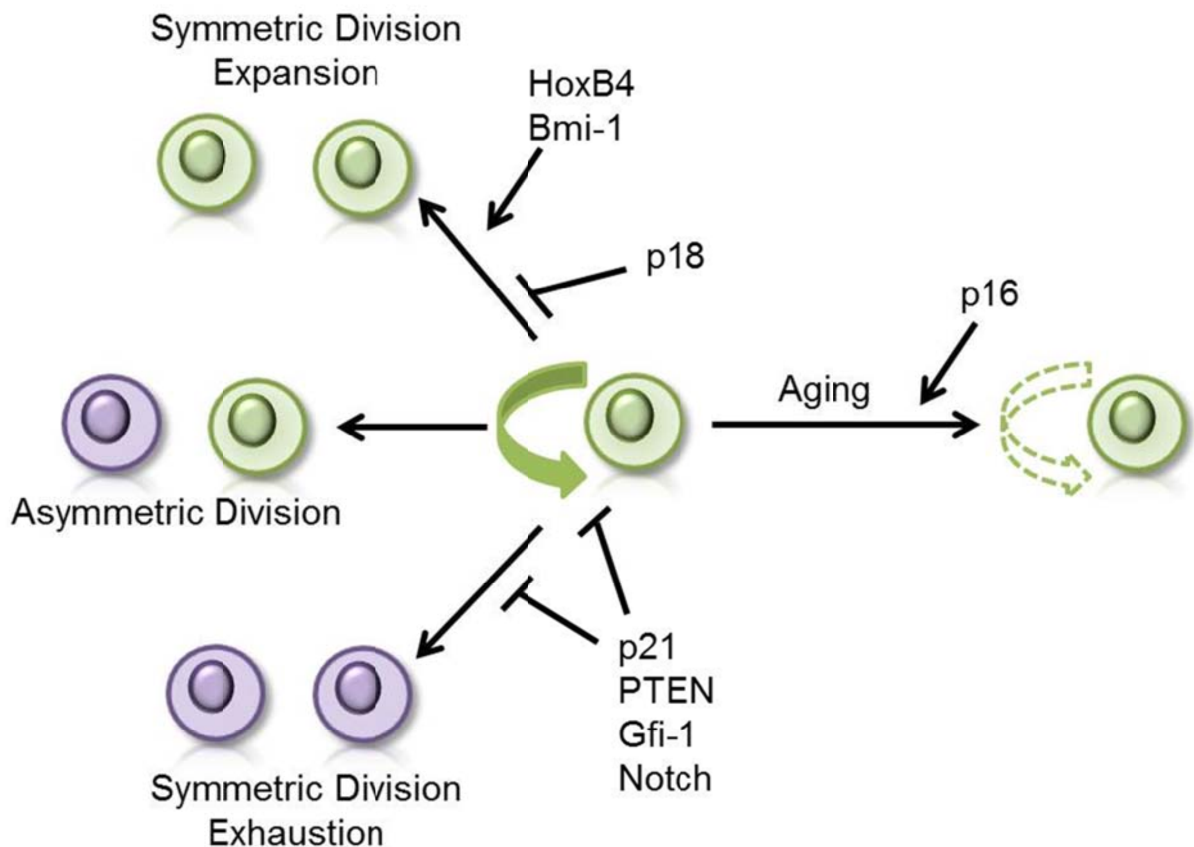


Figure 1-3. Hematopoietic Stem Cell Divisions and Fate Choice

Hematopoietic stem cell (green) function can be described in terms of the balance between self-renewal and differentiation during cell division. Molecules such as p21 that increase the proportion of cycling hematopoietic stem cells tend to lead to loss of stem cell function in i by a preponderance of exhaustive symmetric divisions yielding two more differentiated daughter cells (purple). Alternatively stem cell expansion is governed by molecules that tend not to increase hematopoietic stem cell cycling under homeostatic conditions. As stem cells age they lose functionality, indicated by the dashed arrow, a process which involves the cyclin dependent kinase inhibitor p16.

leukemia induction as well as increased hematopoietic stem cell cycling and loss of functionality[54, 89]. Wnt signaling, *via* β-catenin, can expand the hematopoietic stem cell pool likely through activation of both intracellular HoxB4 and expression of Notch at the cell surface[90]. Wnt signaling is particularly dependent on Notch to act as an inhibitor of differentiation indicating that an Wnt induced prolifeation would lead to stem cell exhaustion if not for its inhibition by Notch[91]. Hematopoietic stem cells lacking Bmi-1 only transiently contribute to hematopoiesis and have a decreased ability to self-renewal which can be compensated for by forced expression of p16 and p19[92]. Thus multiple unique genetic programs regulate hematopoietic stem cells, the coordination of which during hematopoiesis and maintenance of the stem cell pool, has not been fully addressed.

1.4 DNA METHYLATION AND THE DNA METHYLTRANSFERASES

1.4.1 The DNA Methyltransferase Family

The data summarized in Figure 1-3 indicates that precise regulation of gene expression levels, both within hematopoietic stem cells and their progeny, is necessary for their maintenance and function. Given that epigenetic regulation of gene expression by DNA methylation has been shown to play a role in the differentiation and self-renewal of a number of pluripotent cell types[2-4] investigation of DNA methylation in hematopoietic stem cells is warranted. DNA methylation of CpG dinucleotides in mammals is initiated and maintained by a family of DNA methyltransferases diagrammed in Figure 1-4. DNMT1 was the first cloned

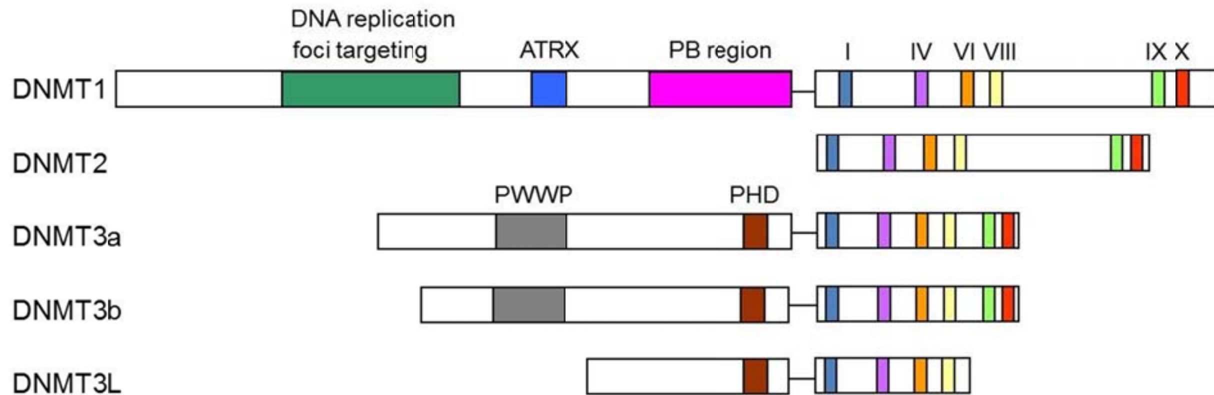


Figure 1-4. Mammalian DNA Methyltransferases

DNA methylation in mammals is carried out and maintained by the DNA methyltransferase family shown above. Each member of the family contains core, conserved catalytic motifs in the C-terminus (labeled I-X) and an N-terminus that plays a role in DNA binding and protein:protein interactions. The N-terminal domains indicated are discussed in the text below.

mammalian DNA methyltransferase based on its catalytic activity in murine erythroleukemia cells[93, 94]. DNMT1 is considered a maintenance methyltransferase in that it shows a preference for hemi-methylated DNA such as that found in the cell after DNA replication. DNMT3a and DNMT3b, cloned based on their sequence homology to bacterial methyltransferases[95], are considered *de novo* methyltransferases and have equal affinity for hemi-methylated and unmethylated target DNA sequences. Two other DNA methyltransferase family members, DNMT2 and DNMT3L, have also been identified based on their homology with bacterial methyltransferases. DNMT2 has been shown to play a role in tRNA processing[96] while DNMT3L lacks methyltransferase activity but can enhance the activity of DNMT3a and DNMT3b in certain cell populations[97].

All members of the DNA methyltransferase family have core catalytic motifs that are shared from bacteria through mammals[98]. DNA methylation proceeds through a covalent intermediate formed between a cysteine residue in the enzyme and the target cytosine base which is flipped out of the DNA backbone. This is followed by transfer of a methyl group to the C-5 position in cytosine from an adenosine methionine donor[99]. The N terminal domains of mammalian methyltransferases have not been fully characterized. Consistent with its role in methylating daughter strands during DNA replication, two regions in the N-terminus of DNMT1 have been shown to be important for localization to the replication fork: a DNA replication foci targeting domain and a polybromo homology domain[98]. The N-terminus of DNMT1 also contains a cysteine rich ATRX domain important for DNA binding[98]. The proline-tryptophan-tryptophan-proline (PWWP) motif in DNMT3a and DNMT3b has been crystallized and shown to enhance binding to pericentric heterochromatin[100, 101]. Disruption of this domain blocks the ability of both DNMT3a and DNMT3b to methylate major satellite repeats within pericentric heterochromatin although the PWWP domain of DNMT3b shows a greater binding affinity for nucleosomal DNA than that of DNMT3a[101]. The enhanced chromatin DNA binding of DNMT3b as compared to DNMT3a may also be due to an I662N substitution in the active site loop of mammalian DNMT3b which lies in close proximity to the phosphate backbone of the target DNA sequence[102]. Consistent with the enhanced binding of DNMT3b to heterochromatin DNT3b also shows increased methyltransferase activity on reconstituted nucelosomal DNA as compared to DNMT3a[103]. The pleckstrin homology domain (PHD in Figure 1-4) has been shown to recruit histone deacetylases and therefore can allow DNMT3a and DNMT3b to act as transcriptional repressors in the absence of their DNA methyltansferase activity[104]. These differences in molecular enzymology may explain the unique activities of DNMT3a and DNMT3b in multiple cell types as discussed below.

1.4.2 DNA Methylation and Regulation of Gene Expression

DNA methylation occurs at CpG dinucleotides which are present at only one-fifth of their expected frequency of one out of every sixteen dinucleotide pairs in the human genome [105]. Approximately one percent of all cytosines are methylated which corresponds with nearly seventy to eighty percent of all CpG dinucleotides[98, 106]. Methylation of a gene's CpG island, a clustering of CpG dinucleotides in the promoter and 5' region, is associated with transcriptional silencing *via* direct inhibition of transcription factor binding or recruitment of components of the chromatin modifying machinery[107]. However the majority of CpG islands are unmethylated, despite a high degree of methylation throughout the genome, even in genes that show a distinct tissue specific expression pattern and methylation of the CpG island is not a prerequisite for gene silencing[108]. Variations in DNA methylation have recently been proposed to occur most often at CpG shores, genome regions within 2.5 kilobases on either the 5' or 3' end of a CpG island. Comparing genome wide methylation differences between tissues or normal tissue and cancerous lesions from the same tissue[108], as well as embryonic and induced pluripotent stem cells[109], have shown that the majority of differentially methylated regions reside in these CpG shores. Moreover methylation within these CpG shore regions of genes is associated with gene expression rather than repression[108, 109].

How then does DNA methylation induce expression of a gene? A study of DNMT3a in neural precursor cells indicated an interplay between DNA methylation and histone modifications may generate a permissive chromatin state in the presence of DNA methylation. . Genes that were down regulated in DNMT3a knock-out cells generally were hypo-methylated in their respective CpG shores. This hypo-methylation was associated with increased H3K27 trimethylation, a repressive histone methylation mark. Methylation of the genomic DNA by DNMT3a and not the presence of DNMT3a itself, allowed for PcG2 dependent changes in histone methylation[110]. Genome wide microarray analysis of changes in methylation during hematopoietic development also demonstrates that a majority of lineage dependent changes in DNA methylation to occur in close proximity to, but

not overlapping, the identified CpG island of the gene[111]. This suggests a close interplay between DNA methylation and the histone code and that methylation carried out by the *de novo* methyltransferases may allow for gene specific regulation of expression.

1.4.3 *De Novo* Methylation in Development and Cancer

The roles of the DNA methyltransferases during development and tumorigenesis are summarized in Table 1-1. . DNMT3b is expressed early during embryogenesis in totipotent cells of the inner cell mass and epiblast while DNMT3a is expressed after E10.5[112]. This may correlate with the different phenotypes of the respective knock-out mice; DNMT3b knock-out mice die *in utero* around day E12.5 while DNMT3a knock-out mice die within four weeks after birth[9]. Loss of DNMT1 is also embryonic lethal and associated with global hypomethylation[113]. Loss of both DNMT3a and DNMT3b from embryonic stem cells leads to a loss of DNA methylation at both repetitive and distinct sites within the genome that can be restored by re-introduction of either

Table 1-1. Summary of DNA Methyltransferase Family Members in Development and Cancer

	Knockout Mouse Phenotype	ES cell phenotype	Cancer phenotype
DNMT1	Death at E10.5 Global Demethylation [113]	Microsatellite instability [114]	Mitotic defects in HCT116 cancer cells [115]
DNMT3a	Die four weeks after birth [9]	Loss of methylation with DNMT3b deletion [9]	Enhanced melanoma growth and metastasis [116]
DNMT3b	Death at E12.5 [9]	Loss of methylation with DNMT3a deletion [9]	Enhanced APC induced colon cancer growth [117]
DNMT3L	Hypogonadism, azoospermia in males [118] Heterozygous females infertile [119]	Loss of methylation accelerated in female ES cells [120]	Enhanced human embryonal carcinoma growth [121]

Phenotypes indicated for embryonic stem (ES) cells and cancer are those observed following deletion of the DNMT in the indicated cell or cancer type.

DNMT3a or DNMT3b, but not DNMT1[2]. This data suggests that in embryonic stem cells methylation of certain loci is maintained by DNMT3a and DNMT3b apart from the maintenance methyltransferase activity of DNMT1 and assigns relatively unique roles for the *de novo* methyltransferases in this population of stem cells.

Expression of DNMT3a and DNMT3b, but not DNMT1, was also shown to be necessary for the formation of teratomas following injection of embryonic stem cells into nude mice[2]. This data correlates with evidence that cancer cells can maintain hyper-methylation of the genome in the absence of DNMT1[122]. However depletion of DNMT3b but not DNMT3a from human cancer cells resulted in apoptosis and re-expression of hyper-methylated genes due to their demethylation[123]. Double knockdown of DNMT1 and DNMT3b abrogated almost all methyltransferase activity in human colon cancer cells allowing for enhanced de-methylation of hyper-methylated genomic sequences[124]. DNMT3b has also been shown to play a role in tumor initiation in an Adenomatous Polyposis Coli ($APC^{min/+}$) model of intestinal neoplasia. Inactivation of DNMT3b led to a decrease in macroadenoma formation[117] while overexpression of DNMT3b but not DNMT3a increased the number and size of adenomas in $APC^{min/+}$ mice[125]. Aberrant splicing of DNMT3b may also contribute to the hypermethylator phenotype in cancer cells[126, 127]. Taken together with the data from embryonic stem cells the changes in the expression of the *de novo* methyltransferases DNMT3a and DNMT3b can alter DNA methylation levels independent of DNMT1 and these two molecules have unique roles within different cell types.

1.4.4. ICF Syndrome

Somatic mutations in DNMT3b in humans are associated with an autosomal recessive syndrome characterized by immunodeficiency, centromeric region instability, and facial (ICF) abnormalities[128]. These mutations have been shown to decrease the methyltransferase activity of

DNMT3b by ten to fifty fold but not completely abrogate its activity[129] consistent with the embryonic lethality of DNMT3b knockout mice[9]. The centromeric region instability is generally thought to be caused by hypo-methylation of the pericentromeric regions of chromosomes 1, 9 and 16[130]. However only a small decrease in overall genomic DNA methylation levels have been observed[130]. Wide variability is seen in the range of symptoms perhaps due to differences in the residual level of methyltransferase activity as well as the fact the not all patients showing the characteristic features of ICF syndrome have demonstrable mutations in DNMT3b[131-133]. ICF syndrome can be corrected by bone marrow transplantation[134] indicating a cell intrinsic defect in hematopoietic cells in the absence of DNMT3b. ICF syndrome suggests a role for DNA methylation in hematopoiesis as well as a unique role for DNMT3b within this system.

1.4.5 DNA Methylation and Hematopoiesis

While the maintenance methyltransferase DNMT1 is expressed in all hematopoietic lineages[8] DNMT3a and DNMT3b have been shown to be uniquely expressed in hematopoietic stem cells of mice[8] while other studies have shown increased expression of DNMT3b alone in hematopoietic stem cells of mice[7] and humans[135]. Overexpression of DNMT1, DNMT3a, and DNMT3b has been observed in AML and CML cell lines and primary cells[135]. In addition demethylating agents, such as 5-aza-deoxycytidine, have proven to be effective in myelodysplatic syndromes and certain leukemias[136]. Taken together with the expression data of the DNA methyltransferase family, increased DNA methylation appears to be associated with leukemogenesis. Indeed we have shown T cell leukemogenesis in a p18-null background is commonly associated with methylation of the promoter of p15 and/or p16 [137]. These results underscore the potential role of DNA methylation and in particular the *de novo* methyltransferases, due to their unique expression in the most immature cells in the bone marrow, in regulating hematopoietic stem cell function.

A role for DNMT1 in hematopoiesis has not been clearly defined. Broske *et al* demonstrated that loss of DNMT1 by Cre mediated recombination led to bone marrow failure and death of the animals by three weeks[6]. Mice with one hypomorphic DNMT1 allele demonstrated an increased frequency of long-term hematopoietic stem cells and decreased frequency of highly proliferative progenitors in the bone marrow under homeostatic conditions[6]. When transplanted into irradiated recipients hematopoietic stem cells containing the hypomorphic DNMT1 allele failed to recapitulate hematopoiesis following serial transplantation indicating a self-renewal defect in hematopoietic stem cells in the absence of DNMT1[6]. Also while DNMT1 hypomorphic mice had normal myeloid cell frequencies in the bone marrow and blood, lymphoid progenitor and differentiated cells in these mice were nearly absent[6]. In contrast Trowbridge *et al* did not observe any changes in hematopoiesis under homeostatic conditions with DNMT1 deletion but did observe engraftment and self-renewal defects when these cells were transplanted into irradiated recipients[5]. Unlike Broske *et al* this engraftment defect was found to occur primarily because inadequate generation of the myeloid lineage[5]. While Broske *et al* did show changes in DNA methylation after loss of DNMT1 at two myeloid restricted genes neither work proposed changes in gene expression or associated DNA methylation to explain the observed self-renewal defect[5, 6]. A similar study with DNMT3a and DNMT3b failed to identify any molecular targets underlying hematopoietic stem cell dysfunction in the absence of both *de novo* methyltransferases[8]. An assessment of methylation changes near and within CpG islands has identified a number of genes, including DNMT3b, that may be regulated by DNA methylation during hematopoiesis[111], however the DNA methyltransferases responsible for these changes have not been addressed.

1.5 GOALS OF DISSERTATION RESEARCH AND SUMMARY OF FINDINGS

Hematopoietic stem cells reside in a specialized niche within the bone marrow of adults and can be isolated by flow cytometry. These cells are largely defined functionally by their ability to engraft in irradiated recipients. While a number of different genetic programs have been shown to regulate hematopoietic stem cell function, the coordination of expression of these programs during hematopoietic development has yet to be fully elucidated. DNA methylation is an epigenetic means of regulation of gene expression that is maintained and carried out by a family of DNA methyltransferases. A role for DNA methylation in the hematopoietic system has been suggested by studies of DNMT1 within these cells and observations of the changes in DNA methylation during hematopoietic development. Of the DNA methyltransferse family the de novo methyltransferases DNMT3a and DNMT3b appear to play roles in other stem cell types as well as in cancer intiation and maintenance. In particular, DNMT3b is uniquely expressed with hematopoietic stem cells and inherited mutations in DNMT3b lead to a syndrome characterized by immunodeficiency. Therefore this study proposes to elucidate the role of DNMT3b in hematopoietic stem cells.

The findings presented in Chapter 2 confirm the overexpression of DNMT3b in hematopoietic stem cells of mice. Targeted knockdown of DNMT3b by short, hairpin RNA limits hematopoietic engraftment of bone marrow cells in both the blood and bone marrow of recipient mice and is associated with a decreased proliferative capacity in vitro. Specific recombination of floxed DNMT3b alleles indicates a cell intrinsic effect of DNMT3b on hematopoietic stem cell self-renewal and enhanced apoptosis in the absence of DNMT3b in vivo. This effect was stem cell specific, as single cell culture or transplantation of hematopoietic stem cells, demonstrated a defect in proliferation or engraftment, respectively. Moreover the function of DNMT3b in hematopoietic cells requires its methyltransferase activity and loss of DNMT3b is associated with down-regulation of MLL expression

in stem cell enriched bone marrow cells concurrent with demethylation of this locus. Taken together this data indicates a cell intrinsic role for DNMT3b in regulating hematopoietic stem cell function via maintenance of MLL expression.

All of the experiments in this thesis were performed by Matthew Boyer, with the assistance of Hui Yu and Richard XuFeng.

2.0 DNMT3B'S ROLE IN HEMATOPOIETIC STEM CELLS

2.1 INTRODUCTION

Hematopoiesis proceeds from a bone marrow resident population of hematopoietic stem cells which must balance self-renewal versus differentiation in order to maintain a stem cell pool that can produce all lineages within the blood over the lifetime of an organism. Distinct molecular programs in hematopoietic stem cells are the key to maintenance of hematopoiesis under homeostatic conditions and their dysregulation can lead to a number of pathological states, including leukemia. However the mechanism by which these molecular programs are regulated remain largely undefined.

Epigenetic regulation of gene expression by DNA methylation has been shown to play a role in a number of pluripotent cell types[3, 9], including hematopoietic stem cells[111]. DNA methylation is initiated and maintained in mammals by a family of DNA methyltransferases[98]. Of these the *de novo* mehyltransferase DNMT3b is uniquely overexpressed in murine hematopoietic stem cells as compared to DNMT3a[138]. Aging murine hematopoietic stem cells exhibit epigenetic dysregulation including down-regulation of DNMT3b which correlates with a decrease in functionality[139]. Bone marrow transplantation in humans was shown to correct the immunodeficiency in a small cohort of patients with mutations in the DNMT3b gene[140], suggesting a hematopoietic stem cell intrinsic defect in these patients. Using hematopoietic stem cells from mice with floxed DNMT3a and/or DNMT3b, it was recently shown that loss of both methyltransferases blocked engraftment in irradiated recipients[8]. However the cellular and molecular mechanisms underlying this defect were not vigorously addressed[8]. Taken together, this data strongly indicates a role for DNMT3b in regulating hematopoietic stem cell function.

By knock-down of DNMT3b with a retrovirally delivered shRNA or Cre mediated recombination of floxed DNMT3b alleles work presented in this thesis demonstrates a critical role for DNMT3b in hematopoiesis in mice. Loss of DNMT3b leads to limited reconstitution of hematopoiesis in irradiated recipients associated with a proliferative defect in vitro and a failure of hematopoietic stem cell self-renewal in vivo. Targeted deletion of DNMT3b in hematopoietic stem cells leads to decreased engraftment following transplantation and decreased proliferation in vitro. DNMT3b function in hematopoietic cells requires the methyltransferase activity of the enzyme and the defects in hematopoiesis are associated with loss of DNA methylation and decreased expression of MLL. Therefore DNMT3b is necessary for maintenance of the proliferative ability and engraftment capacity of hematopoietic cells and hematopoiesis is dependent upon appropriate DNA methylation.

2.2 MATERIALS AND METHODS

2.2.1 Mice

DNMT3b$^{lox/lox}$ mice in a C57BL/6 background were a kind gift of En Li. Wild-type C57Bl/6 and B6.Cg-Tg(CAG-cre/Esr1)5Amc/J (ER-Cre) mice were purchased from the Jackson Laboratory. ER-Cre mice were crossed to our DNMT3b$^{lox/lox}$ mice which were bred in-house. Excision of floxed DNMT3b alleles in DNMT3b$^{lox/lox}$ ER-Cre mice was accomplished by oral administration of 5 mg tamoxifen (Sigma) in 0.1 mL of peanut oil daily for 5 days. In vitro excision of floxed DNMT3b alleles was accomplished by treatement with 1 μM 4-hydroxytamoxifen. Mice were genotyped by PCR primers flanking the either loxP insertion site. All mice were handled in accordance to institutional guidelines for animal care.

2.2.2 Retroviral Vectors

A U6 promoter construct was cloned from the lentiviral vector pLL3.7, a kind gift from Tyler Jacks, into MSCV-pgk-GFP, a kind gift from Guy Sauvageau, to generate MSCV-U6. A 19 bp shRNA with an 8 bp loop targeting DNMT3b was cloned into the HpaI and XhoI sites of MSCV-U6. Replication incompetent retroviral particles were generated by transient transfection of 5 micrograms of MSCV-shRNA or MSCV-U6 along with 2.5 micrograms of the packaging plasmid pKat and 2.5 micrograms of the envelope plasmid pVSV-g into 293T cells with Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. Forty-eight hours after transfection retrovirus containing supernatants were collected, spun at 1300 rpm for 10 minutes, passed through a 0.45 micron filter and used for transduction.

2.2.3 Retroviral Transduction

Lineage$^-$ or c-kit$^+$ bone marrow cells from 8-12 week old wild-type C57Bl/6 or DNMT3b$^{lox/lox}$ ER-Cre mice were flushed from the long bones and enriched with the MACS Streptavidin or MACS CD117 Enrichment Kit and Separation columns (Miltenyi Biotec) per the manufacturer's instructions. The cells were pre-stimulated overnight with 50 ng/ml SCF, 10 ng/ml TPO and 10 ng/ml Flt-3L (all from Peprotech) in X-Vivo-15 (Lonza) at 37 ^oC, 5% CO_2. 1 to 5 x 10^5 cells were plated per well in a 24 well plate pre-coated with retronectin (Takara) for four hours and spun at 1700 rpm for 30 minutes with 80% retroviral supernatant in X-Vivo 15 with cytokines. Following overnight culture at 32 ^oC, 5% Co2 the cells were harvested and cultured in fresh media for 48 hours prior to cell sorting or use *in vitro* or *in vivo* assays.

2.2.4 Bone Marrow Cell and Hematopoietic Stem Cell Transplantation.

Sixteen hours before transplantation, 6 to 8-week-old recipient C57Bl/6 mice were lethally irradiated to 9.5 Gy at 82 cGy/min on a ^{137}Cs radiator. The indicated cell populations in each experiment were sorted and injected into the tail veins of recipients. Recipient mice were supplied with sterile food and acidified water. Multilineage engraftment in peripheral blood was monitored monthly for 3 months by flow cytometry and bone marrow engraftment measured after sacrificing the animal at 3 months. Secondary transplantation was performed by collecting donor cells from primary recipients and injecting, *via* the tail vein, into irradiated recipients.

2.2.5 Flow Cytometry

Cultured and freshly harvested bone marrow cells were stained with a Lineage cocktail containing equal amounts of anti-CD3, anti-CD4, anti-CD8, anti-CD11b, anti-B220, anti-Ter119, and anti-Gr-1 conjugated to PE-Cy7, anti-ckit conjugated to PE or APC, anti-Sca-1 PE-Cy5.5, anti-CD34 FITC, anti-FcRγ Pacific Blue, and anti Il-7R APC as appropriate and sorted by MoFlo or analyzed on a Cyan ADP flow cytometer.

2.2.6 Liquid Culture

1,000 GFP^{+} cells were sorted forty eight hours after culture into four wells of a 96-well plate per group containing X-Vivo 15 plus 50 ng/ml SCF, 10 ng/ml TPO and 10 ng/ml Flt-3L. Cells were cultured for 14 days during which time aliquots of each well were taken and counted.

2.2.7 Colony Forming Cell (CFC) Assay

GFP^+ cells were sorted forty-eight hours after transduction, resuspended in semisolid methylcellulose medium M3434 (StemCell Technologies) and plated in triplicate in 24-well plates at 1,000 cells/well. The cells were cultured at 37 °C, 5% CO_2 and colonies counted after 7–11 days in culture. For the secondary CFC assay primary colonies were harvested by triplicate washes with 1X PBS, spun down, and counted. 5,000 cells were then re-plated in fresh M3434 media and cultured as above for another 7-11 days and the colonies counted.

2.2.8 Quantitative Real-Time RT-PCR

Five thousand hematopoietic cells from 6- to 8-week-old C57Bl/6 mice were directly sorted into cell lysis buffer and the RNA was extracted using Absolute RNA extraction kits (STRATAGENE) according to the manufacturer's instructions. cDNA was synthesized using SuperScript II (Invitrogen) according to the manufacturer's instructions and real-time RT-PCR was performed using DynamoSYBR green qPCR kit (Finnzymes) on a PTC-200 thermo cycler (MJ Research) according to the manufacturer's instructions.

2.2.9 Annexin V Staining

1×10^5 to 5×10^6 bone marrow cells were first stained with antibodies for hematopoietic stem cells as described in 2.2.5 and then stained with Annexin V PE and 7-AAD using the Annexin V:PE Apoptosis Detection Kit (BD Pharmingen) per the manufacturer's instructions. Apoptotic cells that are either Annexin V positive or Annexin V/7-AAD double positive were analyzed on a Cyan ADP flow cytometer.

2.2.10 BrdU Staining

Mice were given an intraperitoneal injection of 0.5 mg of BrdU in PBS sixteen hours prior to sacrificing and collection of the bone marrow. Cells were first stained with antibodies for hematopoietic stem cells as described in 2.2.5 and then fixed, permeabilized and stained with anti-BrdU FITC using the BrdU Flow kit from BD Pharmingen according to the manufacturer's instructions and analyzed on a Cyan ADP flow cytometer.

2.2.11 Bisulfite Sequencing

The DNA from $1x10^6$ whole bone marrow cells was collected by the DNEasy Extraction Kit (Qiagen) per the manufacturer's instructions and re-suspended in 200 microliters of TE. Forty microliters was subsequently bisulfite treated using the EpiTect Bisulfite Kit (Qiagen) per the manufacturer's instructions. One microliter of the bisulfite treated DNA was then amplified by nested PCR, the product isolated by gel electrophoresis, and collected by Wizard SV Gel and PCR Purification Kit (Promega) per the manufacturer's instructions. The amplified fragment was then cloned with the pGEM-T Easy Vector System (Promega) per the manufacturer's instructions and sequenced.

2.3 RESULTS

2.3.1 DNMT3b is Uniquely Expressed in a Highly Enriched Hematopoietic Stem Cell Population

In order to confirm previous studies showing up-regulation of DNMT3b in hematopoietic stem cells[7, 141] stem, progenitor and mature cell populations were isolated from the bone marrow of C57Bl/6 mice by flow cytometry and the relative expression of DNMT1, DNMT3a, and DNMT3b measured in each by quantitative real-time RT-PCR. In long-term hematopoietic stem cells ($CD34^{-}LKS$) the expression of DNMT3b mRNA is 25.1 and 12.8 times higher than that of DNMT1 and DNMT3a, respectively, and approximately 4.5-fold higher than the expression of DNMT3b mRNA in any progenitor or mature hematopoietic population (Figure 2-1). This suggests a specific role for DNMT3b in hematopoietic stem cells as compared to more differentiated cell types as well as a specific function of DNMT3b as compared to other members of the DNA methyltransferase family within these cells.

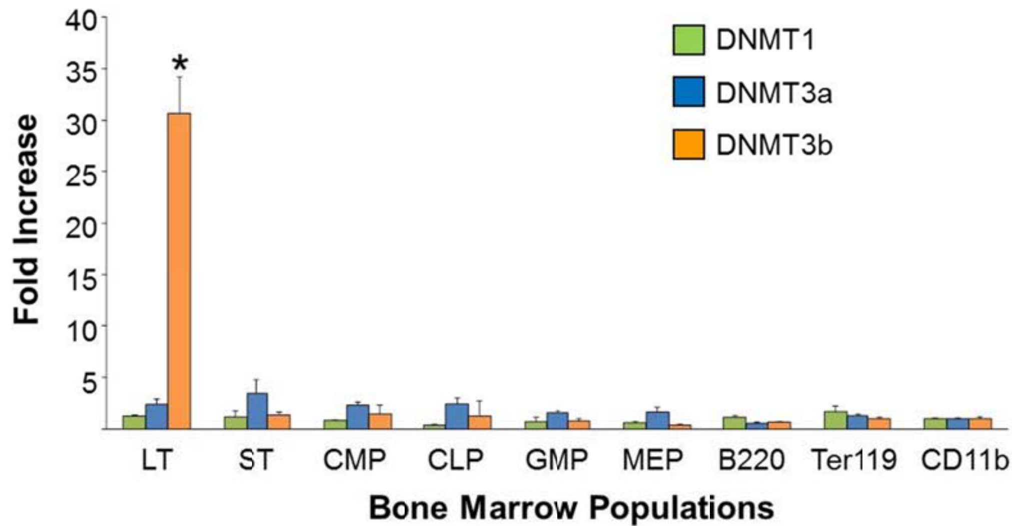


Figure 2-1. DNMT3b is Highly Expressed in Hematopoietic Stem Cells

The relative expression level of DNMT1, DNMT3a, and DNMT3b as determined by quantitative real-time RT-PCR. The expression level of each methyltransferase was first normalized to the expression of β-actin within that population and then to the level of expression in $CD11b^{+}$ cells. LT, $CD34^{-}$LKS; ST, $CD34^{+}$LKS; CMP, $LKS^{-}CD34^{lo}FcR\gamma^{lo}$; CLP, $LKS^{-}Il\text{-}7R^{+}$; GMP, $LKS^{-}CD34^{hi}FcR\gamma^{hi}$; MEP, $LKS^{-}CD34^{hi}FcR\gamma^{lo}$; B220, $B220^{+}$ cells; Ter119, $Ter119^{+}$ cells; CD11b, $CD11b^{+}$ cells. DNMT3b is significantly over-expressed in long term hematopoietic stem cells as compared to any other cell type tested and to the other members of the DNA methyltransferase family, DNMT1 and DNMT3a (* = $p < 0.05$).

2.3.2 DNMT3b Knockdown Limits Hematopoietic Reconstitution in Competitive Bone Marrow Transplantation

In order to assess the role of DNMT3b in hematopoietic stem cells a murine stem cell virus (MSCV) harboring a short, hairpin RNA (shRNA) under the control of a U6 promoter with GFP as a marker of transduced cells was generated (Figure 2-2a). Transduction of stem cell enriched Lineage$^-$ (Lin$^-$) bone marrow cells with the DNMT3b shRNA produced an approximately 93% reduction in DNMT3b expression at the RNA level by quantitative real-time, RT-PCR (Figure 2-2b) and significant loss of DNMT3b protein expression by Western blot when DNMT3b shRNA was transduced into the murine hematopoietic progenitor cell line 32D (Figure 2-2c). Although a significant decrease in DNMT3a was observed at the RNA level (Figure 2-2b) a similar decrease was not observed at the protein level (Figure 2-2c) suggesting the relative specificity of the transduced shRNA for DNMT3b.

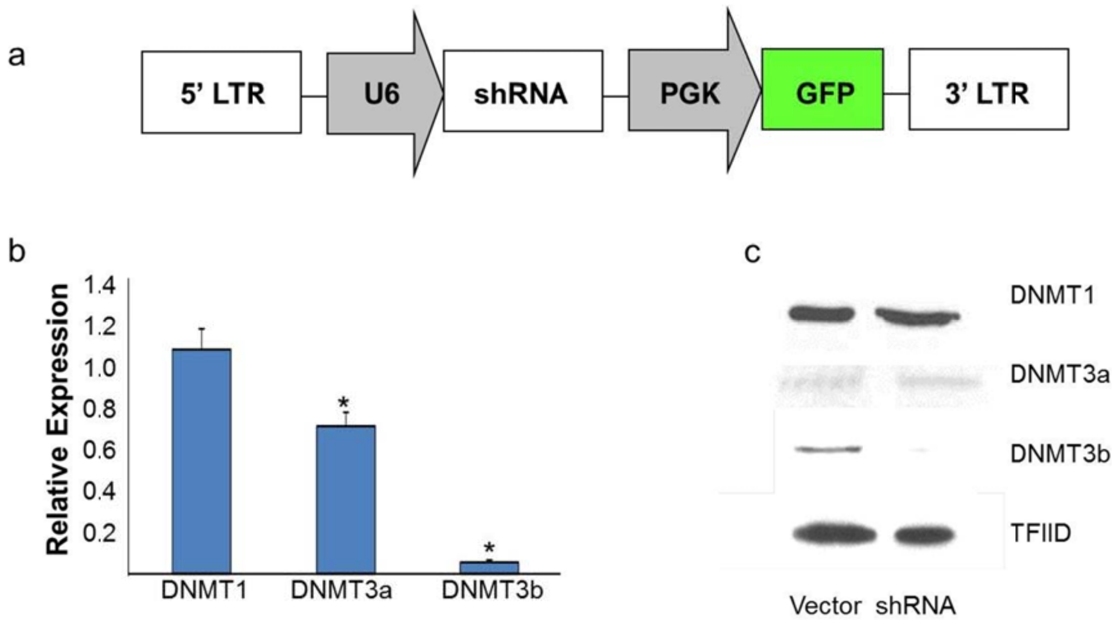


Figure 2-2. Knockdown of DNMT3b Expression by a Retrovirally Delivered shRNA

(a) Diagram of the MSCV vector harboring a shRNA targeting DNMT3b. LTR, 5' or 3' long terminal repeats; U6, U6 promoter; PGK, human phosphoglycerate kinase promoter; GFP, green fluorescent protein. (b) Expression of DNMT1, DNMT3a, and DNMT3b in Lineage^{-} cells transduced with shRNA as compared to controls. Expression levels were first normalized to β-actin and then the expression within the vector transduced group. Both DNMT3a and DNMT3b show significant decreases in expression in the shRNA transduced group however DNMT3b is more significantly reduced (* = $p < 0.05$). (c) Western blotting for protein expression of DNMT1, DNMT3a, and DNMT3b in nuclear extracts from the hematopoietic progenitor cell line 32D stably expressing GFP alone (Vector) or DNMT3b shRNA (shRNA). TFIID (Tata binding protein) was used as a protein loading control.

To assess hematopoietic function following knockdown of DNMT3b, $1x10^5$ retrovirally transduced $Lineage^-$ bone marrow cells sorted for GFP expression were transplanted along with $1x10^5$ freshly isolated whole bone marrow cells as competitors into 9.5 Gray irradiated recipients as outlined in Figure 2-3a. Bone marrow cells transduced with DNMT3b shRNA showed significantly limited engraftment ability in the peripheral blood of irradiated recipients at two (42.0 vs. 7.1) and three (61.4 vs. 2.83) months post-transplantation as compared to vector transduced cells (Figure 2-3b). When compared to the engraftment in mice receiving vector transduced cells at one month post-transplantation shRNA transduced bone marrow cells had a 3.2-times increased contribution to the myeloid (CD11b+) compartment which decreased to 0.07-times by three months post-transplantation (Figure 2-3c). shRNA and vector transduced cells showed approximately equivalent engraftment within CD3+ T cells and an increase in B220+ cells over the course of the transplantation (Figure 2-3c) indicating the overall defect in engraftment is largely due to limited long-term myelopoiesis from cells in which DNMT3b has been knocked down.

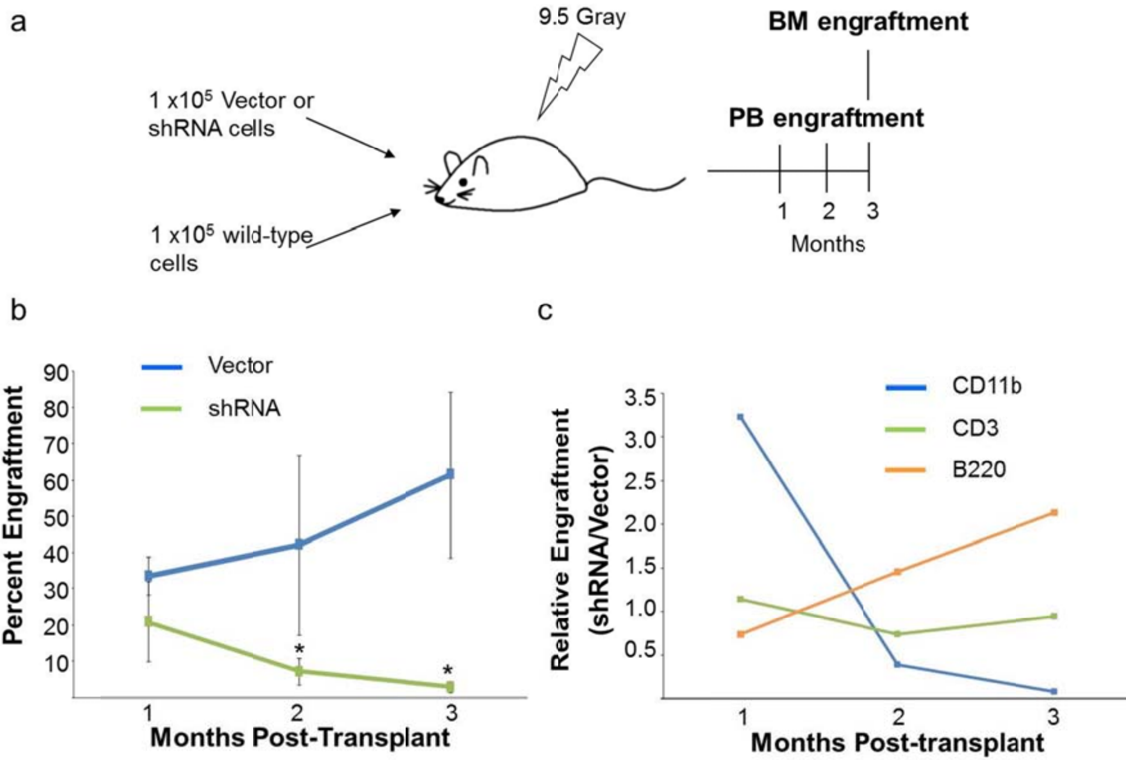
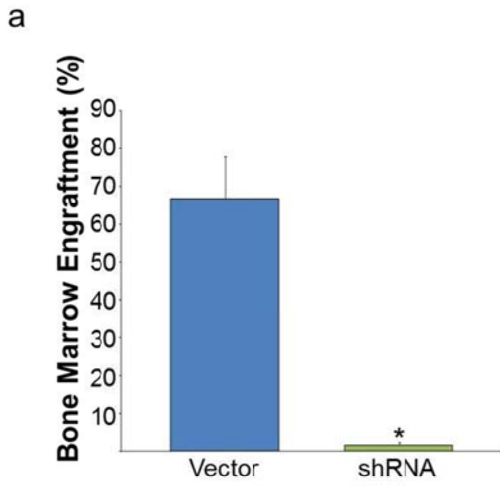


Figure 2-3. Limited Peripheral Blood Engraftment of DNMT3b shRNA Transduced Bone Marrow Cells

(a) Lineage depleted bone marrow cells were enriched and transduced with Vector or DNMT3b shRNA retroviruses and 1×10^5 GFP+ cells were sorted by flow cytometry and transplanted into 9.5 Gray irradiated mice along with 1×10^5 freshly isolated competitor bone marrow cells. (b) Engraftment in the peripheral blood of mice transplanted with vector transduced or DNMT3b shRNA transduced bone marrow cells as a percentage of total engraftment of donor plus competitor cells. There is a significant decrease in engraftment at 2 and 3 months post-transplantation (n=5 mice per group, * = $p < 0.05$). (c) Relative engraftment in the myeloid (CD11b) B cell (B220), and T cell lineages (CD3) of shRNA transduced to vector transduced cells. Peripheral blood cells were first gated on expression of donor-derived CD45.1 then expression of each lineage marker.

Three months after transplantation engraftment within the bone marrow of the recipient mice was determined by flow cytometry. shRNA transduced bone marrow cells contributed to only 1.5% of the transplanted cells in the bone marrow as compared to vector transduced cells which constituted 66.7% of transplanted cells (Figure 2-4a). While there was a similar percent of engraftment within the stem and progenitor enriched Lineage^{-} and LKS populations a significant decrease in engraftment within the LKS^{-} progenitor population was seen as a percentage of total engraftment (0.6 vs 0.03%, Figure 2-4b). However there was a significant decrease in the absolute number of all three stem and progenitor populations assessed (Figure 2-4c). Taken together with the peripheral blood engraftment the absolute lack of stem and progenitor cells is suggestive of a proliferative defect in hematopoietic stem cells in the absence of DNMT3b. Moreover given that the myeloid compartment is the most accurate depiction of the hematopoietic stem cell activity in the peripheral blood and the lack of engraftment within this compartment following DNMT3b knock-down as compared to vector control cells, DNMT3b may have a direct effect on hematopoietic stem cells themselves.

a



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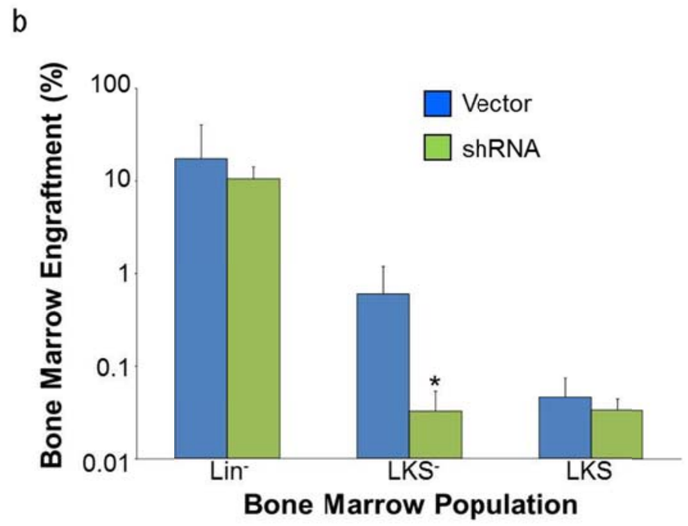

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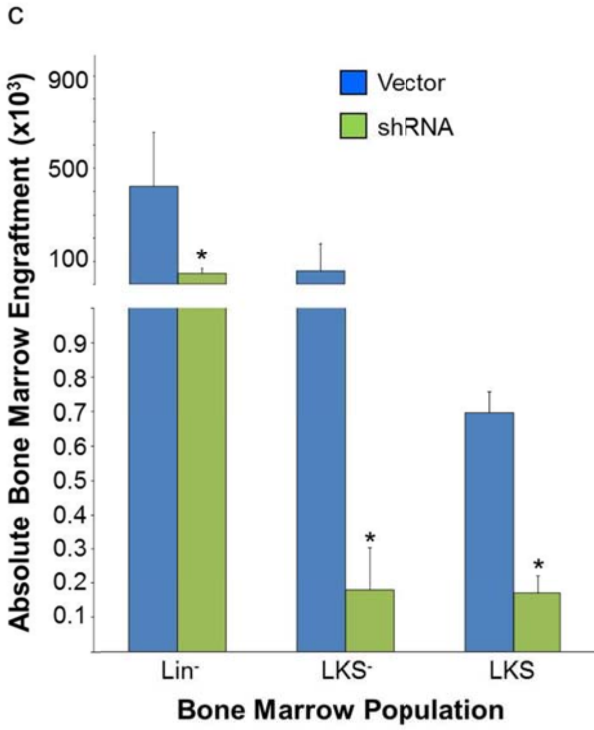


Figure 2-4. Limited Bone Marrow Engraftment of DNMT3b shRNA Transduced Bone Marrow Cells

(a) Total bone marrow engraftment as a percentage of competitor and donor engraftment of recipient mice (n=5 mice per group, * = $p < 0.05$). (b) Percent engraftment within the donor derived cells in the bone marrow of recipient mice. Bone marrow cells were first gated on CD45.1 expression and then the percentage of cells within the indicated populations was determined by flow cytometry. Lin^-, Lineage $^-$; LKS^- $Lineage^-ckit^+Sca\text{-}1^-$; LKS, $Lineage^-c\text{-}kit^+Sca\text{-}1^+$. A significant decrease in the LKS- progenitor population is seen (n=5 mice per group, * = $p < 0.05$) (c) Absolute engraftment was calculated per femur by counting the recovered cells and then multiplying by the percent engraftment in (a) and the percent engraftment in the indicated lineages as described in (b). All three progenitor and stem cell enriched populations showed significant decreases in shRNA transduced cell recipient mice (n=5 mice per group, * = $p < 0.05$).

2.3.3 DNMT3b Knockdown Limits Hematopoietic Progenitor Proliferation *In Vitro*

To address the cellular mechanism underlying the observed engraftment defect *in vivo* the stem cell enriched Lineage$^-$ population of bone marrow cells was cultured *in vitro* with limited cytokine support. Over the course of the two weeks in culture vector transduced cells increased almost 6-fold whereas the DNMT3b shRNA transduced cells failed to proliferate (Figure 2-5a, b). This failure to proliferate was associated with loss of the more immature Lineage$^-$Sca-1$^+$ population (Figure 2-6a) and an increase in apoptotic cells within this population as determined by Annexin V staining (Figure 2-6b) after fourteen days of culture. This data indicates the observed engraftment defect *in vivo* was likely a result of decreased proliferation concomitant with increased apoptosis in shRNA transduced hematopoietic cells.

When plated in methylcellulose media immediately after sorting for GFP expression, DNMT3b shRNA transduced Lineage$^-$ bone marrow cells generated more colonies (Figure 2-7a) similar to the relatively increased myeloid engraftment at one month following transplantation (Figure 2-3c). When five thousand of these cells were re-plated in fresh media, however, DNMT3b shRNA transduced cells lost almost all of their colony-forming ability (Figure 2-7b). This serial colony-forming ability has been employed as an indicator of the function of highly proliferative progenitors[142] again suggesting a proliferative defect in hematopoietic cells in the absence of DNMT3b.

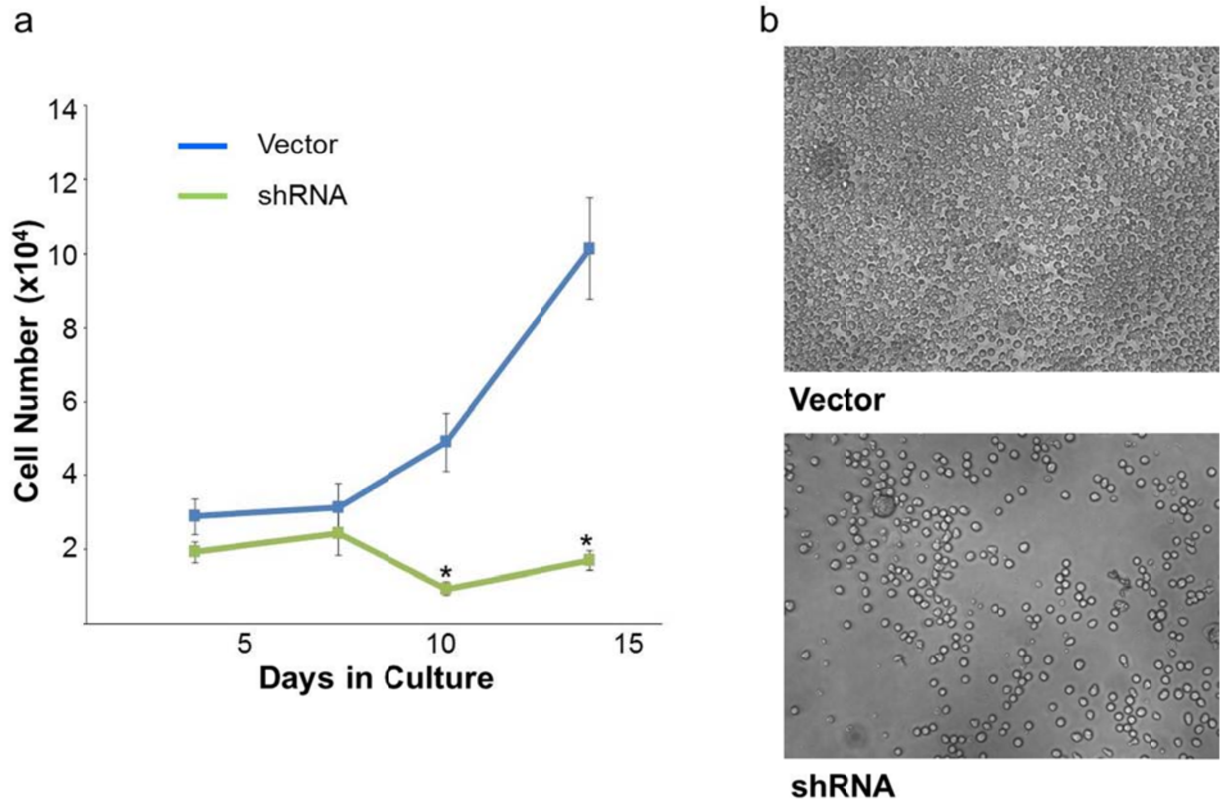


Figure 2-5. Hematopoietic Stem Cell Enriched Bone Marrow Cells Fail to Proliferate During *In Vitro* Culture

(a) Growth of vector or DMT3b shRNA Lineage$^-$ bone marrow cells in liquid culture over two weeks. Data shown is the average of four individual wells (* = $p < 0.05$). (b) Images of a representative well after two weeks of culture.

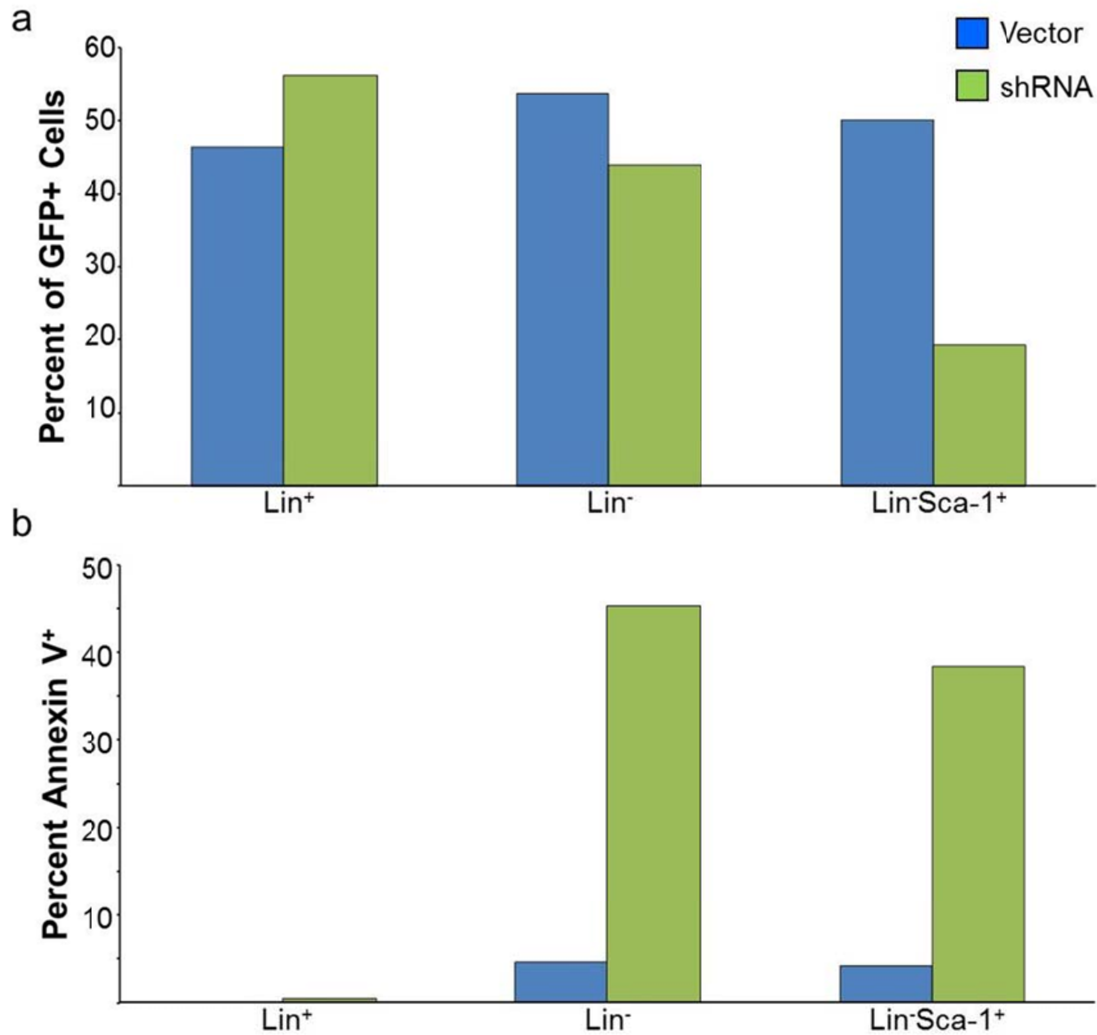


Figure 2-6. Increased Apoptosis Within the Lineage$^-$Sca-1$^+$ Population of Cells During *In Vitro* Culture

(a) Analysis of the immunophenotype of cells counted in Figure 2-5 after two weeks of culture. There is a decrease in the more immature population of Lin$^-$Sca-1$^+$ cells. (b) Annexin V of hematopoietic stem and progenitor populations after 2 weeks of culture. Concurrent with a loss of the more immature Lin$^-$Sca-1$^+$ cells a dramatic increase in apoptosis within these cells was observed.

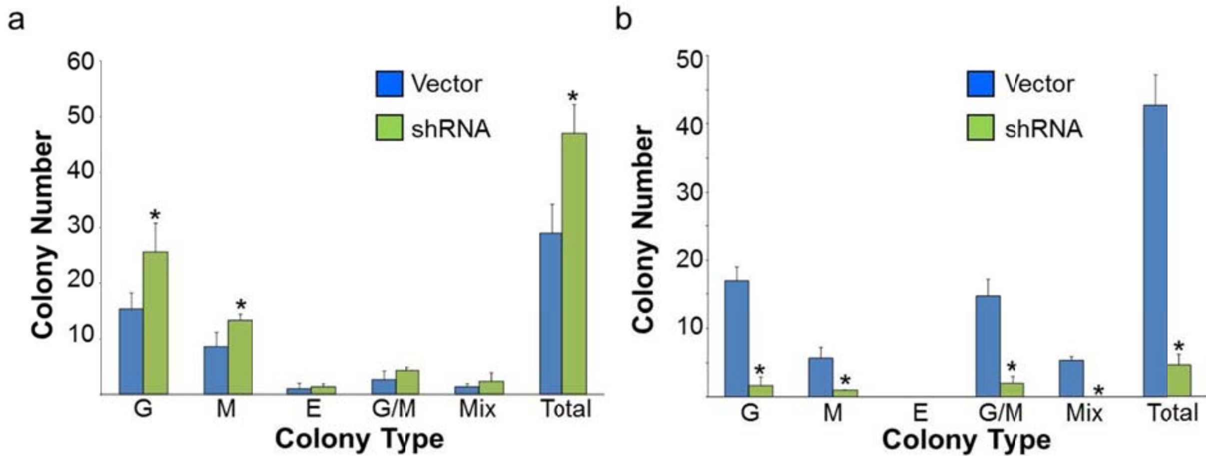


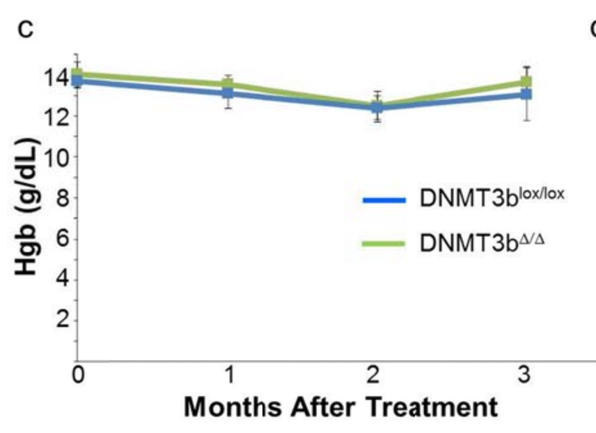
Figure 2-7. Limited Hematopoietic Progenitor Proliferation Following DNMT3b Knockdown

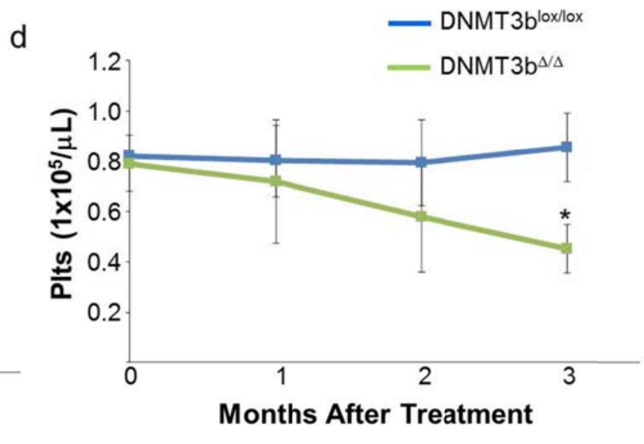
(a) The colony forming ability of cells transduced with DNMT3b shRNA after 10 days of culture in methylcellulose media. G, granulocyte; M, monocyte; E, erythrocyte; GM, granulocyte and monocyte; Mix, granulocyte, monocyte, and erythrocyte. DNMT3b shRNA transduced cells generated more total colonies due to an increased number of granulocytic and monocytic colonies (Average of four wells, * = $p < 0.05$). (b) Secondary colony formation from the cells cultured in (a). Almost all the colony forming ability of DNMT3b shRNA transduced cells was lost during serial culture (Average of four wells, * = $p < 0.05$).

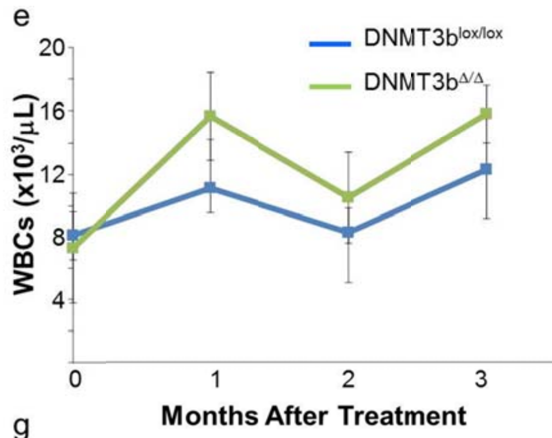
2.3.4 Specific DNMT3b Deletion Reduces Hematopoietic Cell Numbers Under Homeostatic Conditions

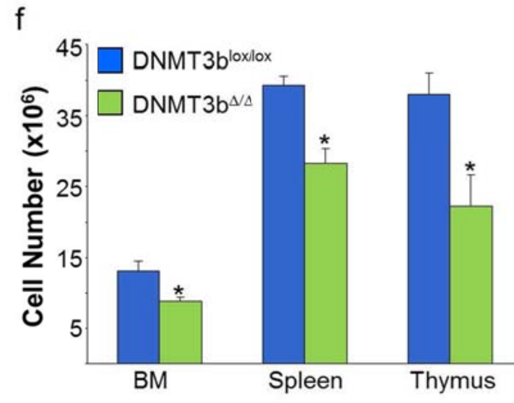
To control for any potential off-target effects of the DNMT3b shRNA employed above, mice harboring floxed DNMT3b alleles along with Cre under the control of an estrogen responsive promoter were bred. Treatment of these mice with tamoxifen allows for efficient deletion of DNMT3b (Figure 2-8a). To test the role of DNMT3b under homeostatic conditions DNMT3b$^{lox/lox}$ ER-Cre^{+} mice were treated with tamoxifen (DNMT3b$^{\Delta/\Delta}$) and blood counts determined for three months (Figure 2-8b). Tamoxifen treated DNMT3b$^{lox/lox}$ ER-Cre^{-} (DNMT3b$^{lox/lox}$) mice were used as controls. Conditional deletion of DNMT3b did not affect the hemoglobin concentration in the blood of DNMT3b$^{\Delta/\Delta}$ mice as compared to controls (Figure 2-8c) but did significantly decrease the platelet concentration (Figure 2-8d) at 3 months after tamoxifen treatment. Loss of DNMT3b also lead to an increased number of white blood cells in the blood, although not significantly (Figure 2-8e). Three months after tamoxifen treatment the mice were sacrificed and the bone marrow, spleen and thymus analyzed. There was a significant decrease in the cellularity of the bone marrow, spleen and thymus of DNMT3b$^{\Delta/\Delta}$ mice as compared to controls (Figure 2-8f). Similar to the transplant experiments with cells transduced with DNMT3b shRNA, although there was a similar percentage of progenitor and stem populations in the bone marrow (Figure 2-8g), the absolute number of both populations was significantly decreased (Figure 2-8h) in DNMT3b$^{\Delta/\Delta}$ mice as compared to controls.

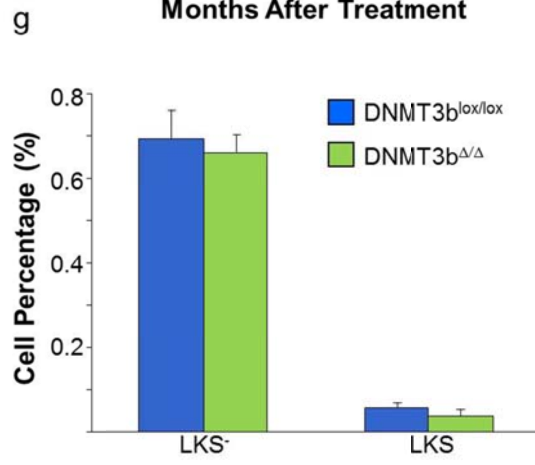
a
lox
Δ
Cre-
Cre+
b
BM cellularity
PB counts
DNMT3blox/lox ER-Cre
0
1
2
3
Months
tam


c
Hgb (g/dL)
DNMT3blox/lox
DNMT3bΔ/Δ
Months After Treatment


d
DNMT3blox/lox
DNMT3bΔ/Δ
Plts (1x10⁵/μL)
*
Months After Treatment


e
DNMT3b$^{lox/lox}$
DNMT3b$^{\Delta/\Delta}$
20
16
12
8
4
WBCs (x10^3/μL)
0
1
2
3
Months After Treatment


f
DNMT3b$^{lox/lox}$
DNMT3b$^{\Delta/\Delta}$
45
35
25
15
5
Cell Number (x10^6)
*
*
*
BM
Spleen
Thymus


g
DNMT3b$^{lox/lox}$
DNMT3b$^{\Delta/\Delta}$
0.8
0.6
0.4
0.2
Cell Percentage (%)
LKS$^-$
LKS

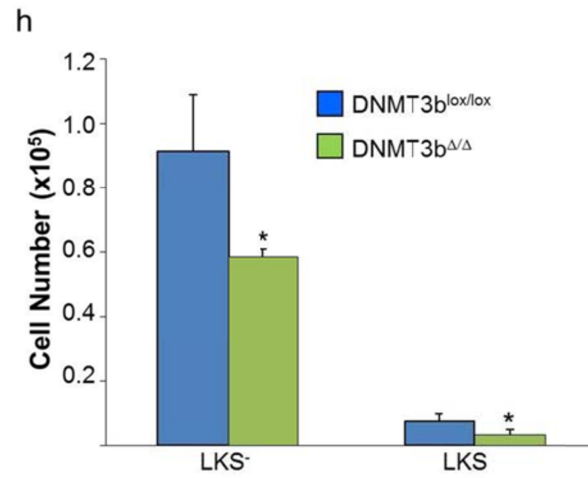
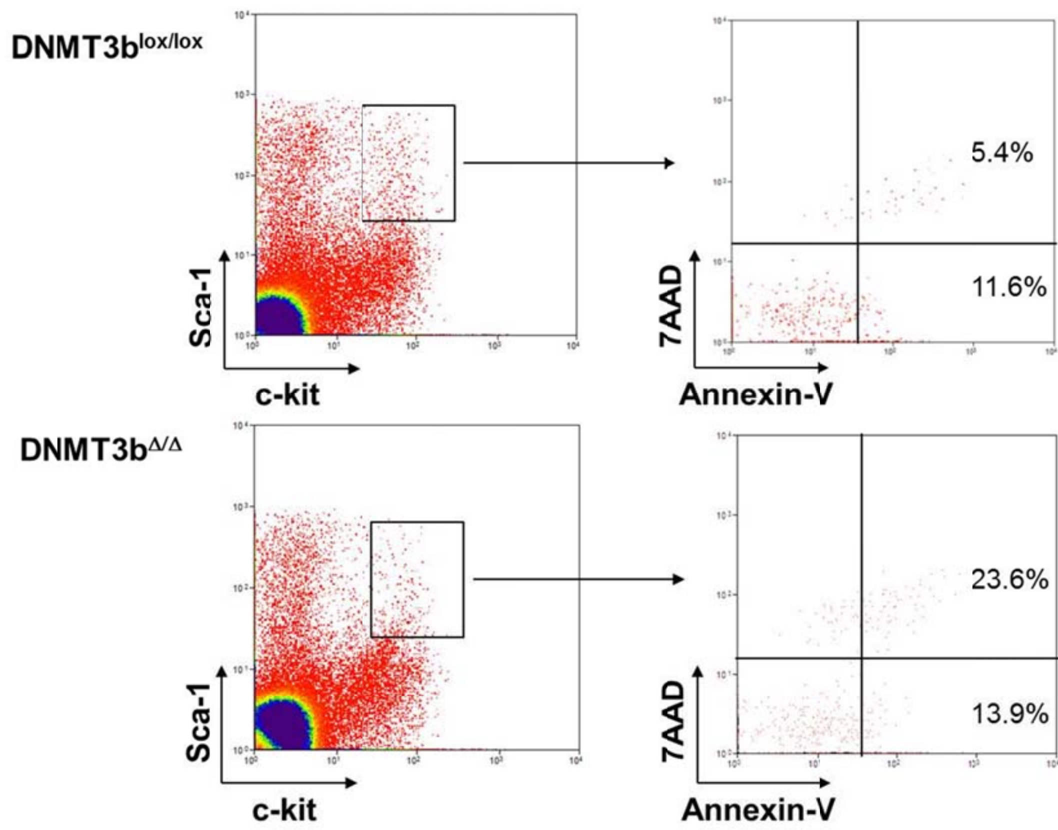

h
DNMT3b$^{lox/lox}$
DNMT3b$^{\Delta/\Delta}$
1.2
1.0
0.8
0.6
0.4
0.2
Cell Number (x10^5)
*
*
LKS$^-$
LKS

Figure 2-8. DNMT3b Deletion Reduces Hematopoietic Cell Numbers Under Homeostatic Conditions

(a) Efficient recombination of the floxed DNMT3b allele in bone marrow cells following tamoxifen treatments. Lox, floxed allele; Δ, recombined allele. (b) Diagram of tamoxifen treatment and analysis of peripheral blood and bone marrow. Hemoglobin (c), platelet (d), and white blood cell (e) counts in the peripheral blood of $DNMT3b^{lox/lox}$ and $DNMT3b^{\Delta/\Delta}$ mice (n=10 mice per group, * = $p < 0.05$). (f) Cellularity of one femur (BM), spleen, and thymus of $DNMT3b^{lox/lox}$ and $DNMT3b^{\Delta/\Delta}$ mice. There is a significant decrease in the cellularity of each hematopoietic organ in $DNMT3b^{\Delta/\Delta}$ mice as compared to controls (n=10 mice per group, * = $p < 0.05$). (g) Percentage of progenitor and stem cell enriched LKS^- and LKS cells within the bone marrow of $DNMT3b^{lox/lox}$ and $DNMT3b^{\Delta/\Delta}$ mice. (h) Absolute number of progenitor and stem cell enriched LKS^- and LKS cells within one femur from $DNMT3b^{lox/lox}$ and $DNMT3b^{\Delta/\Delta}$ mice. Values were calculated by multiplying the total cell number in (f) by the percentage of each cell type in (g). There is a significant decrease in the absolute numbers of both hematopoietic progenitor and stem cell populations (* = $p < 0.05$).

This decrease in the absolute number of hematopoietic stem cells in the bone marrow of DNMT3b$^{\Delta/\Delta}$ mice was associated with an increase in the apoptotic fraction within LKS cells (37.9% vs. 17.0%) by Annexin V and 7-AAD staining as compared to DNMT3b$^{lox/lox}$ controls (Figure 2-9a). LKS cells from DNMT3b$^{lox/lox}$ and DNMT3b$^{\Delta/\Delta}$ mice exhibited similar uptake of BrdU sixteen hours after a single intraperitoneal injection indicating equivalent rates of hematopoietic stem cell cycling within these two groups (Figure 2-9b). This data provides evidence that loss of hematopoietic stem cell numbers following DNMT3b excision is likely due to an increased rate of apoptosis as observed *in vitro* (Figure 2-6b) rather than changes in the cell cycle.

a



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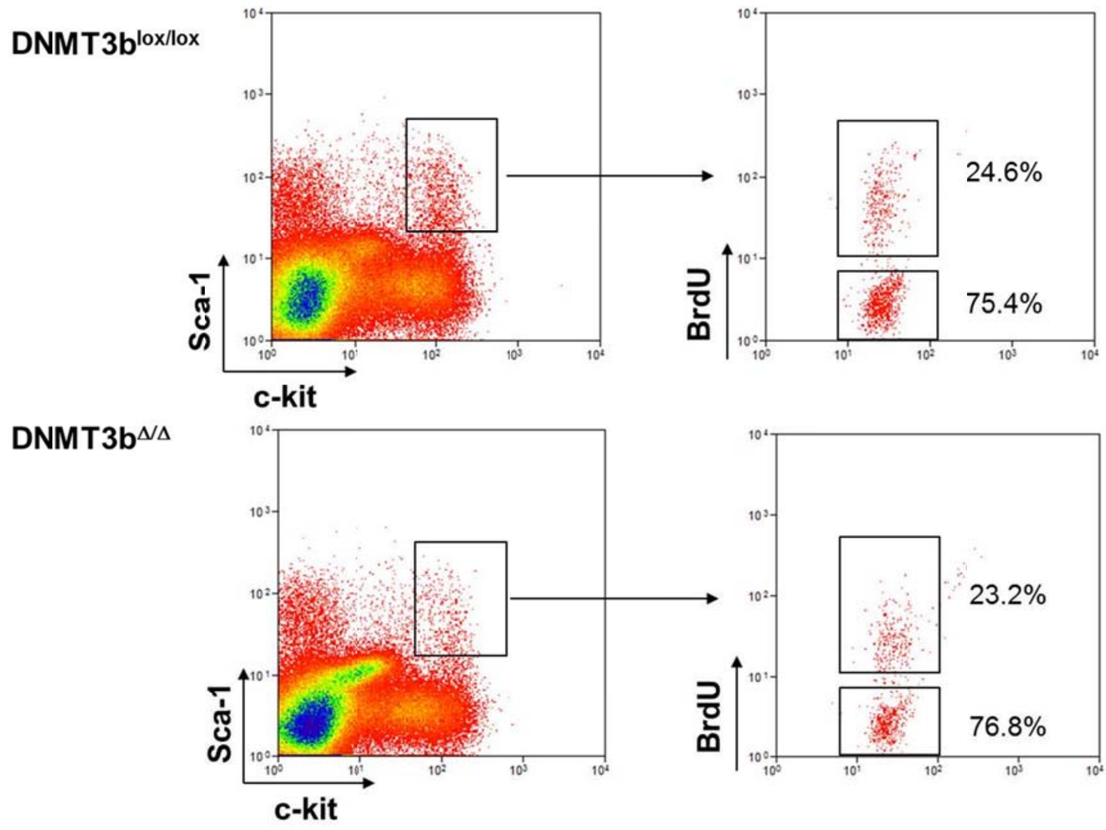

Figure 2-9. Increased Apoptosis in Hematopoietic Stem Cells in DNMT3b$^{\Delta/\Delta}$ Mice

(a) Apoptosis within the hematopoietic stem cell enriched LKS population of bone marrow cells as measured by annexin V and 7-AAD staining. Percentages in the scatter plot indicate the percentage of events within that region. (b) Cell cycle analysis of the hematopoietic stem cell enriched LKS population of bone marrow cells as measured by flow cytomtetry analysis of BrdU uptake. Percentages in the scatter plot indicate the percentage of events within that region.

2.3.5 DNMT3b Limits Hematopoietic Stem Cell Self-Renewal *Via* a Cell Intrinsic Mechanism

In order to determine a cell intrinsic and specific role for DNMT3b in hematopoietic cells, as compared to cells of the hematopoietic niche, hematopoietic cells of irradiated wild-type mice were reconstituted with bone marrow from DNMT3b$^{lox/lox}$ ER-Cre mice (Figure 2-10a). After allowing for stable engraftment for three months, donor DNMT3b$^{lox/lox}$ ER-Cre cells comprised more than ninety-seven percent of the myeloid (CD11b+) cells in the blood (Figure 2-10b). Half of the mice were then treated with tamoxifen and the engraftment in the peripheral blood monitored for another three months. Deletion of DNMT3b by Cre mediated recombination led to a significant decrease in peripheral blood engraftment within the myeloid compartment at two (98.7% vs 35.2%) and three (99.1% vs 28.1%) months post Cre induction (Figure 2-10b).

This decrease in contribution to the myeloid compartment of the blood was paralleled by an approximately sixty percent decrease in the percentage of DNMT3b$^{\Delta/\Delta}$ cells in the bone marrow and approximately thirty percent decrease in SLAM^{+} (CD150^{+}CD48^{-}) hematopoietic stem cells as compared to DNMT3b$^{lox/lox}$ controls (Figure 2-11a). Within the donor cells in the bone marrow there was a slight percentage increase in CD34^{-}LKS defined long-term hematopoietic stem cells, similar to the relative preservation of SLAM^{+} hematopoietic stem cells as compared to overall engraftment (Figure 2-11b). As a percentage of transplanted cells a decrease in common myeloid and granulocyte monocyte progenitors was observed similar to the more profound effect on progenitor (LKS^{-}) engraftment seen with knockdown of DNMT3b with a retrovirally delivered shRNA (Figure 2-4b). Taken together this data indicates impaired hematopoiesis in the absence of DNMT3b due to cell intrinsic defect.

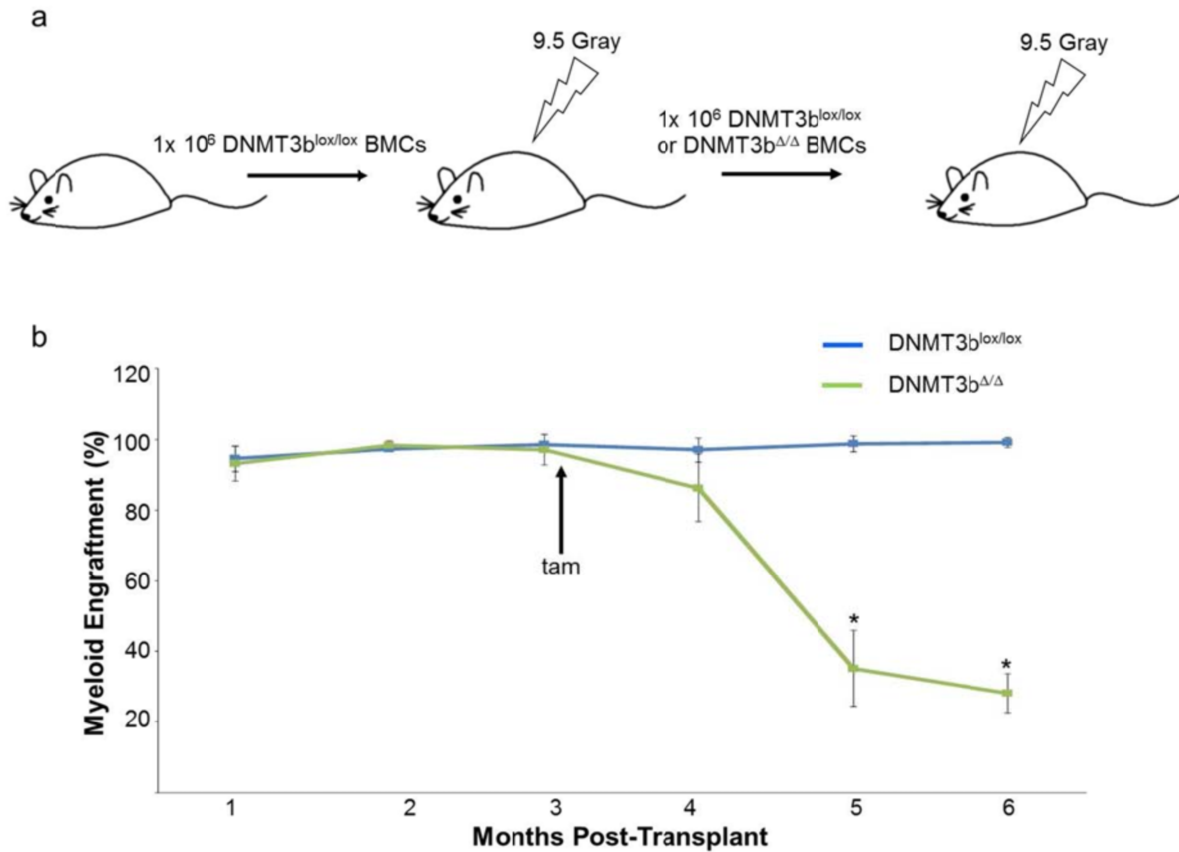

Figure 2-10. DNMT3b is a Cell Intrinsic Regulator of Hematopoietic Stem Cells

(a) Generation of mice carrying floxed DNMT3b alleles within the hematopoietic compartment only. At 3 months post-transplanation mice were treated with tamoxifen and peripheral blood engraftment followed for another 3 months. Donor derived bone marrow cells were then transplanted into lethally irradiated secondary recipients. (b) Engraftment with the myeloid ($CD11b^+$) compartment in peripheral blood before and after DNMT3b deletion as indicated by the arrow. Following DNMT3b deletion there was a significant loss of contribution of DNMT3b D/D cells to the myeloid cells within the blood (n=9-10 mice, * = $p < 0.05$).

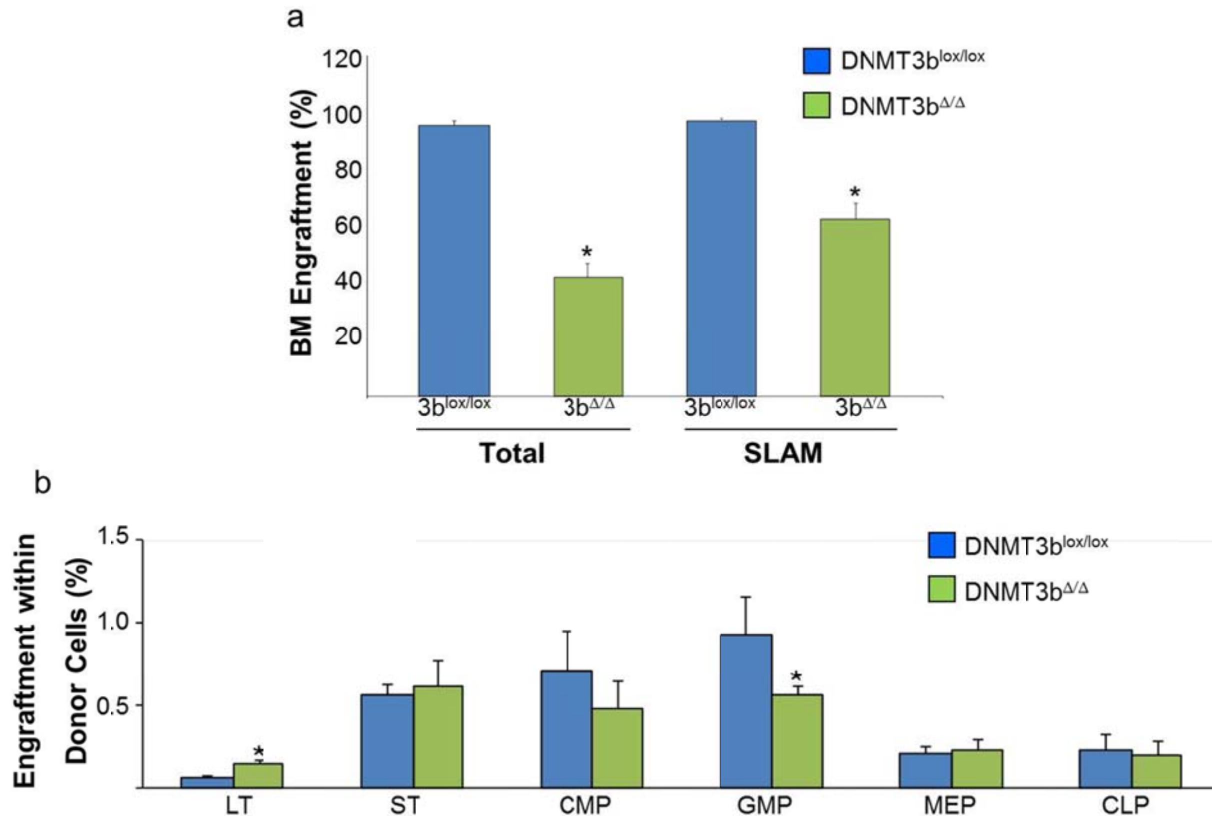

Figure 2-11. DNMT3b Deletion Limits Bone Marrow Engraftment in a Cell Intrinsic Manner

(a) Total percent engraftment in the bone marrow and percent engraftment within SLAM$^+$ cells of DNMT3b$^{lox/lox}$ and DNMT3b$^{\Delta/\Delta}$ mice. Engraftment of DNMT3b$^{\Delta/\Delta}$ cells in each population is significantly decreased as compared to DNMT3b$^{lox/lox}$ mice (n=9-10 mice,* = p <0.05). (b) Percentage of cells within the donor population of cells. Cells were first gated on donor-derived cells and then engraftment within the indicated stem and progenitor populations (as described in Figure 2-1) was determined. A significant increase in long term hematopoietic stem cells was observed along with a significant decrease in granulocyte monocyte progenitors (n=9-10 mice, * = p <0.05).

Given that the LKS phenotype becomes less reliable for stem cell identification following transplantation[58] a secondary transplant was employed to test the self-renewal of hematopoietic stem cells in the absence of DNMT3b. Prior to transplantation, analysis of the recombination of DNMT3b indicated efficient deletion in DNMT3b$^{\Delta/\Delta}$ mice (Figure 2-12a). Secondary recipient mice transplanted with one million DNMT3b$^{\Delta/\Delta}$ bone marrow cells had an average engraftment within the blood of four percent at one month post-transplantation while DNMT3b$^{lox/lox}$ cells constituted ninety-one percent of peripheral blood cells (Figure 2-12b). This engraftment defect was not due to loss of a particular cell type as DNMT3b$^{lox/lox}$ and DNMT3b$^{\Delta/\Delta}$ had equivalent engraftment in all three lineages in the blood (Figure 2-12c). Similar to the engraftment within the peripheral blood, DNMT3b$^{lox/lox}$ cells constituted nearly sixty percent of the bone marrow whereas engraftment in DNMT3b$^{\Delta/\Delta}$ recipient mice was less than three percent (Figure 2-12d). This difference in engraftment in secondary recipients correlated with a re-emergence of the DNMT3b lox allele in the transplanted DNMT3b$^{\Delta/\Delta}$ bone marrow cells following one month of engraftment (Figure 2-12e). This suggests that the observed engraftment, even at a low level, was at least partly due to residual cells that retained at least one DNMT3b allele after tamoxifen treatment and that these cells can out-compete cells in which both alleles of DNMT3b are deleted. Nevertheless this data indicates a self-renewal defect in hematopoietic stem cells following DNMT3b deletion and confirms a cell intrinsic defect in hematopoietic stem cell function observed following DNMT3b deletion.

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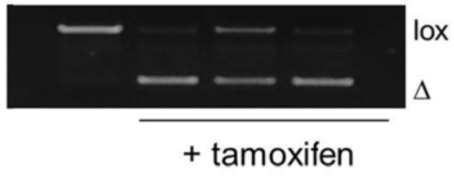
a



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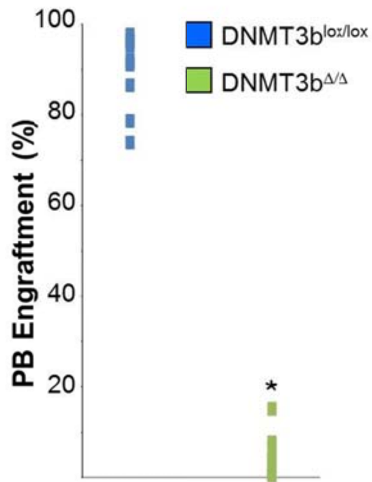

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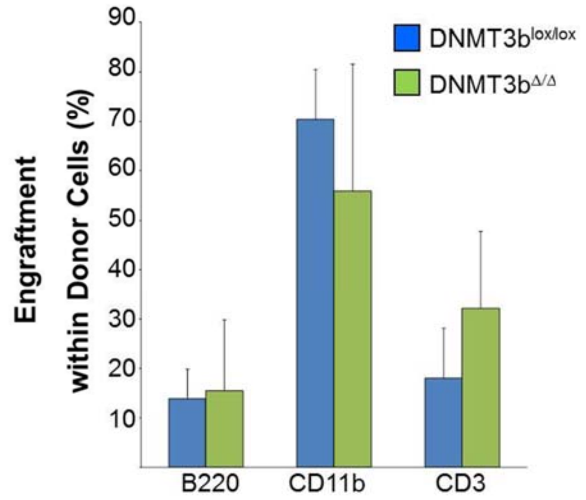
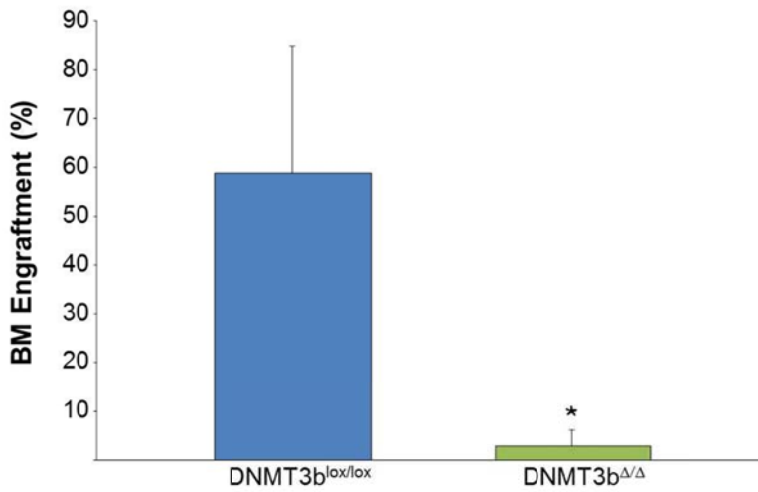


d



e

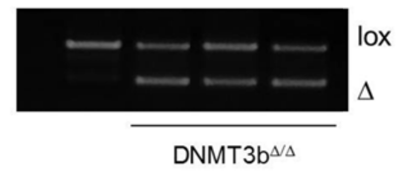
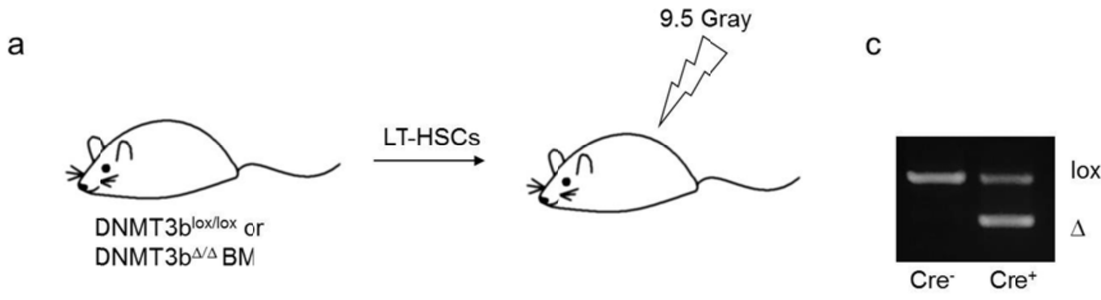


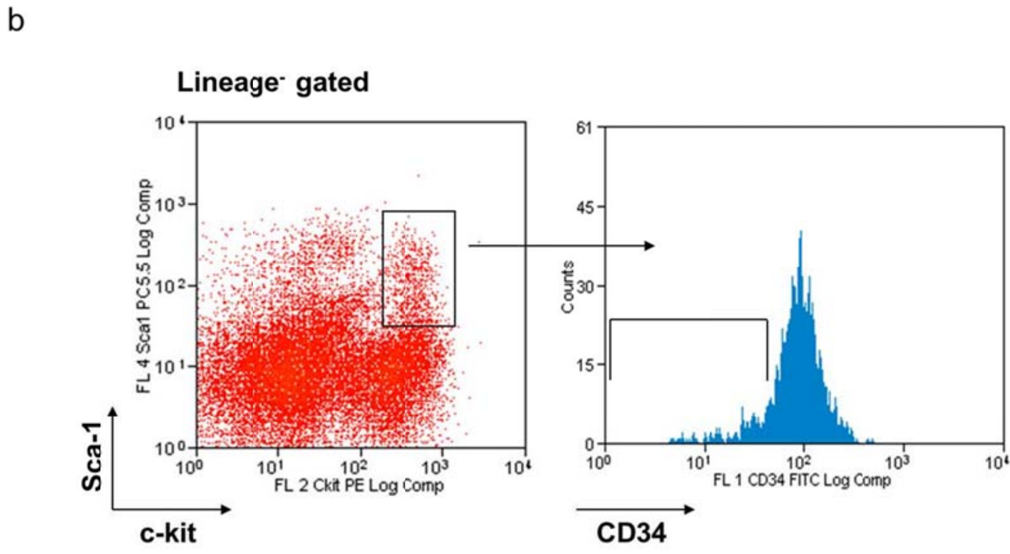
Figure 2-12. DNMT3b Deletion Limits Hematopoietic Stem Cell Self-Renewal

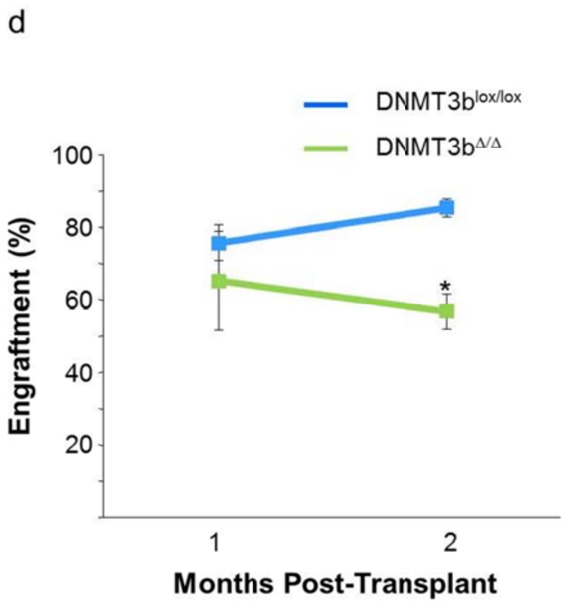
(a) Recombination of the floxed DNMT3b allele in tamoxifen treated primary recipients. The DNMT3b genotype of three individual mice tamoxifen treated mice as well as one control is shown. (b) Peripheral blood engraftment in secondary recipients transplanted with either $1x10^6$ DNMT3b$^{lox/lox}$ or DNMT3b$^{\Delta/\Delta}$ bone marrow cells. A significant decrease in total engrafted cells was observed at one month post-transplantation (n=10 mice per group, * = $p < 0.05$). (c) Engraftment within B cell (B220), myeloid (CD11b), and T cell (CD3) lineages within the blood of secondary recipients transplanted with DNMT3b$^{lox/lox}$ or DNMT3b$^{\Delta/\Delta}$ bone marrow cells. (d) Total percent engraftment with the bone marrow of secondary recipients transplanted with DNMT3b$^{lox/lox}$ or DNMT3b$^{\Delta/\Delta}$ bone marrow cells. A significant decrease in engraftment of DNMT3b$^{\Delta/\Delta}$ cells (n=10 mice per group, * = $p < 0.05$) was observed. (e) Genotype of donor derived bone marrow cells in secondary recipients. The DNMT3b genotype of three individual DNMT3b$^{\Delta/\Delta}$ recipients as well as one control is shown. An increase in the lox allele is observed in DNMT3b$^{\Delta/\Delta}$ secondary recipients as compared to primary recipients.

2.3.6 DNMT3b Maintains the Functionality and Proliferative Capacity of Hematopoietic Stem Cells

In order to test the role of DNMT3b in hematopoietic stem cells more directly five thousand $CD34^-$LKS long-term hematopoietic stem cells were sorted from $DNMT3b^{lox/lox}$ ER-Cre mice immediately after tamoxifen treatment and transplanted along with $5x10^5$ whole bone marrow competitor cells into 9.5 Gray irradiated recipients (Figure 2-13a,b). Recombination of the floxed DNMT3b allele after tamoxifen treatment was confirmed by PCR (Figure 2-13c). At two months post-transplantation $DNMT3b^{\Delta/\Delta}$ cells only contributed to 56.8% of the engrafted cells in the peripheral blood while DNMT3b lox/lox cells made up 85.3% of engrafted cells (Figure 2-13d). This defect in engraftment at two months correlated with a decrease in the percentage of $DNMT3b^{\Delta/\Delta}$ derived myeloid cells within the peripheral blood (Figure 2-13e) similar to the results seen following targeting of DNMT3b with a retrovirally delivered shRNA (Figure 2-3c).


a
9.5 Gray
c
LT-HSCs
DNMT3b$^{lox/lox}$ or
DNMT3b$^{\Delta/\Delta}$ BM
lox
Δ
Cre⁻
Cre⁺


b
Lineage⁻ gated
FL 4 Sca1 PC5.5 Log Comp
FL 2 Ckit PE Log Comp
Counts
FL 1 CD34 FITC Log Comp
Sca-1
c-kit
CD34


d
DNMT3b$^{lox/lox}$
DNMT3b$^{\Delta/\Delta}$
Engraftment (%)
Months Post-Transplant

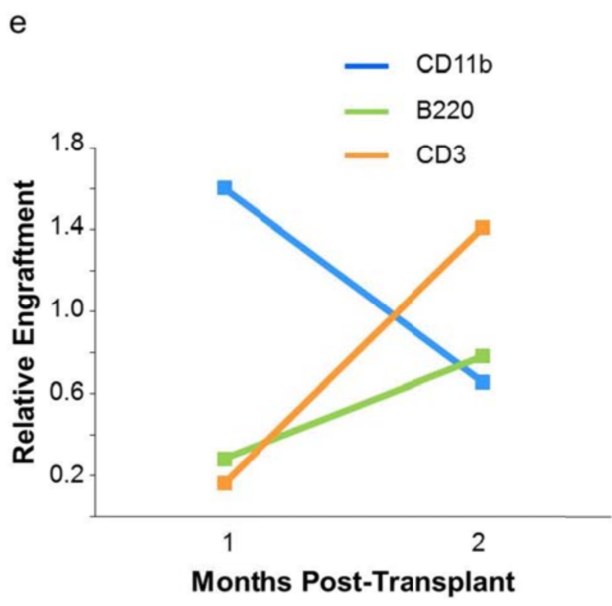
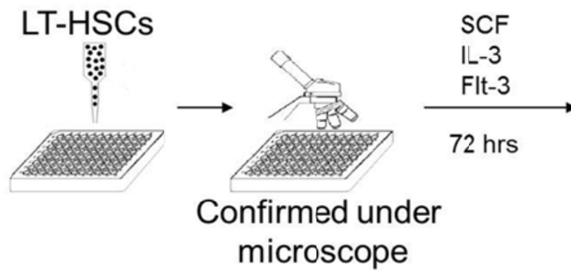

e
CD11b
B220
CD3
Relative Engraftment
Months Post-Transplant

Figure 2-13. DNMT3b is Required for Long-Term Hematopoietic Stem Cell Engraftment *In Vivo*

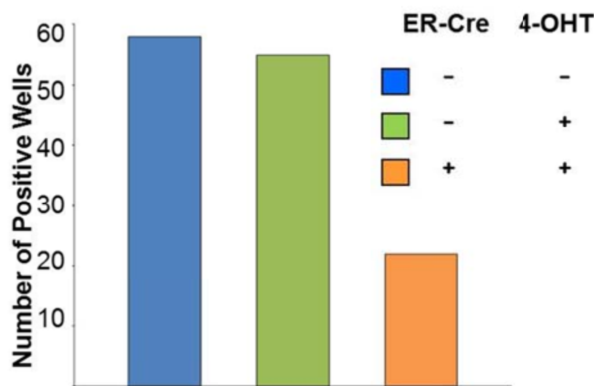
(a) Diagram of the long-term hematopoietic stem cell transplantation. 5,000 long-term hematopoietic stem cells were sorted from DNMT3b$^{lox/lox}$ ER-Cre^{-} or DNMT3b$^{lox/lox}$ ER-Cre^{+} mice following tamoxifen treatment and transplanted along with 5x10^{5} whole bone marrow cells from wild-type mice into 9.5 Gray irradiated recipients. (b) Flow cytometry strategy for isolation of long-term hematopoietic stem cells. (c) PCR confirmation of deletion of floxed DNMT3b alleles within the DNMT3b$^{\Delta/\Delta}$ group. (d) Peripheral blood engraftment of donor-derived cells. DNMT3b$^{\Delta/\Delta}$ cells had a significantly lower engraftment at 2 months post transplantation (n= 5 mice per group, * = $p < 0.05$). (e) Contribution to myeloid (CD11b), B cell (B220), and T cell (CD3) populations within the blood at one and two months post transplantation. The decrease in overall engraftment in DNMT3b$^{\Delta/\Delta}$ recipient mice shown in (d) is paralleled by a decrease in myeloid engraftment as compared to DNMT3b$^{lox/lox}$ recipients.

To further confirm a defect following DNMT3b loss at the hematopoietic stem cell level single $CD34^{-}$LKS long-term hematopoietic stem cells were sorted into one well of a 96 well plate and cultured with cytokines favoring cellular proliferation (Figure 2-14a). After seventy-two hours of culture approximately fifty-seven of ninety-six wells that originally contained one $DNMT3b^{lox/lox}$ $ER\text{-}Cre^{+}$ or tamoxifen treated $DNMT3b^{lox/lox}$ $ER\text{-}Cre^{-}$ cell still contained at least one cell and of these nearly fifty-eight percent (thirty-three out of fifty seven) contained at least five cells (Figure 2-14b, c). In contrast only twenty out of ninety-six wells that originally contained one $DNMT3b^{lox/lox}$ $ER\text{-}Cre^{+}$ cell treated with tamoxifen still contained one cell after seventy-two hours and the majority of these wells (40.9%) contained only one cell (Figure 2-14b,c). This data indicates a proliferative defect at the single cell level for highly purified hematopoietic stem cells and provides a potential cellular mechanism for the observed defect in stem cell self-renewal observed *in vivo*.

a



b



c

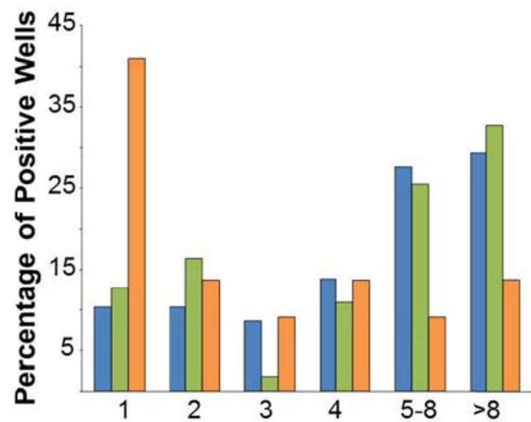
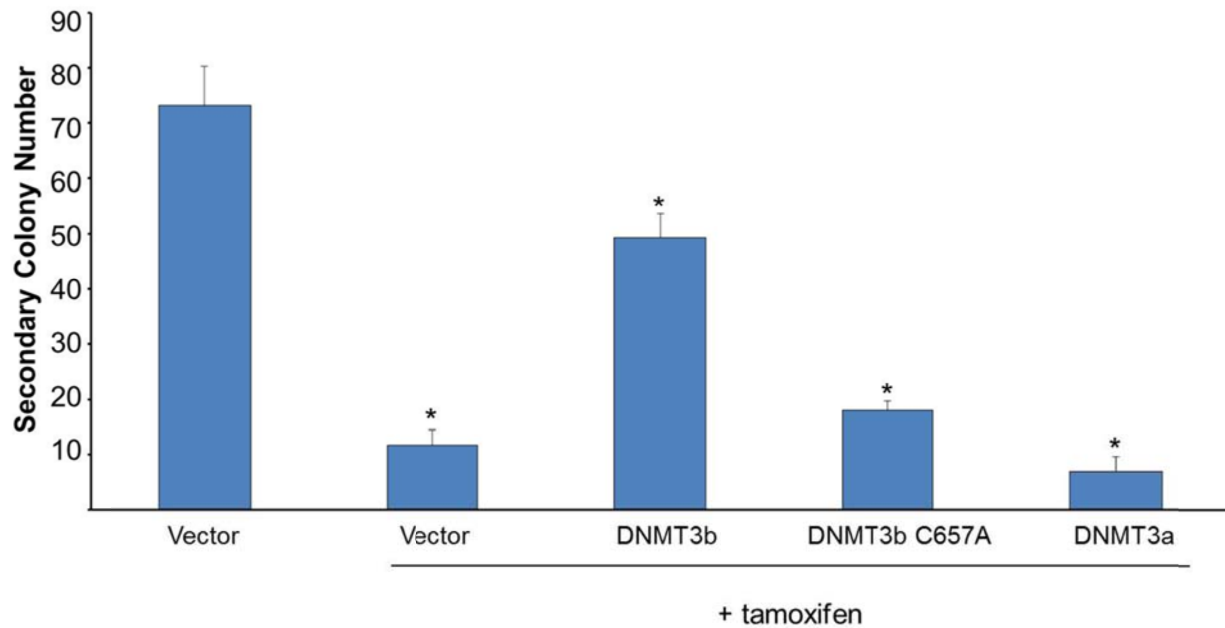


Figure 2-14. Long-Term Hematopoietic Stem Cells Fail to Proliferate *In Vitro* in the Absence of DNMT3b

(a) Single cell culture experimental design. (b) Number of wells (out of 96) containing at least one cell after 72 hours of culture. 4-OHT, 4-hydroxytamoxifen. (c) Percentage of wells with at least one cell (b) with the indicated number of cells. The majority of wells in which DNMT3b was deleted by Cre-induction contained only one cell ($p<0.05$ by χ^2 test).

2.3.7 DNMT3b Function in Hematopoietic Cells Requires Its Methyltransferase Activity

In an attempt to define a molecular mechanism underlying the functional defect in hematopoietic stem cells the ability of wild-type DNMT3b, a mutant DNMT3b lacking catalytic activity, and DNMT3a to rescue the colony forming ability of DNMT3b$^{\Delta/\Delta}$ hematopoietic cells was analyzed. DNMT3b$^{\Delta/\Delta}$ cells transduced with an MSCV vector alone produced significantly fewer colonies in a secondary CFC assay as compared to MSCV transduced DNMT3b$^{lox/lox}$ cells (Figure 2-15). Only wild-type DNMT3b cDNA, and not DNMT3b^{C657A} or wild-type DNMT3a, was able to rescue the colony forming ability of DNMT3b$^{\Delta/\Delta}$ cells (Figure 2-15). This data indicates that DNMT3b activity in hematopoietic progenitors requires the methyltransferase activity of DNMT3b and DNMT3a can not compensate for DNMT3b deletion.

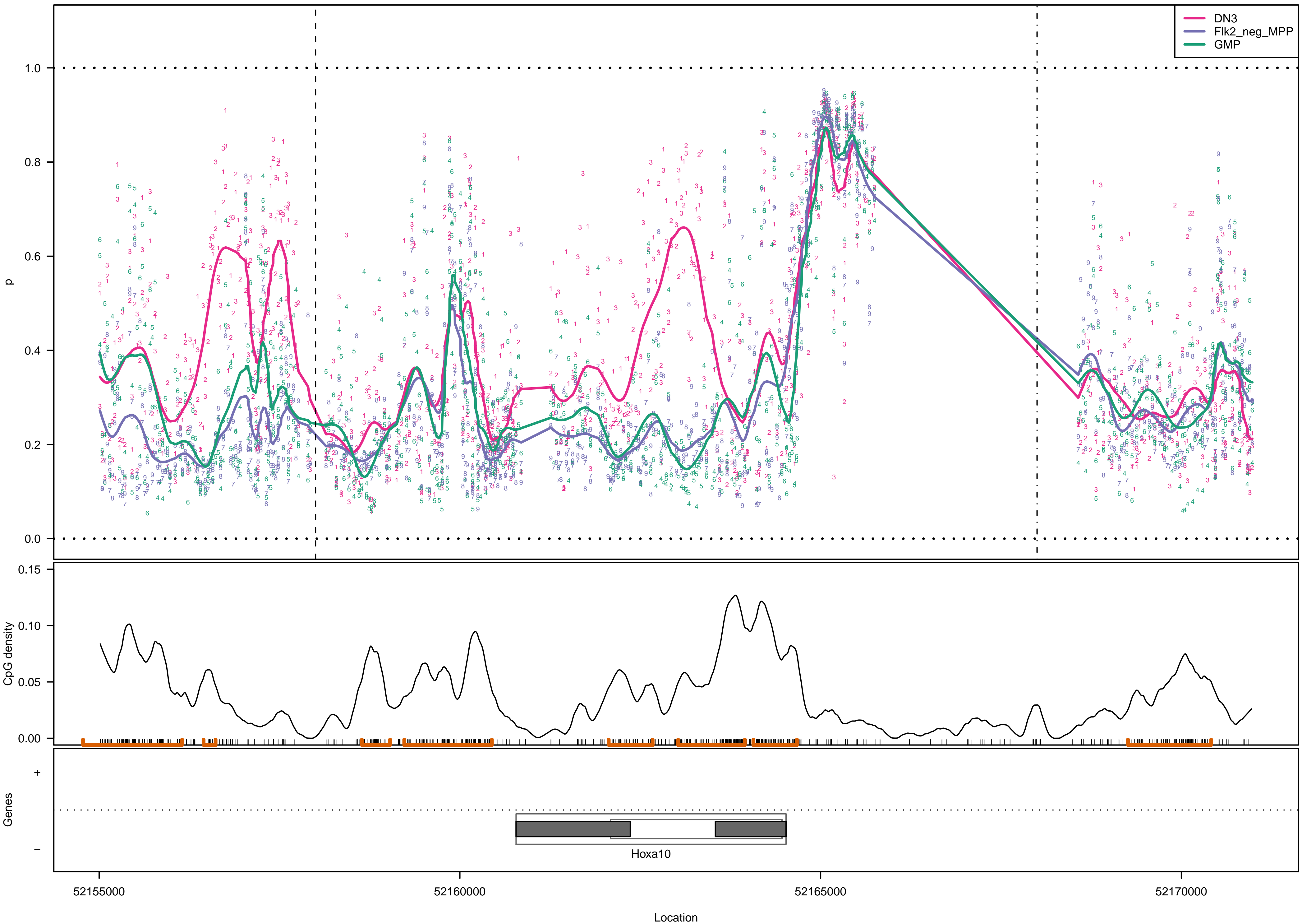


Fgure 2-15. DNMT3b Functions in Hematopoiesis *Via* Its Methyltransferase Activity

Total secondary colony formation for cells transduced with MSCV constructs expressing GFP alone or methyltransferase cDNAs. Vector, MSCV control; DNMT3b, wild-type DNMT3b cDNA; DNMT3b C657A, mutant DNMT3b lacking methyltransferase activity; DNMT3a, wild-type DNMT3a; +tamoxfien, DNMT3b$^{\Delta/\Delta}$ cells. Although transduction with wild-type DNMT3b could not completely rescue colony formation, cells transduced with a mutant DNMT3b or DNMT3a formed significantly fewer colonies (Average of four wells, *= $p < 0.05$).

2.3.8 Decreased MLL Expression in DNMT3b$^{\Delta/\Delta}$ Cells is Associated with Loss of DNA Methylation

In order to identify potential targets of DNMT3b methyltransferase activity an analysis of previously reported changes in DNA methylation during hematopoiesis[111] was undertaken. It was assumed that loss of DNMT3b would lead to a decrease in DNA methylation and, as observed in embryonic stem cells, defective DNA methylation would lead to ineffective gene silencing during differentiation[2]. Nine potential candidate genes were identified that had highly methylated regions in close proximity to their first exon similar to HoxA10 which is highly methylated just 5' of an associated CpG island in its first exon (Figure 2-16). The expression level of Gata2, HoxA6, HoxA9, HoxA10, HLF, Meis1, MLL, Prdm16, and Nkx2-3 was determined by quantitative real-time RT-PCR in the stem cell enriched LKS bone marrow cells as well as two myeloid progenitor populations, common myeloid and granulocyte-monocyte progenitors from DNMT3b$^{lox/lox}$ and DNMT3b$^{\Delta/\Delta}$ mice three months after tamoxifen induction of DNMT3b recombination. Of these genes HoxA9, HoxA10, Meis1, and MLL showed decreased expression in DNMT3b$^{\Delta/\Delta}$ LKS cells as compared to DNMT3b$^{lox/lox}$ controls (Figure 2-17a). To determine if this decrease in expression was due to changes in DNA methylation bisulfite sequencing of highly methylated regions in MLL and HoxA10 was performed in whole bone marrow cells. Loss of DNMT3b lead to a decrease in methylation at the MLL locus from 91.1% to 70.3% while no change was observed in DNA methylation at the HoxA10 locus (Figure 2-17b). This data implicates MLL as a downstream target of DNMT3b and loss of DNMT3b may in turn silence its expression in a hematopoietic stem cell enriched compartment.

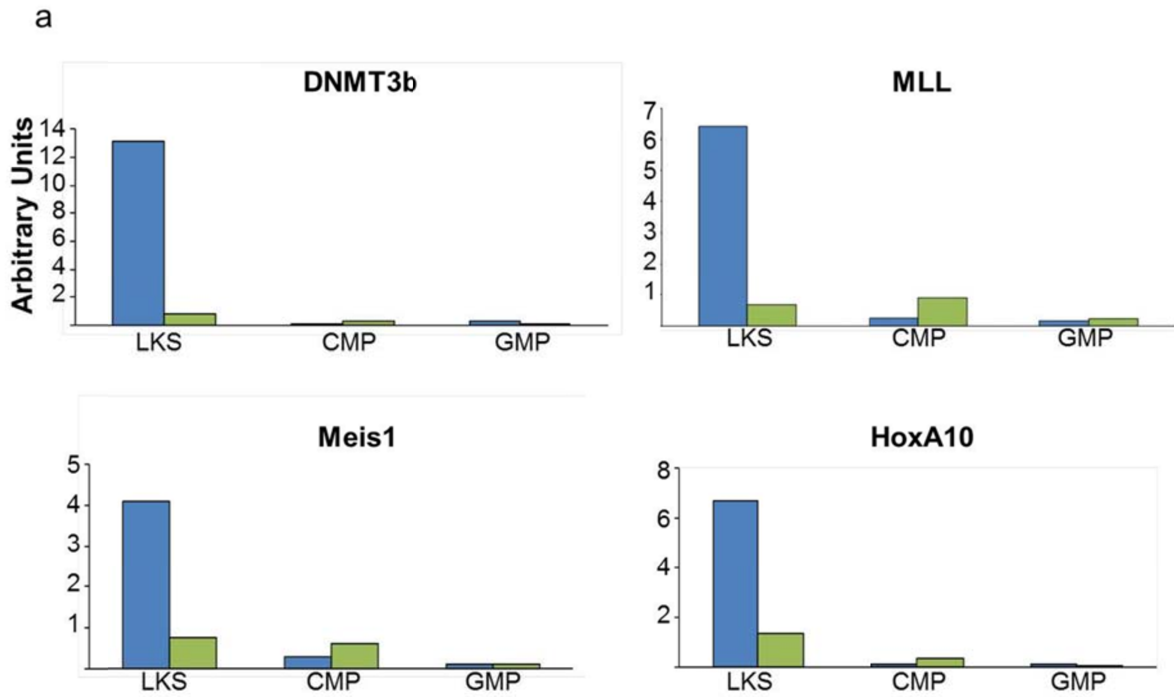


ID:1--chr6:52158000-52168000
DN3
Flk2_neg_MPP
GMP
p
1.0
0.8
0.6
0.4
0.2
0.0
CpG density
0.15
0.10
0.05
0.00
Genes
+
–
Hoxa10
52155000
52160000
52165000
52170000
Location

Figure 2-16. Methylation of HoxA10 in Hematopoietic Progenitors

Diagram of the methylation at HoxA10 as found by Ji *et al* [111]. Bottom panel shows the alignment of HoxA10 on the negative strand of chromosome 9, the 5' end of the gene is at the right of the page. Grey boxes represent exons and the underlaid white box indicates the coding sequence of the gene. The middle panel displays the corresponding CpG density with each horizontal black line indicating one CpG and orange bars a potential CpG island. The top panel shows the methylation of each CpG for double negative 3 (DN3) T cell precursors, Flk2 negative multipotent progenitors (Flk2_neg_MPP) and granulocyte-monocyte progenitors (GMP). There is a high level of DNA methylation just to the 5' end of the first exon of HoxA10.

a



b

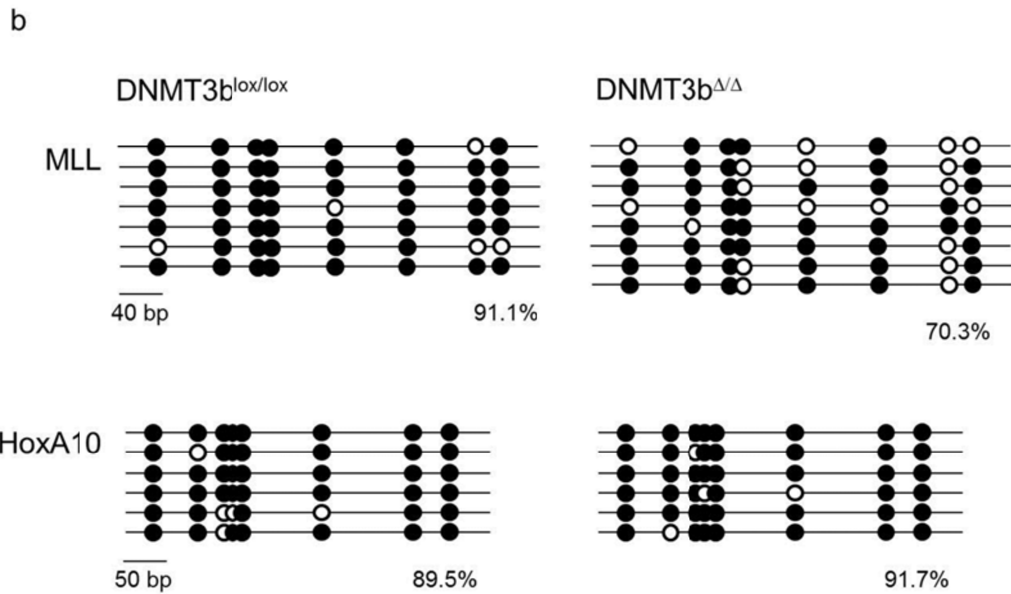


Figure 2-17. DNMT3b Loss Causes a Decrease in MLL Expression Associated with Demethylation.

(a) Expression of DNMT3b, MLL, Meis1, and HoxA10 in hematopoietic stem and progenitor cells of DNMT3b$^{lox/lox}$ and DNMT3b$^{\Delta/\Delta}$ mice. Expression was measured by quantitative real-time RT-PCR and normalization to the level of β-actin. LKS, Lineage-c-kit+Sca-1+ stem cell enriched bone marrow; CMP, common myeloid progenitors; GMP, granulocyte-monocyte progenitors. (b) Methylation of the MLL and HoxA10 locus in whole bone marrow cells from DNMT3b$^{lox/lox}$ and DNMT3b$^{\Delta/\Delta}$ mice. The percentage of methylated CpGs (black circles) is indicated.

2.4 CONCLUSIONS

The data presented in this thesis demonstrates that hematopoiesis is dependent upon DNMT3b by a cell intrinsic mechanism. Underlying this defect in hematopoiesis is a loss of hematopoietic stem cell self-renewal as evidenced by the failure of DNMT3b$^{\Delta/\Delta}$ cells to engraft in secondary recipients. This decreased self-renewal is associated with a decreased proliferative capacity at the single cell level *in vitro* as well as increased apoptosis both *in vivo* and *in vitro*. While we can not rule out an effect of DNMT3b on other hematopoietic stem cell functions such as homing or retention within the bone marrow niche the presence of equivalent engraftment to control cells after one month of transplantation make a role for DNMT3b in hematopoietic stem cell homing or retention in the bone marrow unlikely.

DNMT3b may also play a cell extrinsic role in maintenance hematopoietic stem cells by regulating the hematopoietic stem cell niche. Irradiation of the bone marrow can induce expansion of transplanted cells and this, rather than a lack of space within the marrow in non-ablated recipients, may cause the enhanced engraftment of transplanted bone marrow in irradiated recipients[143]. In addition radiation has been shown to induce changes in the expression of the *de novo* methyltransferases DNMT3a and DNMT3b[144], and the former of the two may mediate bystander effects following irradiation[145]. Loss of DNMT1 has also been shown to enhance both whole bone marrow and hematopoietic stem cell engraftment in the setting of non-ablative transplantation[5]. Thus depletion of DNMT3b from the hematopoietic niche in the bone marrow or spleen may account for the observed expansion of white blood cells in the peripheral blood (Figure 2-8e).

A defined role for DNMT1 in hematopoietic cells remains obscure[5, 6]. While Broske *et al* demonstrated frank bone marrow failure in the absence of DNMT1[6] and Trowbridge *et al* found no effect of DNMT1 loss on hematopoietic cells under homeostatic conditions[5] both studies

demonstrated limited engraftment in irradiated hosts following loss of DNMT1. In both studies this was associated with a more profound effect on hematopoietic progenitors (LKS$^-$ cells) as opposed to more immature stem cell populations and neither assessed the role of DNM1 at the single cell level in hematopoietic stem or progenitor cells[5, 6]. Thus DNMT3b may play a specific role in the maintenance of DNA methylation in hematopoietic stem cells that is unique from DNMT1 which is considered a maintenance methyltransferase. Moreover these studies failed to identify changes in expression of genes associated with hematopoietic stem cell function and alterations in DNA methylation. This may be due to the fact that gene expression changes in these studies were assessed within four days of induction of DNMT1 loss and a passive mechanism of DNA demethylation may have not led to changes in gene expression within this time frame.

The function of DNMT3b within hematopoietic stem cells appears to require the methyltransferase activity of DNMT3b, and not DNMT3a, and a decrease in methylation of MLL, leading to its loss of expression, may underlie this defect. Our data suggests a model whereby DNMT3b is necessary to maintain the expression of MLL in hematopoietic stem cells. Although numerous mechanisms have been identified by which active, i.e. enzyme mediated, DNA methylation may occur[146], the assessed MLL locus remains methylated throughout progenitor development [111] and down-regulation of its expression during hematopoietic differentiation does not appear to be correlated with decreased levels of DNA methylation. It is likely then that the observed demethylation occurs through a passive process during hematopoietic stem cell division in the absence of DNMT3b, similar to a proposed role for DNMT3b in certain cancer cells[124] and embryonic stem cells[2]. With each division the degree of methylation becomes less due to a failure of DNMT3b to methylate the daughter DNA strand. Thus individual bisulfite-treated clones with a relatively high level of methylation may represent cells derived from hematopoietic stem cells that have undergone fewer cell divisions. It is likely that DNA methylation itself, and not the level of DNMT3b, determines the phenotype of DNMT3b$^{\Delta/\Delta}$ cells. A similar mechanism of gene regulation was observed in neural precursor cells in the absence of DNMT3a; changes in the methylation of the

gene and not its occupancy by DNMT3a were correlated with changes in gene expression[110]. Moreover as this process is passive and requires cell division, conditions which enhance cell division would elicit a more dramatic phenotype from DNMT3b$^{\Delta/\Delta}$ cells. This may explain while transplantation into irradiated mice yielded enhanced hematopoietic stem cell defictis following DNMT3b deletion than under homeostatic conditions where the replicative demands on hematopoietic stem cells are limited.

Conditional deletion of MLL produces a similar phenotype observed in this study. MLL deletion leads to a loss of hematopoietic cells in bone marrow chimeras and decreased proliferation of myeloid progenitors[85]. In another study MLL deletion lead to normal hematopoiesis under homeostatic conditions but defective engraftment in irradiated recipients[86]. However it is difficult to determine the kinetics by which demethylation of the MLL locus effects its expression, ie the point at which loss of methylation induces changes in MLL expression that has a functional impact on hematopoietic stem cell function. The homeobox proteins HoxA9 and HoxA10 have been shown to regulated by MLL in hematopoietic cells[85, 87, 147] and loss of HoxA gene expression may underlie the observed hematopoietic defect following DNMT3b deletion. Interestingly MLL is a H3K4 methyltransferase, the permissive histone modification that was shown to lost in the absence of DNMT3a in neural progenitor cells[110], underscoring an interplay between DNA methylation and histone modifications during hematopoiesis. Further investigation of the genetic programs regulated by DNA methylation in hematopoietic stem cells may yield potential targets for therapeutic manipulation of these cells.

2.5 FUTURE STUDIES

A role for DNMT3b in the hematopoietic niche is suggested by the increased number of white blood cells in the peripheral blood of mice lacking DNMT3b under homeostatic conditions (Figure 2-8e) and increased engraftment of wild-type bone marrow under non-ablative conditions in DNMT1-null mice[5]. Therefore it would be interesting to determine a role for DNMT3b in the hematopoietic niche by transplantation of wild-type bone marrow cells into DNMT3b$^{\Delta/\Delta}$ recipient mice without pre-conditioning with irradiation. This work may also be extended to transplantation in the setting of irradiation given that the de novo methyltransferases appear to mediate bystander effects in other cell types following irradiation[145].

A previous study analyzing changes in DNA methylation during hematopoiesis relied on a microarray platform of isolated CpG islands[111]. However regions of differential methylated between cell types may actually lie outside of these islands, at CpG shores[108, 110]. Therefore an unbiased assessment of changes in DNA methylation during hematopoiesis is warranted. Also the analysis of the genes identified by Ji *et al* indicates that the areas of high levels of DNA methylation may maintain that level of methylation during hematopoietic development, as is seen for HoxA10 (Figure 2-16), and down regulation of gene expression may not be associated with changes in DNA methylation. Thus it may be more worthwhile to examine genome-wide changes in methylation following disruption of one or more of the DNA methyltransferases within the hematopoietic stem cell compartment in order to determine the regions that may be important for gene expression but are not necessarily altered during its silencing.

A complete assessment of the role DNA methylation plays in regulation of MLL expression and subsequently in hematopoietic stem cell function is difficult. One method to define MLL role's would be a rescue experiment to determine if MLL can restore hematopoietic engraftment following loss of DNMT3b. However overexpression of MLL in progenitor populations can lead to

immortalization [148] indicating that any experiments involving introduction of MLL would require tight regulation of its expression. Changes in gene expression following loss of methylation may be achieved by MLL promoter constructs driving luciferase expression, however an interplay between DNA methylation and histone modifications likely exists, such that it would be necessary to transfect cells with a nucleosomal rather than naked DNA.

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