2008

GENETIC REGULATION OF HEMATOPOIETIC STEM CELL AGING

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ABSTRACT OF DISSERTATION

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GENETIC REGULATION OF HEMATOPOIETIC STEM CELL AGING

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
ERIN J. OAKLEY

Lexington, Kentucky

Co-Directors: Dr. Gary Van Zant, Professor of Medicine and Physiology
and Dr. Michael Reid, Professor of Physiology

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It is well documented that both quantitative and qualitative changes in the murine hematopoietic stem cell (HSC) population occur with age. In mice, the effect of aging on stem cells is highly strain-specific, thus suggesting genetic regulation plays a role in HSC aging. In C57BL/6 (B6) mice, the HSC population steadily increases with age, whereas in DBA/2 (D2) mice, this population declines. Our lab has previously mapped a quantitative trait locus (QTL) to murine chromosome 2 that is associated with the variation in frequency of HSCs between aged B6 and D2 mice. In these dissertation studies, I first aim to characterize the congenic mouse model which was generated by introgressing D2 alleles in the QTL onto a B6 background. Using a surrogate assay to mimic aging, I analyzed the cell cycle, apoptotic and self-renewal capabilities of congenic and B6 HSCs and show that D2 alleles in the QTL affect the apoptotic and self-renewal capabilities of HSCs. In the second aim of these studies, I used oligonucleotide arrays to compare the differential expression of B6 and congenic cells using a population enriched for primitive stem and progenitor cells. Extensive analysis of the expression arrays pointed to two strong candidates, the genes encoding Retinoblastoma like protein 1 (p107) and Sorting nexin 5 (Snx5). B6 alleles were associated with increased p107 and Snx5 expression in old HSCs therefore both genes were hypothesized to be positive regulators of stem cell number in aged mice. Finally, in the third aim of these studies, I show that the individual overexpression of p107 and Snx5 in congeic HSCs increases day35 cobblestone area forming cell (CAFC) numbers, therefore confirming their roles as positive regulators of HSC number *in vitro*. These studies uncover novel roles for p107 and Snx5 in the regulation of HSC numbers and provide additional clues in the complex regulation of HSC aging.

KEYWORDS: Hematopoietic Stem Cells, Aging, Genetic Regulation, QTL analysis, DNA damage.

Erin J. Oakley

September 11, 2008

GENETIC REGULATION OF HEMATOPOIETIC STEM CELL AGING

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For Brad and Ace and my Parents

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CHAPTER ONE

Introduction

Properties of Hematopoietic Stem Cells and Organization of the HSC Hierarchy

Hematopoietic stem cells (HSCs) are described conceptually as pluripotent cells with the potential to generate all of the mature blood cells in the body over the lifetime of an organism. In the early 1960s, Siminovitch *et al.* provided what remain today the two functional criteria for defining HSCs: the capacity for multi-lineage differentiation and the ability to self-renew to give rise to more HSCs (Siminovitch *et al.*, 1963; Till and Mccullough, 1961). Adult hematopoietic stem cells comprise approximately 0.01% of the bone marrow compartment in which they reside, yet they are responsible for the continuous replenishment of mature blood cells lost through attrition and routine somatic cell aging (Geiger and Van Zant, 2002). A delicate balance between self-renewal, differentiation, and cell death must be maintained to ensure not only hematopoietic homeostasis, but also organismal homeostasis and longevity.

Mouse HSC populations have been subdivided into three populations 1) long-term HSCs (LT-HSCs), which have extensive self-renewal capacity, 2) short-term HSCs (ST-HSCs), which have a limited self-renewal capacity, and 3) multipotent progenitor cells, which do not self-renew and ultimately differentiate into either myeloid or lymphoid precursor cells (Figure 1.1). Thus the differences in developmental potential and the linear relationship of stem and progenitor cells represent a well-established biological hierarchy. LT-HSCs possess high proliferation potential and can generate all eight mature hematopoietic lineages: B and T lymphocytes, erythrocytes, megakaryocytes/platelets, basophils/mast cells, eosinophils, neutrophils, and monocytes/macrophages (Szilvassy, 2003). In mice, a single LT-HSC can regenerate and maintain the hematopoietic system of an irradiated host, demonstrating the extensive proliferation and long-term multi-lineage reconstituting activity of this primitive cell population (Osawa *et al.*, 1996). Multipotent progenitor cells give rise to clonogenic cells that are able to differentiate into cells of a single hematopoietic lineage (Abramson *et al.*, 1977). Common lymphoid progenitors possess a lymphoid-restricted capacity and

produce B and T cells and natural killer cells (Kondo *et al.*, 1997), while common myeloid progenitors give rise to megakaryocyte/erythrocyte or granulocyte/macrophage cells (Akashi *et al.*, 2000). A small reserve of HSCs carry the burden of replenishing millions of mature cells daily throughout the lifetime of an individual in order to prevent immunosenescence or aplasia. There is no compelling evidence that stem cell reserves decline sufficiently in old age to threaten adequate blood cell production (Rando, 2006). However, studies do indicate that aged tissues have a diminished capacity to return to a homeostatic state after exposure to stress or injury, therefore indicating a defect in stem cell function during the aging process. Since the HSC population provides an ideal model to study stem cell aging, it is necessary to elucidate the mechanisms of hematopoietic aging and expand the findings to other tissues and organ systems.

Theories of Aging and Age Related Epigenomic Changes

There are two major theories of organismal aging: evolutionary and damage based. The former suggests that natural selection favors a genetic composition that enhances reproductive fitness and fecundity. According to the theory, since the effect of this genetic repertoire on vitality following the reproductive lifespan is immaterial to the propagation of the species, genes have been selected and accumulated which favor reproductive success but have negative effects later in life, thus limiting lifespan. Simply put, aging genes have not been selected based on their value for fitness, but are most likely by-products of evolution (Vijg and Suh, 2005). This idea has been termed antagonistic pleiotrophy, and can be used to explain how genetic traits can have both beneficial and deleterious effects for a species (Campisi, 1997). Campisi and others propose that cellular senescence, for example, is antagonistically pleiotropic, suggesting that the senescence response is a selected phenotype that prevents cancer during early life, while at later times contributes to age related pathologies (Campisi, 1997; Krtolica and Campisi, 2002). The second theory of aging argues that accumulated cellular damage over a lifetime limits the effective functioning of key organ systems. Poorly repaired damage of chromosomal DNA, stress-related aberrations in structural enzymes or protein turnover, and/or deletions in mitochondrial DNA, for example, may compromise organ function and in turn limit longevity. Given the extremely complex phenotype of aging,

numerous other theories such as the free radial theory of aging (Harman, 1956) and protein damage accumulation theory (Levine, 2002) have been postulated in an attempt to explain what aging is and why it happens. A common theme among many of these theories is to take a reductionist approach and focus attention at the molecular level in hopes of understanding the aging of organisms through the aging of their components. In our quest to understand the aging process, we must face reality and succumb to the notion that aging is a multifactorial process; therefore it's likely that all of the aforementioned processes factor into this phenomenon.

An important theme emerging in the field of aging research is the role of epigenetic alterations in aging mammalian tissues. As mentioned above, genetic instability in the form of direct DNA mutations, translocations, and/or deletions has been proposed to accumulate with age. However, studies reveal that there is yet another class of heritable DNA alterations that are not associated with direct changes in primary nucleotide sequences. These epigenetic changes can alter gene expression patterns simply by treating the same sequence of DNA differently at the level of the individual, organ, cell lineage or even differentiation state (Mager and Bartolomei, 2005). Epigenetic modifications, most commonly in the form of changes in the methylation status of DNA and biochemical modifications of core histones, have been linked to the aging process and are increasingly recognized as part of normal and pathologic aging physiology (Issa, 2003). Manel Esteller's group studied the epigenetic profiles of 80 pairs of monozygotic twins ranging in age from 3-74 years old and found that older twins exhibited large differences in their overall content and distribution of 5-methylcytosine DNA and histone acetylation compared to young twins which were largely indistinguishable epigenetically (Fraga *et al.*, 2005). The epigenetic changes that accumulated with age had a dramatic effect on gene expression, thus the authors propose that a so-called "epigenetic drift" accompanies the aging process. Epigenetic modifications can result in the cumulative loss of gene regulation over time, ultimately impairing cellular and tissue function. Further, recent data suggest that epigenetic disruption of tissue specific stem and progenitor cells may play a role in cancer development (Feinberg *et al.*, 2006). The correlation between aging and loss of

epigenetic control is consistent with the established connection between aging and cancer (Figure 1.2).

HSCs as a Model for Stem Cell Aging

When studying aging it is important to choose an appropriate model system. For instance, cells (such as skin and blood) that undergo continuous turnover are removed from circulation long before they have time to feel the effects of aging, and certainly long before they could exert an effect on tissue function. The predominant substrates for aging, thus it seems, would be long-lived cells in the organism, namely tissue specific stem cells, since this population is exposed to both intrinsic and extrinsic effectors of aging throughout the lifespan of an individual. Within the last several years, stem cells have been discovered in a growing list of adult tissues. There is new-found appreciation that cell turnover and tissue replacement are not the sole province of the hematopoietic system, the gut lining, and the skin as had been the dogma for many years (Potten and Loeffler, 1990). The limited lifespan of somatic cells implies that a constant replacement of aged cells must occur in order for any tissue to maintain its optimal function. With this in mind, it has been hypothesized that the aging or functional failure of tissue-specific stem cells, which fulfill this job, may limit tissue repair and renewal, therefore contributing to overall organismal aging (Krtolica, 2005; Van Zant and Liang, 2003). Because of the unprecedented experimental model systems that are available for the exploration of HSCs, stem cell aging research in the field of hematology has been the subject of extensive studies. Indeed, the hematopoietic system has served as an important model for advancing our understanding of stem cell biology and its association with aging. It is likely that the same broad concepts that define and characterize blood-forming stem cells will apply to stem cell populations found elsewhere. In view of the importance of HSCs for maintaining immune function (preventing immunosenescence) and in a broader sense tissue homeostasis and longevity, there is a critical need to better understand the mechanisms involved not only in HSC aging, but aging in other stem cell compartments as well.

Evidence for HSC Aging

Despite their ability to self-renew and maintain steady-state hematopoietic function in aged mice and humans, a close examination of the available data shows that age-related changes in the HSC population do occur, ultimately resulting in a decline in stem cell function (Harrison, 1972; Rothstein, 1993). For example, when testing HSC function *in vivo* using competitive repopulation experiments in which the repopulating ability of HSCs is tested in irradiated recipients, adult bone marrow has a decreased ability to repopulate myeloablated recipient mice compared to fetal liver (Harrison *et al.*, 1997). Also, Kim et al. observed a defect in B-cell engraftment and a differentiation pattern skewed toward the myeloid lineage in recipients of transplants from old HSC donors compared to young donors (Kim *et al.*, 2003). In agreement with this finding, microarray analyses comparing the gene expression profile of young and old HSCs showed a marked upregulation of myeloid-associated genes and downregulation of lymphoid genes with age (Rossi *et al.*, 2005). Indeed the increased propensity of HSCs to produce myeloid cells with age may be involved in the increase in myeloproliferative diseases seen in the elderly (Warren and Rossi, 2008). Further, HSCs from old mice are less efficient at homing to and engrafting in the bone marrow of young irradiated recipients (Liang *et al.*, 2005; Morrison *et al.*, 1996). Additional evidence suggests that the proliferative response of murine HSCs to early acting cytokines also declines with age (Henckaerts *et al.*, 2004). Similar results have been observed in human HSCs, in that a progressive decline in function as well as HSC number occurs with increasing age (Figure 1.3) (Lansdorp *et al.*, 1994).

Recent evidence suggests that DNA damage accumulation contributes significantly to the decline in stem cell function with age (Nijnik *et al.*, 2007; Rossi *et al.*, 2007). Rossi et al. examined hematopoietic stem cell function with age in mice deficient in various genomic maintenance pathways, and showed that, although HSC reserves were not depleted with age, stem cell functional capacity was severely compromised under conditions of stress in old versus young cells. They hypothesized that the accumulation of DNA damage in HSCs during aging may be limiting the function of these cells in response to injury. In accordance with their hypothesis, they showed that 82% LT-HSCs from old wild-type mice stained positively for γ- H2AX (an indicator of DNA double-

stranded breaks), while young LT-HSCs were largely devoid of the damage marker (Rossi *et al.*, 2007).

Epigenetic alterations have also been implicated in HSC aging (Chambers *et al.*, 2007b; Rossi *et al.*, 2005). Chambers et al. performed microarray analyses on highly purified HSCs from mice aged 2 to 21 months and found a remarkable downregulation of genes involved in chromatin remodeling and DNA repair. Genes involved in transcriptional silencing via chromatin remodeling (Smarca4 and Amarcb1) as well as histone deacetylases (Hdac1, -5, and -6) and a DNA methyltransferace (Dnmt3b) were downregulated in aged cells. They also showed that several chromosomal regions changed with age in a coordinated manner resulting in an overall increase in transcriptional activity. They propose that chromatin dysregulation and epigenetic changes drive the loss of cellular function and ultimately drive the aging process in HSCs. Consistent with these data, Polycomb proteins (transcriptional repressors), such as Ezh2 and Bmi-1, have been shown to influence self-renewal and cell fate determination decisions in HSCs (Lessard *et al.*, 1998; Park *et al.*, 2003). Ezh2 and Bmi-1 act as transcriptional repressors that stabilize the self-renewing HSC population, thus downregulation of these genes with age would likely compromise the functional quality of the HSC pool.

Another mechanism contributing to the functional failure of HSCs is cellular senescence, a process that results in the irreversible loss of cell division. Many stimuli have been shown to induce the senescence response including, but not limited to, telomere erosion, certain types of DNA damage, such as DNA breaks and oxidative lesions, epigenetic changes to chromatin organization, as well as exposure to ionizing irradiation (Campisi, 2005; Wang *et al.*, 2006). There is increasing evidence that senescent cells accumulate with age. Senescence-associated β-galactosidase, an enzyme commonly used as a marker to detect the senescent phenotype, was shown to increase with age in various mammalian tissues (Krtolica and Campisi, 2002). Since stem cells are capable of self-renewal and produce progeny to replenish worn-out and damaged cells in aged tissues, the induction of stem cell senescence may compromise tissue renewal by depletion of stem or progenitor cell pools and thus promote age-related pathologies.

It is apparent that the HSC compartment undergoes considerable age-related changes, however it is not yet clear whether theses changes are intrinsic to the cells themselves or whether they occur due to alterations in the hematopoietic microenvironment, commonly referred to as the HSC niche. Several studies have shown that the systemic milieu regulates stem cell decline during aging. Liang et al. showed that HSCs have a reduced ability to home to the bone marrow and spleen after transplantation into old versus young recipients (Liang *et al.*, 2005). Further experiments demonstrated that the muscle stem cell niche adversely effects stem cell function as evidenced by the restoration of old stem cell regenerative potential upon exposure to a young systemic microenvironment (Conboy *et al.*, 2005; Conboy and Rando, 2005). It has also been reported that the spermatogonial stem cell niche deteriorates with age, causing the failure to support an appropriate balance between stem cell self-renewal and differentiation and ultimately resulting in age-related infertility in mice (Ryu *et al.*, 2006). It is likely that age-related HSC changes derive from intrinsic alterations as well as extrinsic alterations of specific subtypes of cells in the HSC niche, thus adding another layer of complexity to the stem cell aging process (Figure 1.4).

Genetic Regulation of HSCs

Cell intrinsic aging in B6 and DBA mice

Inbred strains of laboratory mice provide excellent models to study HSC properties, such that strain specific phenotypic differences can be directly attributed to underlying genetic differences in the stem cells. Specifically, the genetic regulation of the age-related decline in stem cell function has been demonstrated extensively by comparing this divergent phenotype among various mouse strains. Stem cells in the relatively long-lived C57BL/6 (B6) mouse strain, measured by *in vitro* CAFC and *in vivo* completive repopulation assays, increase in number during aging, whereas stem cells in the DBA/2 (D2) strain decease over time and display overt signs of aging (Chen *et al.*, 1999; de Haan and Van Zant, 1999; Kamminga *et al.*, 2005; Morrison *et al.*, 1996; Yuan *et al.*, 2005) (Figure 1.5). When equal numbers of bone marrow cells from old and young B6 mice are mixed with standard competitor cells and injected into lethally irradiated

recipients, the old cells have a competitive advantage compared to young cells (Harrison *et al.*, 1989; Morrison *et al.*, 1996). In stark contrast to these results, old stem cell populations in the relatively short-lived D2 mice display a significant competitive disadvantage compared to young cells (Chen *et al.*, 2000; Harrison *et al.*, 1989; Van Zant *et al.*, 1990). In a series of experiments performed by Van Zant et al. (1990) the strain-dependent differences in B6 and D2 stem cell aging were elegantly demonstrated. Eight–cell embryos from B6 and D2 mice were combined *ex vivo* to form chimeric embryos that were implanted into the uteri of pseudopregnant females (Van Zant *et al.*, 1990). All tissues in the resulting chimeric mice consisted of a mixture of B6 and D2 cells. During aging of the chimeric animals, D2 HSC function progressively declined, evidenced by a shift to the B6 genotype in all blood cell lineages by two years of age. The chimeric mice showed complete tolerance to B6 and D2 cells, thus ruling out all possibility that the D2 genotype is selectively eliminated during aging (Mintz and Silvers, 1967). Thus the chimeras allow for simultaneous assessment of two stem cell populations in a common environment, demonstrating cell intrinsic stem cell aging properties in the two genetically different mouse strains. It is probably not a coincidence that D2 hematopoiesis in the chimeras ceased around the time of the strain's natural lifespan. This leaves one to postulate that there may be a link between the viability of HSC populations and the longevity of organisms.

In addition to life span and HSC aging phenotypes, B6 and D2 mice show significant strain-specific variation in multiple HSC paramaters. For example, in contrast to what we observe in old animals, young D2 mice contain higher numbers of hematopoietic stem and progenitor cells when compared to young B6 mice (de Haan and Van Zant, 1999; Geiger *et al.*, 2001). Other HSC strain specific differences include higher proliferation or cycling activity in D2 hematopoietic cells (de Haan *et al.*, 1997; Van Zant *et al.*, 1983), as well as reduced proliferation in response to early-acting cytokines when compared to B6 cells (Henckaerts *et al.*, 2002). Additional studies have shown differences in the percentage of circulating white blood cells, B220+ B cells, as well as CD8+ and CD4+ T cells between B6 and D2 mice (Chen *et al.*, 2002). The myriad differences that exist between the hematopoietic systems of B6 and D2 mice provides a powerful approach to study the genetic control of HSCs.

Forward genetic approach to study stem cell genes

The completion of the mouse and human genome sequencing projects has greatly facilitated our ability to identify and characterize gene function at the level of the whole organism. Specifically, in HSCs, both 'gene-driven' (reverse genetics) and 'phenotype-driven' (forward genetics) approaches have been utilized to identify genes and regulatory pathways that influence stem cell function. Reverse genetics approaches rely on the ability to manipulate the expression of a specific gene in the whole animal using transgenic and knock-out strategies, while forward genetics approaches aim to identify genetic polymorphisms that underlie naturally occurring phenotypes (Appleby and Ramsdell, 2003). Due to the numerous differences in HSC properties that exist between B6 and D2 mice, forward genetics approaches have been extremely successful in identifying quantitative trait loci (QTL) (de Haan *et al.*, 2002; de Haan and Van Zant, 1999; Geiger *et al.*, 2001; Henckaerts *et al.*, 2002) and genes (Liang *et al.*, 2007) that account for strain-specific stem cell properties. The use of recombinant inbred (RI) strains has been vital in our quest to identify QTL and quantitative trait genes (QTG) that underlie various HSC traits. Our lab uses the C57BL/6J cross DBA/2J (BXD) RI strains due to the extensive differences between the hematopoietic systems of the B6 and D2 parental strains from which they were derived. RI lines are formed by the crossing of B6 and D2 parental strains, followed by repeated brother-sister mating (Figure 1.6). During inbreeding, homologous recombination reshuffles the two parental genomes in a distinct fashion, resulting in alleles that become fixed in a unique pattern (de Haan and Williams, 2005). The genome of each BXD strain has been mapped in great detail, thus regions of B6 and D2 derived DNA can be genotyped, resulting in a strain distribution pattern (SDP) for each individual BXD line. Each BXD strain is then phenotyped for the trait of interest to derive a phenotype distribution pattern (PDP). A computer algorithm is used to compare the SDP and PDP and determines the association between a particular trait and B6 or D2 alleles at known marker loci. Significant associations between a trait and a genotype define QTLs that contain the gene(s) that control the trait (Wang *et al.*, 2003). QTLs can only be mapped if the two parental strains have different alleles at a locus of interest, therefore it is necessary to use two strains that show large natural variations in

the parameter of interest (Van Zant, 2003). Once strain specific stem cell phenotypes are identified, linkage analysis using BXD RI strains enables the genetic mapping of the location of the QTL that contributes to the phenotype in question. Using this forward genetic approach, we and others have been very successful at identifying numerous QTL that underlie various HSC traits. QTL on chromosomes 1, 3, and 5 were found to regulate the number of HSCs in young B6 and D2 mice (Geiger *et al.*, 2001), while a locus on chromosome 2 was shown to be the major determinant of stem cell number in old mice (de Haan and Van Zant, 1999). Another QTL on chromosome 18 was linked to the number of stem cells in both young and old animals (Geiger *et al.*, 2001). QTL governing other strain specific HSC traits such as the response of hematopoietic stem and progenitor cells to early acting cytokines (Henckaerts *et al.*, 2002), the fraction of hematopoietic progenitor cells killed by the cytotoxic drug hydroxyurea, as well as the mobilization efficiency of progenitor cells (Geiger *et al.*, 2004) have also been mapped. Interestingly, a QTL that regulates mouse lifespan was mapped to the same location on chromosome 2 that regulates the number of HSCs in old mice (de Haan and Van Zant, 1999; Geiger *et al.*, 2001), suggesting that stem cell pool size may have an effect on longevity and that the same gene(s) may regulate the two traits. More recently, genetic loci involved in controlling gene expression in HSCs have also been identified using a "genetical genomics" approach. Using this strategy, global gene expression data are collected from HSCs isolated from each BXD strain, and mRNA abundance for each gene is treated as an individual trait that can be mapped to a particular region of the genome (Bystrykh *et al.*, 2005). QTLs that are responsible for variation in expression of transcripts can be classified into two groups: *cis*-acting QTLs which are located at the physical position of the transcript itself, and *trans*-acting QTLs that modulate the expression of large numbers of genes throughout the genome. *Cis*-regulated transcripts located in a QTL that controls a particular trait serve as good candidates for quantatittive trait gene identification.

Quantitation and Identification of HSCs and their Progeny

In vitro cobblestone area forming cell (CAFC) assay

It has long been established that the HSC compartment represents a hierarchy of cells, separated on the basis of pluripotency, differentiation potential, and proliferation rate. The CAFC assay is a convenient *in vitro* limiting-dilution assay used to predict the frequency of different hematopoietic cell subsets in this hierarchy growing as "cobblestones" underneath a stromal layer (Ploemacher *et al.*, 1989). A pre-established stromal layer, such as one established by FBMD1 cells, allows for the long-term growth of stem cells in an *in vitro* setting. Primitive stem cells migrate beneath the stromal layer and form colonies (cobblestones) at various time-points over a period of a month or more. Cobblestones are defined as a cluster of at least five non-refractive cells growing beneath the stromal layer. The longer the latency for a cobblestone to appear, the more primitive in nature the cell. Thus, cells that form cobblestones on days 7 to 21 correspond to hematopoietic progenitor cells, while cells that form cobblestones on days 28 and 35, or later, are considered LT-HSCs. CAFC frequencies determined at various culture times show good correlation with stem cell frequencies determined by more expensive and time consuming *in vivo* assays discussed below (Ploemacher *et al.*, 1992; Ploemacher *et al.*, 1991). The CAFC assay is a fast and relatively easy way to quantify stem cells. However, the cell culture conditions used in the assay do not exactly mimic the natural bone marrow microenvironemnt (which has been shown to be very hypoxic) and the assay is not capable of measuring the *in vivo* homing ability of stem cells. Therefore, the gold standard for measuring HSC activity remains *in vivo* repopulation experiments.

In vivo competitive repopulation assays

As mentioned previously, HSCs have two distinct properties; 1) the ability to self-regenerate into cells that are functionally identical to the stem cell from which were derived and 2) they are pluripotent and have the ability to differentiate into all of the eight major lineages of lymphoid, myeloid and erythroid cells. These hallmark HSC properties are measured empirically by their potential to regenerate and sustain the

lymphohematopoietic compartment upon transplantation into lethally irradiated secondary hosts. The first *in vivo* assay for stem cells was introduced by Till and McCulloch (Till and McCulloch, 1961), in which they noticed that cells obtained from the bone marrow or spleen of donor mice formed macroscopic colonies in the spleens of lethally irradiated recipient mice. These spleen-colony forming units (CFU-S) could be enumerated, therefore enabling the frequency of CFU-S in the initial cell inoculation to be quantitated. Although it was later determined that CFU-S were not able to sustain long-term hematopoiesis (Jones *et al.*, 1990), this was the first ground-breaking assay for the functional identification and quantification of HSCs *in vivo*.

The first assay to measure the true multilineage reconstitution potential of HSCs *in vivo* was introduced by Harrison in 1980 (Harrison, 1980). This competitive repopulation assay measures the functional potential of an unknown source of HSCs (test cells) relative to a known number of distinguishable "competitor" cells. When equal numbers of test and competitor cells are co-injected into lethally irradiated mice, theoretically 50% of the hematopoietic cells in the recipient will be regenerated by each population. However, any deviation from the 50% regeneration by one of the donors indicates either a competitive advantage or disadvantage. Although this assay provides information about the repopulating ability of HSCs compared to competitor cells, it cannot distinguish between increased/decreased proliferation of individual HSCs and an actual increase/decrease in absolute HSC numbers. To address this problem the standard competitive repopulation assay was modified to introduce a limiting dilution design, in which graded doses of donor test cells are injected into lethally irradiated, but genetically distinguishable, recipient mice along with a set number of competitor cells to ensure survival of the recipient even when the test cell sample contains little or no repopulating activity (Szilvassy *et al.*, 1990). The number of mice negative for reconstitution in each cell dose is then measured several months later, and the frequency of HSCs (defined as competitive repopulating units, CRU) is calculated using Poisson statistics and the method of maximum likelihood (Szilvassy *et al.*, 1990; Szilvassy *et al.*, 1989).

Both the standard competitive repopulation assay and the CRU assay take advantage of the Ly5 congenic B6 mouse strains whose white blood cells can be distinguished by their expression of the *a* (Ly5.1) or *b* (Ly5.2) forms of the *ptprc*

alloantigen. Typically, wild-type B6 mice expressing the Ly5.2 allele are used as donors and B6.SJL(Boy J) mice expressing the Ly5.1 allele are used for competitor cells and recipients. Test cell (donor) contribution to myeloid and lymphoid cells in the recipient is then quantitated by flow cytometric analysis of cells stained with fluorescently labeled Ly5.1 and Ly5.2 antibodies.

Flow cytometric identification of HSCs

Great advances in flow cytometry over the past several years have allowed the isolation of highly purified primitive stem cell populations. Using fluorescently labeled antibodies directed against specific cell surface receptors, it is possible to identify, quantify, and sort various hematopoietic cell subsets to near purity. Although several approaches have been developed, staining for the presence or absence of cell surface markers is the most commonly used strategy to separate different hematopoietic populations. The general consensus is that primitive stem cells are characterized by the absence of lineage antigens expressed on differentiated cells such as granulocytes, macrophages, B and T lymphocytes, and erythrocytes. Further enrichment of HSCs relies on the presence of Sca-1, a membrane glycoprotein, and c-kit, a tyrosine kinase receptor (Ogawa *et al.*, 1991; Spangrude *et al.*, 1988). The *L*ineage- *S*ca-1+ c-*K*it+ (LSK) cells comprise ~0.1% of the whole bone marrow and are considered to be highly enriched for HSCs.

In the last few years, a new cell surface phenotype based on the presence and absence of SLAM proteins, has been used to purify a population of which ~50% of single cells reconstituted lethally irradiated animals (Kiel *et al.*, 2005). SLAM proteins are a group of 10-11 cell surface receptors that regulate the proliferation and activation of lymphocytes (Howie *et al.*, 2002; Wang *et al.*, 2004). Primitive HSCs are highly enriched within the CD150+ CD48- cells population. Further, SLAM proteins are expressed by many mouse strains and appear to be conserved among HSCs from old, mobilized, and transplanted cells (Yilmaz *et al.*, 2006), thus ensuring the purity of HSCs when examining cells across a variety of contexts.

Another widely used HSC purification strategy relies on the robust ability of HSCs to efflux a variety of compounds (Goodell *et al.*, 1996). Efflux of the fluorescent

DNA binding dye Hoechst 33342 results in a "side population" (SP) of cells that is enriched for HSCs. SP cells were shown to be enriched ~1000 fold for HSC activity, and provided long-tern mulitlineage contribution in lethally irradiated recipients (Goodell *et al.*, 1996). Inclusion of the canonical LSK cell surface markers in combination with the Hoechst 33342 efflux property allows for even further enrichment for HSCs, yielding a population termed SParKLS (LSK cells in the SP) (Lin and Goodell, 2006).

Mapping of a QTL Linked to the Change in HSC Number in Old B6 and DBA Mice

The strain specific HSC aging phenotype between B6 and D2 mice is the focus of this dissertation. As mentioned previously, when using the CAFC assay to measure HSC frequency, our lab found that the stem cell population in long-lived B6 mice increases steadily with age, whereas the frequency of HSCs in the relatively short-lived D2 strain follows a bell shaped curve throughout life, reaching a peak at ~1 year then declining in old age (Figure 1.5) (de Haan and Van Zant, 1999). In order to understand the genetic basis for the variation of HSC numbers in old B6 and D2 mice, we used the aforementioned forward genetic approach and performed genetic linkage analysis in BXD RI strains of mice. Using this approach, we identified a locus on murine chromosome 2 that is significantly linked to the variation in frequency of HSCs in aged B6 and D2 animals. The locus on chromosome 2 was linked to the number of HSCs only in old animals, thus qualifying as a true "aging locus" at which the B6 allele is responsible for the expansion of HSCs in old B6 animals (de Haan and Van Zant, 1999). In order to definitively associate stem cell aging with the QTL identified on chromosome 2, our lab created congenic mice in which the distal region of chromosome 2 containing the QTL was transferred from D2 mice onto a B6 background. The introgressed D2 genomic interval was derived from BXD strain 31 because it phenotypically best demonstrated the decline in HSCs in old age. Using a speed congenic approach, BXD31 mice were backcrossed to the B6 parental strain to expunge D2 DNA located outside of the QTL region (Wakeland *et al.*, 1997). The generation of mice using a speed congenic approach relies on genotyping each generation for the distribution of allelic markers every 10 cM along the entire mouse genome. Mice with the greatest amount of parental DNA and bearing donor alleles at the QTL are then chosen for further backcrossing. The

resulting chromosome 2 congenic mice have a genome that is ~99% B6 with the exception of the introgressed D2 congenic interval spanning 50 Mbp on the end of chromosome 2. Congenic mouse models are very powerful tools when studying QTLs, as they allow for the confirmation that a particular chromosomal locus does influence a particular trait. Essentially one can determine if in fact "the phenotype follows the genotype." We next hypothesized that congenic B.D Ch2 mice (hereafter referred to simply as congenics), in which the QTL interval in B6 mice has been replaced by D2 alleles, would confer the D2 phenotype of decreased HSCs numbers only in aged mice. To test our hypothesis, CAFC d35 frequency was measured in young and old B6 background and congenic mice. At 2 months of age, the frequency of HSCs in congenic bone marrow did not differ significantly from the frequency HSCs in B6 animals. In contrast, at 24 months of age, the frequency of HSCs in congenic bone marrow was a statistically significant 2-fold less than in age-matched B6 mice (Figure 1.7A). Because bone marrow cellularity showed only a modest 20% increase in old animals, the decreased frequency of HSCs in old congenic mice represents a decrease in the absolute number of HSCs in their bone marrow (Geiger *et al.*, 2005).

Because stringent testing of stem cell activity and frequency is best accomplished by *in vivo* transplantation experiments, an additional competitive repopulation experiment was performed in which equal numbers of congenic or B6 bone marrow cells were mixed with an equal number of competitor cells and injected into lethally irradiated mice. In contrast to almost equal contribution to PB at early time points, the contribution of congenic cells to peripheral blood chimerism eleven months post transplant was significantly less than contribution from B6 cells (Figure 1.7B). Thus, *in vitro* and *in vivo* results confirmed the linkage analysis, and demonstrated that the influence exerted by the D2 allele caused a significant reduction in HSC frequency and activity with age (Geiger *et al.*, 2005).

Aging phenotypes in mice are difficult to study, partly because test subjects must age ~2 years before they can be used in an experiment. In order to overcome this hurdle, as well as to determine if DNA integrity influenced stem cell aging, we administered either 1 or 2 Gy of gamma radiation to congenic and B6 animals, a treatment we hypothesized would cause DNA damage and partial depletion of the stem cell pool, thus

creating a prematurely aged the stem cell population (Figure 1.8A). Two weeks post irradiation, we injected the test cells (B6 or congenic) along with an equal number of competitor cells into lethally irradiated mice. As expected, bone marrow cells from mice given no radiation contributed equally to peripheral blood (PB) chimerism in recipient animals up to 20 weeks post transplant. Bone marrow from congenic mice given 1 Gy, which results in ~20-30 chromosomal breaks per genome, displayed an initial defect in contribution to PB chimerism, but by 20 weeks post transplant showed an equivalent contribution to PB as did B6 cells. In contrast, BM from congenic animals receiving 2 Gy contributed only 10% of PB cells at 8 weeks following transplant, and this low contribution remained unchanged for the duration of the experiment, a finding consistent with stem cell exhaustion due to radiation (Figure 1.8B) (Geiger *et al.*, 2005). Thus, although low dose irradiation does not precisely mimic the natural aging process, it does elicit a phenotypic response in young congenic mice that can be measured in a relatively short period of time and confirms the notion that DNA damage in stem cells leads to stem cell aging.

Experimental Goals and Significance

The experimental goal of the work performed in this dissertation was to identify specific gene(s) and molecular pathways underlying HSC aging in two commonly used strains of inbred mice using a forward genetic approach. Three specific aims are proposed to accomplish this goal: 1) to confirm the phenotype of the congenic mouse model in highly purified CD150+CD48- stem cells and to study the mechanistic basis for the difference in HSC numbers in old mice by looking at self-renewal, cell cycle, and apoptosis in B6 and congenic mice using a previously established irradiation study to mimic the natural aging process; 2) to perform microarray analyses using stem and progenitor enriched hematopoietic cells from B6 and congenic mice in order to identify genes that are differentially expressed between the strains that may contribute to the different HSC aging phenotypes; and 3) to overexpress candidate gene(s) identified in the microarray analysis in primary hematopoietic cells and perform functional studies in order to determine whether their overexpression results in quantitative changes in HSC number.

Human HSCs undergo similar age-related changes as do the mouse, thus it is probable that some of the genes and regulatory mechanisms uncovered with our murine model will be applicable to human HSC biology. Therefore these studies are important because they may lead to the identification the genes that regulate and control the number of HSCs in aged humans as well as mice. Further, these studies may identify genes that serve as biomarkers to distinguish an individual's ability to serve as "good" or "bad" bone marrow transplant donors. In view of the importance of stem cells for maintaining immune function and in a broader sense tissue homeostasis and longevity, there is a critical need to better understand the mechanisms involved in HSC aging.

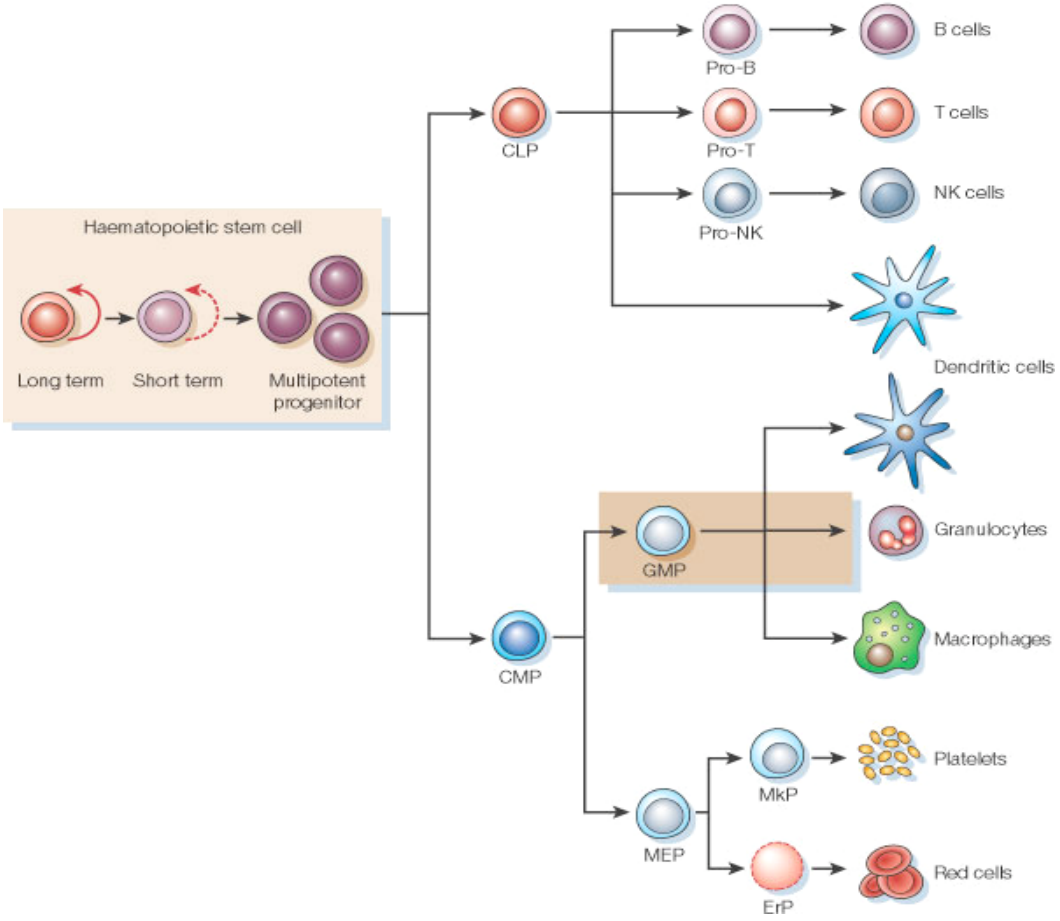


Figure 1.1 The HSC hierarchy.

The HSC compartment can be functionally divided into three populations; long-term HSCs, which have extensive self-renewal capacity, short-term HSCs, which have limited self-renewal capacity, and multipotent progenitor cells which cannot self-renew and give rise to common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CLPs differentiate into pro-B, T, and natural killer (NK) cells, which then give rise to mature B and T cells and NK cells. CMPs differentiate into granulocyte/macrophage progenitors (GMP) and mekaryocyte/erythroid progenitors (GEP). GMPs then give rise to mature macrophages and granulocytes. GEPs differentiate into megakaryocyte

progenitors (MkP) and erythroid progenitors (ErP), which give rise to platelets and erythroblasts, respectively. Both GMPs and CLPs can give rise to mouse dendritic cells. Reprinted by permission from Macmillan Publishers Ltd: [Nature] Reya, T, Morrison, SJ, Clarke MF, Weissman IL. 2001. Stem cells, cancer and cancer stem cells. Nature 414:105-111, copyright (2001).

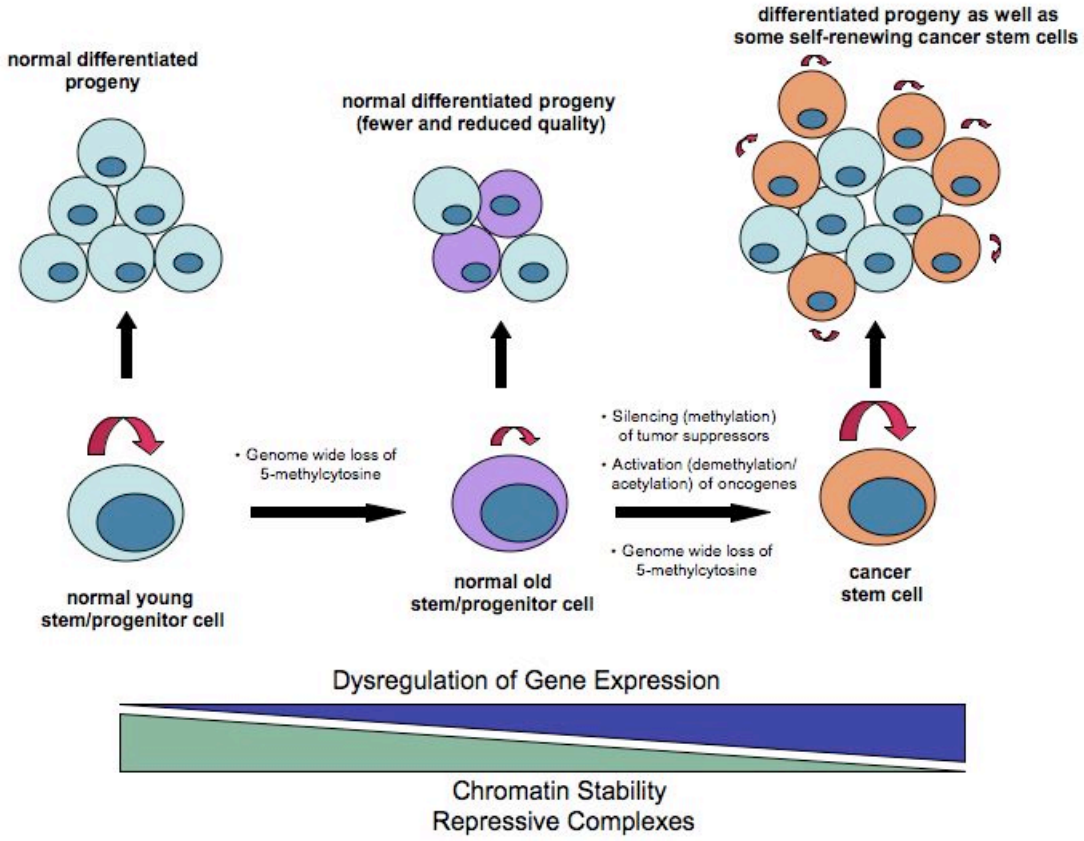


Figure 1.2 Epigenetic changes that accompany aging and tumorigenesis.
Normal young stem cells undergo controlled self-renewal divisions and differentiate to produce large numbers of high-quality mature cells. These well-orchestrated processes rely heavily upon regulated epigenetic modifications of gene expression. However, as stem cells age, their functional attributes decline. They have a reduced capacity for self-renewal and differentiate to give rise to fewer, low-quality daughter cells. A general loss of repression, resulting in dysregulation of gene expression and decreased chromatin stability, also accompany the aging process. It is hypothesized that, in addition to alterations in gene expression and reduced chromatin stability, the aging microenvironment creates conditions permissive to cancer development. Subsequent cancer-promoting epigenetic events, including silencing of tumor suppressor genes and activation of oncogenes promote the transition from a normal to a cancerous state. The cancer stem cell has extensive self-renewal capacity and differentiates to give rise to self-renewing (disease sustaining) as well as terminally differentiated progeny. It is important

to note, however, that the cancer stem cell does not necessarily have to arise from aged stem cell populations, but instead can arise from young stem cells whose normal developmental processes have gone awry. Reprinted by permission from Macmillan Publishers Ltd: [Leukemia] Oakley, E.J., and Van Zant, G. 2007. Unraveling the complex regulation of stem cells: implications for aging and cancer. Leukemia 21:612-621, copyright (2007).

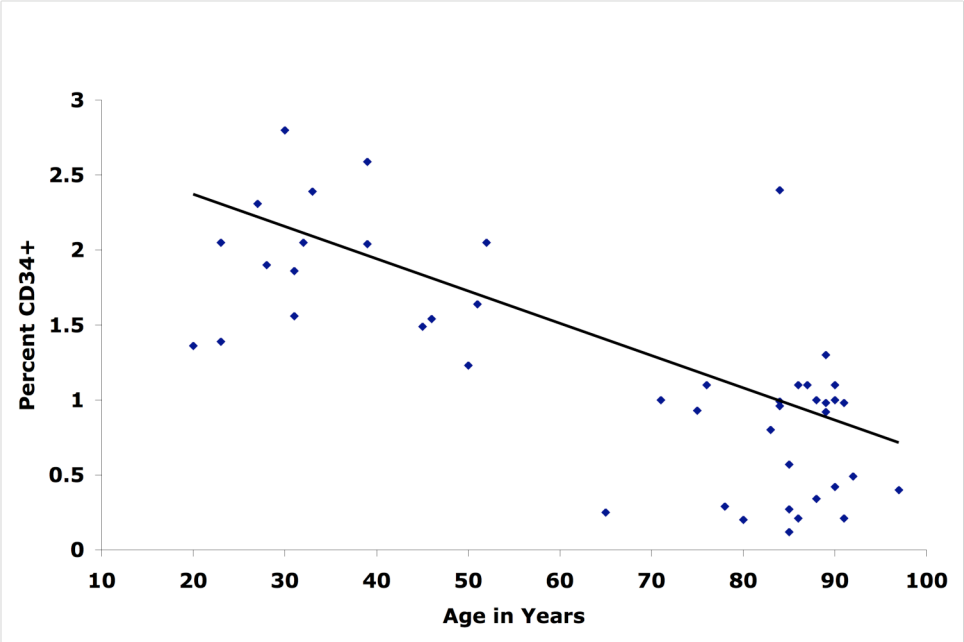


Figure 1.3 Decline in human HSCs with advancing age.

Human HSCs, defined as CD34+ cells, show a steady decline in number as individuals age.

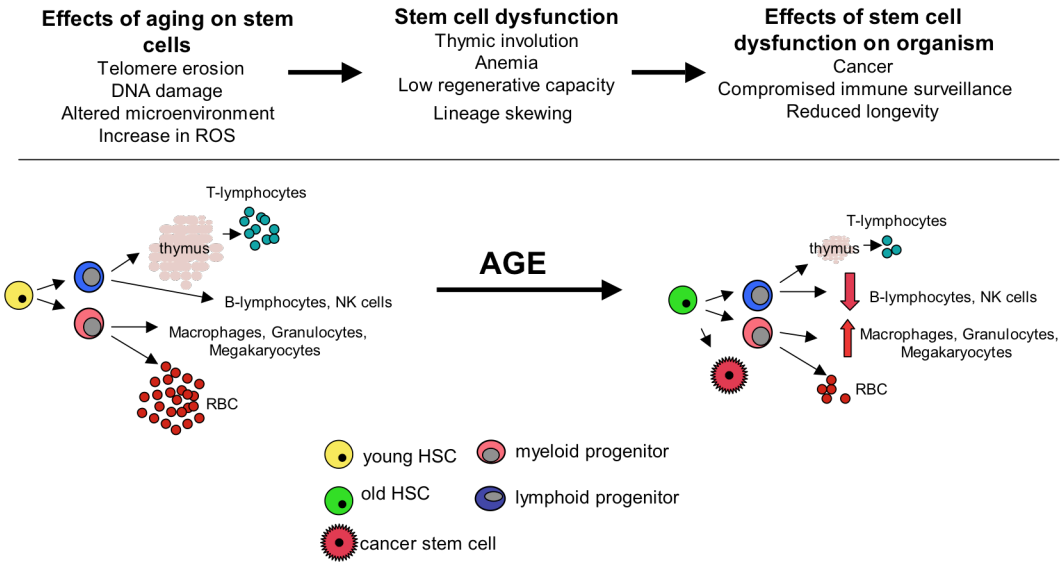


Figure 1.4 Stem cell theory of aging.

Schematic representing the stem cell theory of aging as it pertains to the hematopoietic system. Aging results in the accumulation of damage in the HSC compartment, which in turn leads to compromised function of this system and ultimately reduced health of the entire organism. Adapted from Waterstrat, A., E. J. Oakley, A. Miller, and G. Van Zant. Stem cells and aging. Modified from Telomeres and Telomerase in Aging, Disease, and Cancer. L. Rudolph, ed. Springer, Berlin, 2007.

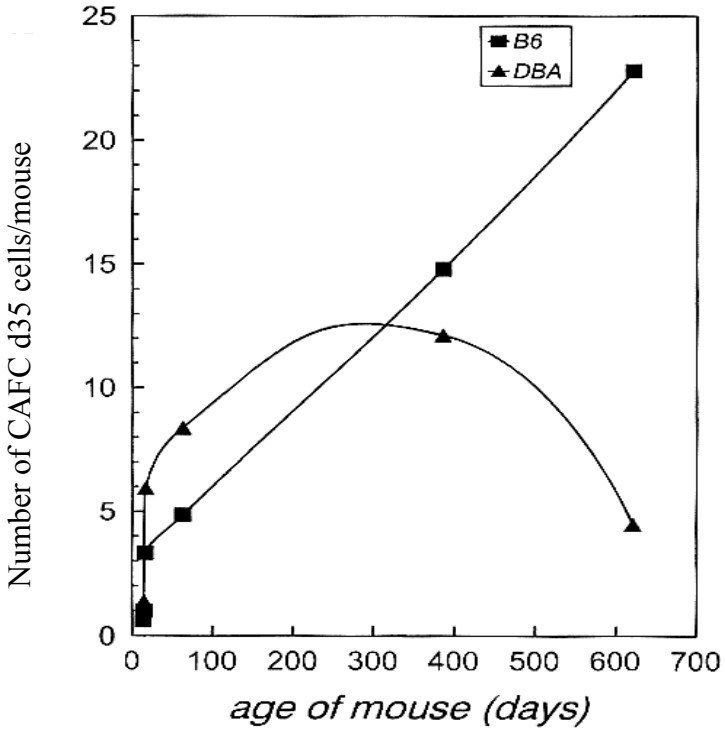


Figure 1.5 Frequency of CAFC day35 cells in young and old B6 and D2 mice.
The frequency of CAFC day35 cells in B6 mice increases in a linear fashion with age, whereas the frequency of CAFC day35 cells in the D2 strain follows a bell shaped curve throughout life, with HSC numbers reaching a peak at ~1 year then declining in old age. Modified from de Haan, G., and Van Zant, G. 1999. Dynamic changes in mouse hematopoietic stem cell numbers during aging. Blood 93:3294-3301.

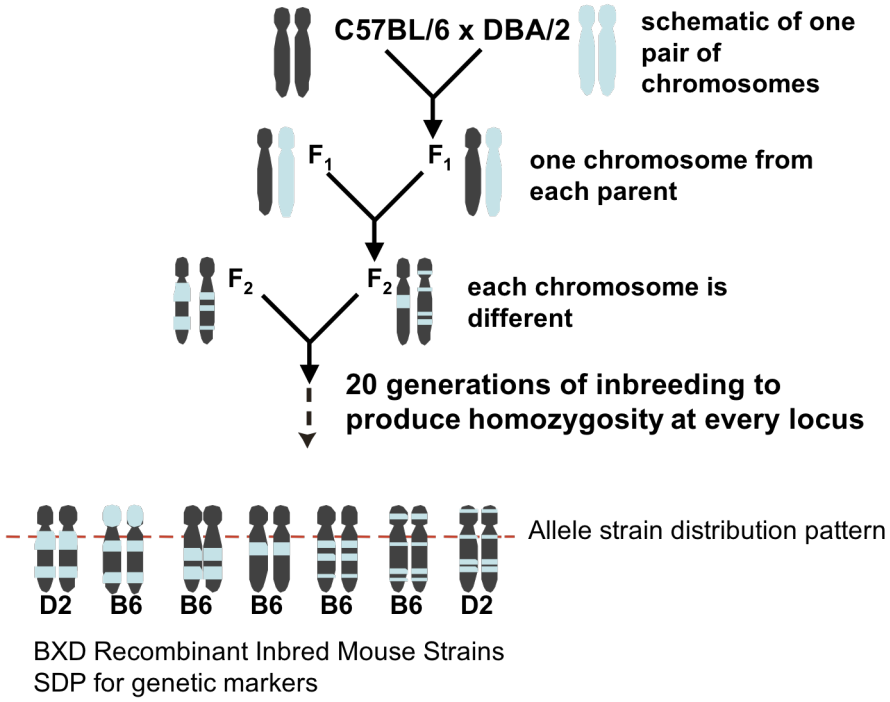
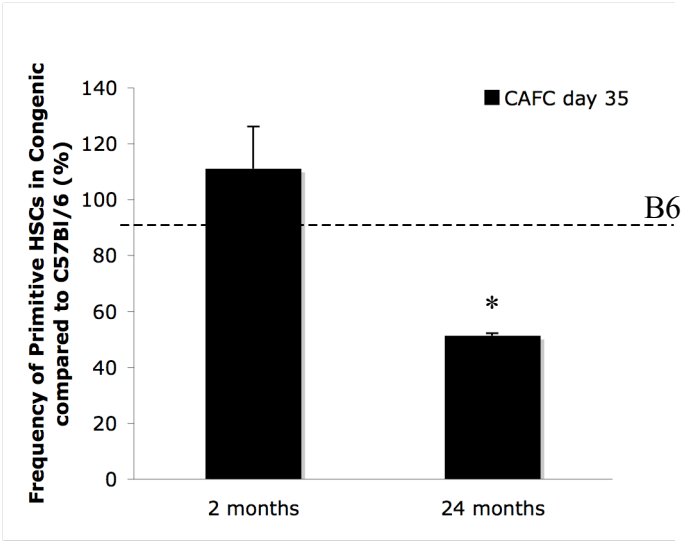


Figure 1.6 Illustration of the derivation of BXD RI strains of mice.
C57BL/6J cross DBA/2J (BXD) RI strains are created by the crossing of B6 and D2 parental strains, followed by repeated brother-sister mating of their progeny for 20 generations. The alleles in each RI line become fixed in a unique fashion, thus each strain becomes a mosaic of the two parental genomes. Phenotypes are correlated with B6 or D2 alleles present at specific markers located throughout the mouse genome. This linkage analysis has enabled the genetic mapping of numerous QTL that influence various HSC phenotypes.

A



B

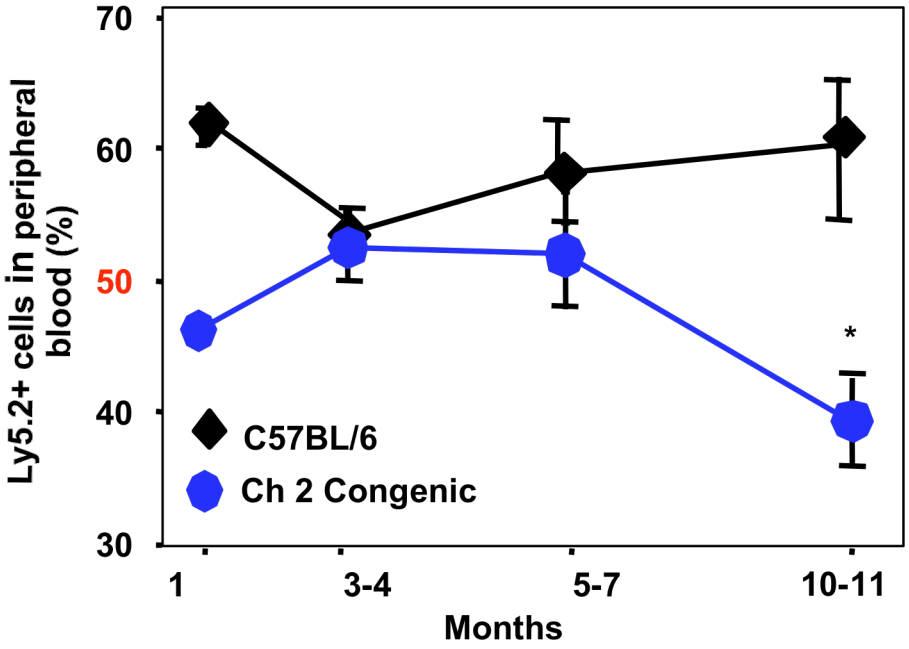


Figure 1.7 *In vitro* and *in vivo* confirmation of the chromosome 2 QTL.
Decreased frequency of LSK cells, as measured by the CAFC assay, in old congenic mice compared with the frequency in old B6 mice ($P < 0.05$) (A). Bone marrow from two animals for each strain was analyzed in six CAFC assays per animal. Equal numbers of competitor and either B6 or congenic bone marrow cells were co-injected into lethally

irradiated recipient mice, and the animals were aged 10 months. Chimerism in the peripheral blood of the recipients was determined at various time points after transplantation. Congenic stem cells displayed a significant competitive disadvantage in aged animals compared to cells in B6 mice ($P < 0.05$) (B). From Geiger, H., Rennebeck, G., and Van Zant, G. 2005. Regulation of hematopoietic stem cell aging in vivo by a distinct genetic element. Proc Natl Acad Sci U S A 102:5102-5107. Copyright (2005) National Academy of Sciences, U.S.A.

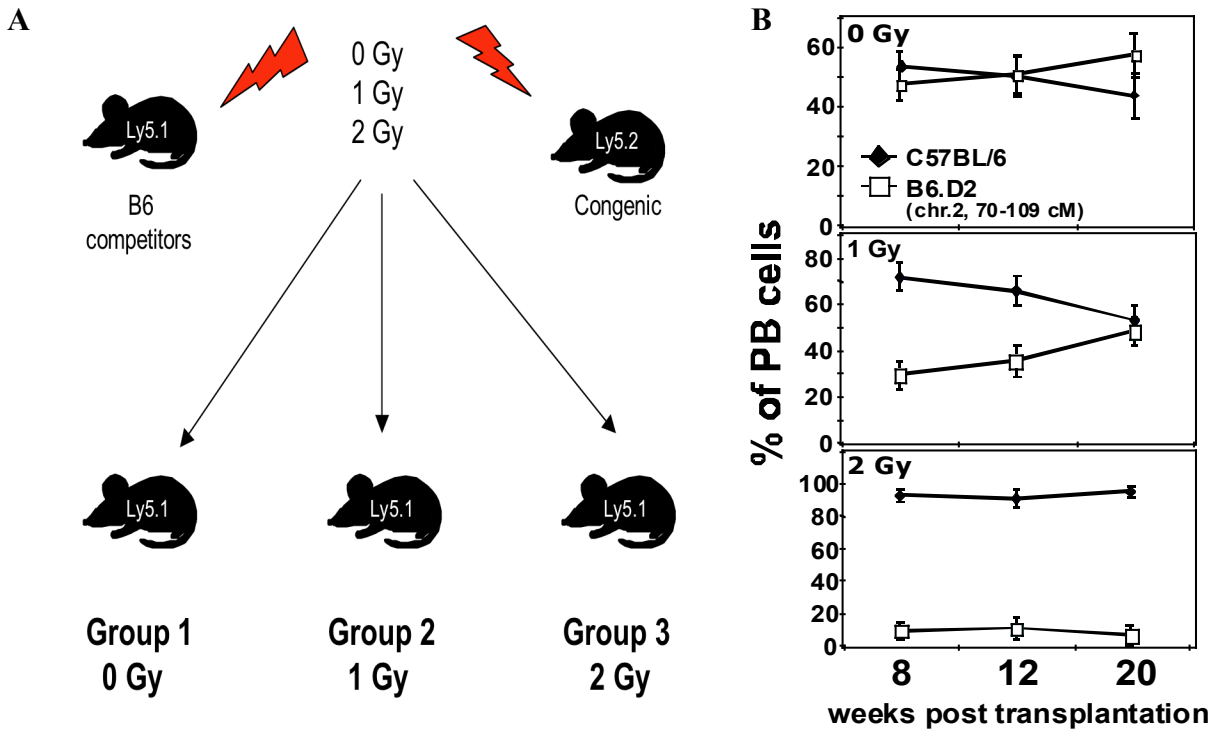


Figure 1.8 Irradiation scheme to mimic HSC aging.

Diagram of the competitive repopulation assay used for these analyses (A). B6 and congenic mice were given 0, 1 or 2 Gy of irradiation, and after a resting period of two weeks, bone marrow cells were harvested and transplanted in equal numbers into lethally irradiated recipient mice. Chimerism in the peripheral blood of the recipients was determined at various time points after transplantation (B). n = 4 mice for each dose. Adapted from Geiger, H., Rennebeck, G., and Van Zant, G. 2005. Regulation of hematopoietic stem cell aging in vivo by a distinct genetic element. Proc Natl Acad Sci U S A 102:5102-5107. Copyright (2005) National Academy of Sciences, U.S.A.

CHAPTER TWO

Analysis of self-renewal, cell cycling, and apoptosis in hematopoietic stem and progenitor cells in congenic and background mice using the surrogate aging assay

Summary

It has previously been established that a quantitative trait locus (QTL) on murine chromosome 2 regulates the change in the number of hematopoietic stem cells (HSCs) in old C57/B6 (B6) and DBA2 (D2) mice. Congenic mice were created by introgressing D2 alleles onto a B6 background, and *in vitro* and *in vivo* studies confirmed that these mice display the D2 phenotype of reduced HSC number and function in old age. Further, a surrogate assay for the HSC aging phenotype was established in which low-dose radiation was administered to young mice to simulate the stress of aging on the HSC population. Young congenic HSCs were found to be extremely sensitive to 2 Gy of irradiation compared to young B6 HSCs. However, the underlying mechanisms that account for differences in HSC number in old B6 and congenic mice as well as the differential radiation sensitivities are not yet known. Therefore in these studies, I investigated the cell cycle, apoptotic, and self-renewal capacity of B6 and congenic HSCs subject to 2 Gy of irradiation using various flow cytometry and serial bone marrow transplantation techniques. I used the surrogate assay to mimic aging with hopes of identifying common mechanisms that may be differentially regulated in aged B6 and congenic mice. Additionally, I further confirmed the phenotype of the congenic mouse model and showed that old congenic mice have 39% fewer CD150+ CD48- than do old B6 mice. I did not detect any significant differences in the percentage of cells in S phase in whole bone marrow, lineage negative (Lin-), or Lin- Sca-1+ c-kit+ (LSK) cells between B6 and congenic mice subject to 2 Gy of irradiation. However, when analyzing the apoptotic status of these cell populations after 2 Gy of irradiation, I demonstrate that B6 Lin- cells undergo significantly more apoptosis (34.7%) than do similarly treated congenic Lin- cells (12.4%). Under steady-state conditions (no irradiation), I observed no difference in the percentage of whole bone marrow, Lin- and LSK cells undergoing

apoptosis between B6 and congenic mice. Finally, serial transplantation experiments revealed a dramatic repopulation advantage in B6 mice treated with 2 Gy of irradiation compared to similarly treated congenic mice in all recipient groups, thus indicating that the D2 alleles in the chromosme 2 QTL confer lower self-renewal capacity of HSCs exposed to 2 Gy of irradiation. In sum these results indicate that the chromosome 2 QTL affects the apoptotic and self-renewal capacity of artificially aged hematopoietic stem and progenitor cells, which may account for the variation in HSC number in old B6 and D2 mice.

Introduction

Hematopoietic stem cells must balance self-renewal, differentiation, and cell death decisions in order to sustain hematopoiesis throughout the lifetime of an organism as well as be poised for extensive proliferation upon demand. The normal fate decisions of stem cells can easily be summarized: a) a stem cell may remain quiescent and not divide at all; b) a stem cell may undergo symmetric cell divisions to generate two new self-renewing stem cells or two new differentiated progeny; c) a stem cell may undergo asymmetric cell divisions and give rise to one new stem cell and one differentiated cell; and finally d) a stem cell may undergo apoptosis and no longer contribute to hematopoiesis (Figure 2.1). There is strong evidence that cell intrinsic factors tightly regulate the survival, differentiation, and proliferation of stem cells, and thus ultimately influence the functional quality and size of the hematopoietic compartment throughout the lifetime of an organism.

The small number of HSCs in an adult mouse must replace approximately 2.4 x 10^8 red bloods cells and 4 x 10^6 non-lymphoid peripheral blood cells each day (Cheshier *et al.*, 1999) as well as be ready to proliferate in response to hematological stresses. According to the clonal-succession hypothesis which was first proposed in 1965, HSCs meet the great demands of hematopoiesis by allowing only one or a few HSC clones to give rise to mature blood cells at any one time (Kay, 1965). According to this model, other HSCs in the pool remain quiescent and do not contribute to hematopoiesis until the proliferative capacity of the active HSC is exhausted. However, Pietrzyk and Bradford later challenged this theory when they analyzed *in vivo* bromodeoxyuridine (BrdU) incorporation rates of day 14 CFU-S and Hoechst low (Ho^{lo}), Rhodanmine^{123} low (Rho^{lo}) enriched HSCs, respectively (Bradford *et al.*, 1997; Pietrzyk *et al.*, 1985). Pietrzyk et al. showed that 75% of progenitors capable of forming day-14 CFU-S had incorporated BrdU over a period of one month (Pietrzyk *et al.*, 1985) and Bradford et al. demonstrated that 89% of $Ho^{lo}Rho^{lo}$ cells incorporated BrdU over a period of 12 weeks (Bradford *et al.*, 1997). Cheshier et al. went on to show that 99% of HSCs incorporated BrdU by the end of six months, thus clearly demonstrating that all HSC do in fact cycle on a regular basis (Cheshier *et al.*, 1999). This high proliferative capacity of HSCs allows for

emergency responses, but under steady state conditions, must be tightly regulated in order to maintain hematopoietic homeostasis.

Perhaps one of the most important factors in sustaining hematopoietic homeostasis and preventing exhaustion of the stem cell pool throughout the lifespan of an organism is maintaining appropriate cell cycle control. Cyclin dependent kinase inhibitors (CKIs) halt cell cycle progression and have been shown to play an important role in stem cell regulation (Cheng *et al.*, 2000; Walkley *et al.*, 2005; Yuan *et al.*, 2004). $p21^{cip1/waf1}$ (p21), a G_1 checkpoint regulator highly expressed in the quiescent fraction of bone marrow cells, has been shown to restrict HSC cycling and thus maintain stem cell quiescence and survival. p21-deficiency increases HSC proliferation and amplifies and the size of the HSC pool under homeostatic conditions in the mouse (Cheng *et al*., 2000). The proportion of quiescent cells in G_0 phase of the cell cycle is reduced in $p21^{-/-}$ mice, and in agreement with these findings, $p21^{-/-}$ mice are more sensitive to 5-flurouracil, a cell cycle specific toxin that kills cycling cells, than wild-type controls. Further, the hematopoietic potential of p21 deficient HSCs was nearly exhausted after two rounds of serial bone marrow transplantation (Cheng *et al.*, 2000). Together these results indicate p21 as a key regulator of stem cell cycling that functions to prevent stem cell exhaustion under prolonged stress (Ezoe *et al.*, 2004).

Unlike p21, $p27^{KIP1}$ (p27) is a CKI whose expression is observed in more mature progenitors cells. The number, cell cycling, and self-renewal of HSCs were found to be normal in p27 deficient mice, however the mice displayed an increase in the number and proliferative capacity of progenitor cells (Cheng *et al.*, 2000). Progenitor cells from p27 null mice were able to expand and regenerate after serial transplantation, thus indicating p27 as an important modulator of the repopulation efficiency but not overall pool size of HSC cells (Cheng *et al.*, 2000). In contrast to p21 studies, yet similar to p27 findings, HSCs deficient in $p18^{INK4C}$ (p18), a member of the INK4 family of CKIs, display diminished cell exhaustion compared to wild-type cells after serial transplantation over three years (Yu *et al.*, 2006). Interestingly, deletion of p18 was able to counteract the accelerated exhaustion of HSC repopulation resulting from p21 deficiency. The advantage imparted to p18 deficient cells is thought to be due to increased self-renewal of the transplanted HSCs in vivo rather than increased donor cell proliferation (Yuan *et al.*,

2004). These studies demonstrate the importance of CKIs in regulating HSC cell cycle kinetics and also indicate the highly divergent effects that cell cycle inhibitors can have on stem cell function.

Unlike p21, p27 and p18, which have not been directly linked to stem cell aging, the stem cell intrinsic effects of $p16^{INK4a}$ have been linked to age-dependent changes in stem cell function. In B6 HSCs, $p16^{INK4a}$ mRNA expression was shown to increase with age. Thus, in order to determine the functional role of this protein, Janzen et al. examined the hematopoietic system of mice deficient in $p16^{INK4a}$ (Janzen *et al.*, 2006). They found that, in contrast to young animals in which no differences were found, the frequency of old HSCs was increased in $p16^{INK4a-/-}$ animals compared to wild-type. They also showed that old $p16^{INK4a-/-}$ HSCs had increased self-renewal capacity when compared to wild-type cells in serial transplantation assays, thus suggesting that the absence of $p16^{INK4a}$ improved HSC function under conditions of stress. They propose that inhibition of $p16^{INK4a}$ may prevent some of the age-related changes that occur in old stem cells.

Several studies suggest that, in addition to cell cycle regulators, apoptosis plays an important role in homeostasis of stem cell numbers both under steady state conditions as well as during the aging process. For example, increased numbers of HSCs were found in the bone marrow of transgenetic mice overexpresssing BCL-2 (an anti-apoptotic protein) in all hematopoietic tissues (Domen *et al.*, 2000). BCL-2 overexpression has also been shown to protect HSCs from radiation- and growth factor deprivation-induced cell death (Domen *et al.*, 2000; Domen *et al.*, 1998). Further, HSCs overexpressing BCL-2 displayed a competitive repopulation advantage in transplantation assay, thus clearly establishing apoptosis as an important mechanism in regulating HSC numbers *in vivo* (Domen, 2001; Domen *et al.*, 2000).

Apoptotic pathways have also been implicated in regulating HSC dynamics in aged stem cell populations. Cellular aging is associated with the accumulation of DNA damage and genomic instability, thus it has been hypothesized that proteins that regulate DNA damage response pathways are key regulators of aging (Dumble *et al.*, 2007). p53 is a tumor suppressor protein that is activated in response to numerous cellular stresses, including DNA damage and oncogenic activation, to induce cell cycle arrest and/or

eventual apoptosis. Mice expressing a hyperactive p53 protein display a heightened tumor resistance, and surprisingly significantly reduced longevity compared to wild-type mice (Tyner *et al.*, 2002). The mutant mice showed generalized organ atrophy and reduced tolerance to stress beginning in middle age that become more apparent in old age. Relevant to this discussion, aged p53 mutant mice also displayed a reduced ability to generate white blood cells after ablation of hematopoietic progenitors with 5-flurouracil. It was hypothesized that reduced HSC function drove the decrease in regenerative capacity. Indeed, subsequent experiments showed that B6 mice expressing hyperactive p53 ($p53^{+/m}$) failed to exhibit age-associated increases in stem cell number (consistent with wild-type B6 mice), and in fact showed a reduced number of HSCs compared to age-matched $p53^{+/+}$ and $p53^{+/-}$ mice. In addition, HSC proliferation was found to be inversely correlated with p53 activity, with $p53^{+/-}$ mice retaining the highest level of proliferative capacity at old age (Dumble *et al.*, 2007). Although the mechanism by which p53 reduces HSC proliferation and functionality is not yet known, a clear role for the pro-apoptotic p53 protein has been established in the HSC aging process. A scenario is favored in which altered p53 dosage affects the self-renewal capacity of HSCs, which overtime results in functional differences in the hematopoietic compartments of mutant and wild-type animals (Dumble *et al.*, 2007).

Perhaps the most important regulators of HSCs are those that govern self-renewal decisions. Self-renewal is crucial for stem cell function because it is required for stem cells to persist throughout the lifetime of the animal. Numerous cell intrinsic transcription factors, transcriptional repressors, and signaling molecules have been indicated as important regulators of stem cell self-renewal and maintenance. For example, homeobox (Hox) genes, which encode transcription factors that regulate segment identity in the developing embryo, have been identified and implicated in the regulation of various stages of hematopoiesis (Abramovich and Humphries, 2005; Abramovich *et al.*, 2005; Lawrence *et al.*, 1996; Lewis, 1978). In particular, HoxB4 has been considered an important regulator of primitive HSCs because it is expressed highly in human CD34+ cells but is rapidly down-regulated upon differentiation (Sauvageau *et al.*, 1994). Overexpression of HoxB4 in the bone marrow compartment was shown to increase HSC numbers, thus suggesting it acts as a positive regulator of HSC self-renewal

(Antonchuk *et al.*, 2002; Krosl *et al.*, 2003; Miyake *et al.*, 2006; Sauvageau *et al.*, 1995). Because maintanence of the HSC compartment is governed by a balance between growth promoting signals as well as signals that promote senescence and apoptosis, one group sought to determine the combined effects of HoxB4 overexpression and p21 down-regulation (Miyake *et al.*, 2006). As mentioned previously, p21 acts to maintain HSCs in a quiescent state, therefore it was hypothesized that p21 deficiency would enhance the HoxB4-mediated increase in self-renewal capacity. Indeed it was found that the simultaneous modulation of p21 and HoxB4 generated a ten- fold increase in the number of repopulating HSCs after 5 days of *ex vivo* culture (Miyake *et al.*, 2006). Another transcription factor, Growth factor independence 1 (Gfi1), controls HSC self-renewal by restricting their proliferation potential (Hock *et al.*, 2004; Zeng *et al.*, 2004). Gfi1 deficiency was shown to increase HSC proliferation and decrease repopulation efficiency during serial transplantation. In fact, Gfi1 is thought to be an upstream regulator of p21, whose expression is decreased in Gfi1 null cells. Factors that govern HSC self-renewal and cellular fate decisions must be tightly controlled both to prevent premature exhaustion and to maintain the integrity of the HSC population.

Clearly, HSC population kinetics are governed by highly orchestrated processes that must remain balanced throughout the lifespan of an organism. The fact that all HSCs are regularly cycling means that the fate of this cell population has to be determined continuously. The regulation of HSC numbers has to be strictly controlled not only to prevent unwanted expansion, but also to prevent premature contraction or aging of this important cell population. Therefore, in order to address the potential mechanisms that contribute to the cell-intrinsic differences in HSC number in old B6 and D2 mice, I investigated the self-renewal, cell cycle, and apoptotic status of young B6 and congenic mice that were given 2 Gy of irradiation. I used a previously established surrogate aging assay, involving low dose irradiation, with hopes of identifying common mechanisms that may be differentially regulated in aged B6 and congenic mice.

Before performing the mechanistic studies I first sought to determine whether the congenic stem cell aging defect was observed in a CD150+ CD48- cell population. Recently, it has been shown that HSCs can be highly enriched using a simple combination of antibodies directed against the SLAM family member proteins CD150

and CD48 (Kiel *et al.*, 2005; Kim *et al.*, 2006; Yilmaz *et al.*, 2006). CD150+ CD48- bone marrow cell populations contain 21% long-term multi-lineage reconstituting cells when intravenously injected into lethally irradiated mice (similar to results obtained with LSK cells) and it has been shown that SLAM family receptor expression is conserved among young and old HSCs (Kiel *et al.*, 2005; Yilmaz *et al.*, 2006). Our lab has previously shown that D2 alleles in the chromosome 2 QTL confer a decrease in CAFC day 35 cells in old age, as well as an *in vivo* competitive disadvantage when compared to B6 alleles (Geiger *et al.*, 2005). To further confirm the phenotype of the congenic mouse model and to ensure that I am studying functionally similar cells in young and old mice, I measured the number of CD150+ CD48- cells in the bone marrow of these mice relative to that of B6.

Materials and Methods

Animals

Young (2-4 months) and old (24-25 months) C57BL/6 (B6) ($Ptprc^b$ [CD 45.2]) mice were purchased from the National Institute of Aging (NIA) Aged Mouse Colony at Harlan Sprague Dawley (Indianapolis, IN). Chromosome 2 congenic mice, with a genetic interval from D2 mice spanning 50 Mbp (130Mbp -180 Mbp) on the distal end of chromosome 2, were generated as described previously (Geiger *et al.*, 2005). Young (2-4 months) and aged (24-27 months) congenic animals were used in the experiments. B6.SJL ($Ptprc^a$ [CD 45.1] were purchased from the Jackson Laboratories and were used as adoptive recipients in serial transplantation assays. All mice were female and were housed in the Division of Laboratory Animal Resources (DLAR) at the University of Kentucky Chandler Medical Center and were maintained under pathogen-free conditions according to NIH guidelines for animal welfare.

Immunofluorescent staining of cells and flow cytometry

Mice were euthanized by CO_2 asphyxiation and the femora and tibiae were removed to a Petri dish containing Hanks Balanced Salt Solution (HBSS, GIBCO) supplemented with 2% fetal bovine serum (FBS, Invitrogen). Femora and tibiae were flushed with 4 mL media to obtain a single cell suspension. Mononucleated cells were obtained via Ficoll gradient separation and Fc receptors were subsequently blocked with anti-CD16/32 (clone 2.4G2, Fc Block) to prevent non-specific staining. For Lineage negative (Lin-) isolation, cells were identified by the lack of cell surface antigens characteristic of cells committed to individual lineages of blood cell development. The lineage cocktail consisted of the following antibodies: CD5 (clone 53-7.3) and CD8a (clone 53-6.7) to identify T cells, CD45R/B220 (clone RA3-6B2) to identify B cells, CD11b/Mac-1 (clone M1/70) and Ly-6G/Gr-1 (clone RB6-8C5) to identify macrophages, and Ter119/Ly-76 (clone Ter-119) to identify erythrocytes. All antibodies were biotinylated, and immunofluorescent staining was achieved with streptavidin-fluorescein isothiocyanate (SA-FITC). Light density bone marrow cells were stained with the above lineage cocktail as well as stem cell specific cell surface markers, phycoerythrin (PE)-

conjugated Ly-6A/E (Sca-1; cloneE13-161.7) and allophycocyanin (APC)-conjugated CD117 (c-kit; clone ne2B8) to identify a population enriched in stem cells (Lin-, Sca-1+, c-kit+ cells or LSK cells). In order to isolate rare CD150+ CD48- cells, whole bone marrow was centrifuged over a Ficoll gradient to enrich for mononucleated cells, and the recovered low-density cells were then labeled with antibodies directed against the SLAM receptors. CD150+ CD48- cells were identified by staining with PE-conjugated anti-mouse CD150 (clone TC15-12F12.2) and FITC-conjugated hampster antimouse CD48 (BCM). In order to determine the number of CD150+ CD48- cells per 2 femora and 2 tibiae, cell counts were performed following Ficoll gradient centrifugation on a hemocytometer. Cell counts were then divided by the number of mice used in the experiment and then multiplied by the percent total CD150+ CD48- cells as determined by flow cytometry staining, thus yielding the total number of CD150+ CD48- cells per 2 femora and 2 tibiae. For each cell preparation, dead cells were excluded by propidium iodide (PI) staining. All monoclonal antibodies were purchased from BD Pharmingen. Cells were analyzed and sorted on a triple-laser FacsVantage (Becton-Dickinson) flow cytometer.

Cell Cycle Analyses

Cell cycle analyses were carried out in young B6 and congenic mice using the *in vivo* BrdU incorporation assay (Dolbeare *et al.*, 1990). BrdU is a thymidine analog that is incorporated into newly synthesized DNA by cells entering and progressing through the S phase of the cell cycle. BrdU can be stained with anti-BrdU fluorescent antibodies in cells to determine the frequency of cells in a population that are synthesizing DNA. Mice were injected intraperitoneally with BrdU (1 mg per kg of body weight) at various time points (0h, 6h, 16h, 3d, 7d) following 2 Gy of gamma irradiation (whole body) delivered from a ^{137}Cs source Mark 1 Irradiator (J.L. Shepherd and Associates) at a dose rate of 1.73 Gy per minute. One-hour post injection the mice were sacrificed and their bone marrow LSK cells were enumerated as described above. Analysis of BrdU incorporation in whole bone marrow, Lin-, and LSK cells was performed using a BrdU Flow Kit (BD Pharmingen) according to the manufacturer's directions. Cells in S phase were identified as having a DNA content intermediate between diploid and tetraploid and

staining positive for BrdU. Flow cytometric analysis was performed on a FACSVantage (Becton-Dickinson). For a given time point, we performed a 3 way ANOVA with strain, cell cycle phase (GO/G1, S, G2/M), and cell population (WBM, Lin-, LSK) as factors. We then determined if there were significant inter-strain differences for a particular time and cell cycle phase.

Apoptotic analysis of cells

Apoptosis is characterized by a variety of morphological features such as blebbing of plasma and nuclear membranes, aggregation of nuclear chromatin, and disruption of cytoskeletal architecture (Israels and Israels, 1999). In the early stages of apoptosis, phosphatidylserine (PS), a membrane phospholipid, is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment (Martin *et al.*, 1995). Annexin V is a Ca^{2+} dependent phopholipid binding protein that has high affinity for PS, and is useful for identifying apoptotic cells with exposed PS (Vermes *et al.*, 1995). Annexin V conjugated to FITC will be used as a probe for flow cytometric analysis of cells undergoing apoptosis.

Young B6 and congenic mice were given 0 or 2 Gy of gamma radiation (whole body) from a ^{137}Cs source Mark 1 Irradiator (J.L. Shepherd and Associates) at a dose rate of 1.73 Gy per minute 2 hours prior to sacrifice. Bone marrow cells were isolated and stained with LSK antibodies as described. Additional staining with FITC-conjugated Annexin V (BD Pharmingen) and 7-AAD (BD Pharmingen) enabled the identification of apoptotic cells. Cells that stain positive for Annexin V and negative for 7-AAD are undergoing apoptosis (Vermes *et al.*, 1995). Three independent experiments were performed using each strain for each treatment (0 versus 2 Gy radiation), with at least two mice for each experiment. Flow cytometric analysis was performed on a triple laser FACSVantage flow cytometer (Becton-Dickinson).

Serial Transplantation Assay to Measure HSC Self-Renewal

Young B6 and congenic mice were irradiated with 0, 1, or 2 Gy of gamma radiation (whole body) from a ^{137}Cs source Mark 1 Irradiator (J.L. Shepherd and Associates) at a dose rate of 1.73 Gy per minute three days prior to sacrifice. Whole

bone marrow cells were isolated from young B6 and congenic mice as described above. These cells (CD45.2) served as "test" cells for individual transplantation assays. Equal numbers ($1x10^6$) of test cells were admixed with CD45.1 competitor cells and injected into lethally irradiated (9 Gy) CD45.1 primary recipients. Marrow was harvested from the primary recipients 20 weeks post transplant and injected into secondary recipients. An identical regimen was repeated once more. At each transplant, peripheral blood, whole bone marrow, Lin-, and LSK cells were analyzed. For peripheral blood anaylsis, mice were bled from the retroorbital sinus under isofluorane anesthesia prior to sacrificing by CO_2 asphyxiation. Erythrocytes were depleted by hypotonic lysis and leukocytes were stained with FITC-conjugated monoclonal anti-CD45.2 (clone ALI4A2) and PE-conjugated monoclonal antibodies (Pharmingen) specific for B (anti-CD45R/B220; clone RA36B2) and T lymphocytes (anti-Thy1.2; clone 30H12) or granulocytes (anti-Ly6G/Gr-1; clone RB6-8C5) and macrophages (anti-CD11b/Mac-1; clone M1/70). Cells were analyzed using a FACScan flow cytometer (Becton-Dickinson). Bone marrow Lin- and LSK cells were stained and analyzed as described above. The self-renewal capacity was determined by comparing the percentage of B6 and congenic derived CD45.2+ cells in recipients at each round of transplantation.

Results

CD150+ CD48- Cells in Congenic Mice

In order to ensure that the HSC aging phenotype is observed in functionally equivalent cell populations, we measured the number of CD150+ CD48- cells in the bone marrow of young and old B6 and congenic mice. As expected, at a young age, I found no significant difference ($P > 0.05$) in the number of CD150+ CD48- cells per 2 femora and 2 tibiae between B6 and congenic mice ($38 \times 10^3 \pm 8 \times 10^3$ cells per mouse and $23 \times 10^3 \pm 9 \times 10^3$ cells per mouse, respectively). However, in old mice, replacement of the B6 alleles at the QTL with D2 alleles in the congenic interval caused a significant decrease in the absolute number of CD150+ CD48- cells per 2 femora and 2 tibiae. Old B6 mice contained 103×10^3 ($\pm 14 \times 10^3$) CD150+ CD48- cells per 2 femora and 2 tibiae whereas old congenic mice contained 59×10^3 ($\pm 8 \times 10^3$) CD150+ CD48- cells per 2 femora and 2 tibiae, a 43% decrease ($P \leq 0.05$) (Figure 2.2A). Moreover, on the basis of frequency, light density marrow from old B6 mice contained 0.25% ± (0.03%) CD150+ CD48- cells, 39% more than that for old congenic mice (0.18% (±0.01%) ($P \leq 0.01$) (Figure 2.2B). The fact that the absolute number of CD150+ CD48- cells increased in old congenic mice compared to young probably reflects the fact that regulation of stem cell aging is a complex quantitative trait that is controlled by multiple loci, in addition to the chromosome 2 QTL, and that body size increases ~50% from 2 months to 2 years. Therefore old congenic mice display an intermediate phenotype when compared to old B6 and D2 mice.

Cell Cycle Analyses

Cell cycling has the ability to influence HSC numbers in two ways; 1) increases or decreases in the number of actively cycling cells can either increase or decrease the total number of HSCs, and 2) increases or decreases in the time it takes to proceed through the cell cycle can subsequently increase or decrease HSC numbers. However, even though increased stem cell cycling has the potential to increase HSC numbers, exhaustion of HSC self-renewal potential, resulting in terminal differentiation or senescence, is an inevitable outcome of a large number of cellular divisions. Additional

studies suggest that engraftment efficiency may be correlated with different phases of the cell cycle. Decreased engraftment potential was observed when HSCs were progressing through S/G2/M phases of the cell cycle, thus suggesting that active cycling negatively correlates with engraftment potential (Bowie *et al.*, 2006; Dykstra and de Haan, 2008; Fleming *et al.*, 1993; Habibian *et al.*, 1998; Vermes *et al.*, 1995). Having previously shown that congenic mice are severely compromised after 2 Gy of irradiation in a transplant setting, I hypothesized that there would be an increase in the number of congenic HSCs in S phase following irradiation, and that this increase in cycling would cause replicative exhaustion of their stem cell pool, and perhaps explain why they cannot sustain hematopoiesis in a competitive transplant setting. Increased cell cycling after a stressor such as irradiaton, may also be indicative of the cells inability to activate appropriate cell cycle checkpoints. This hypothesis can also be extended to old congenic mice, in that increased cell cycling in old HSCs (or throughout the lifespan of the organism) would lead to exhaustion of the stem cell pool as well as decrease engraftment potential in a competitive transplant setting. In order to address the role of the chromosome 2 QTL in HSC cell cycle kinetics, I measured the proportion of HSCs in S phase of the cell cycle using the BrdU incorporation assay (Dolbeare *et al.*, 1990). Young B6 and congenic mice were given 2 Gy of irradiation and the percent of cells in S phase in whole bone marrow, Lin-, and LSK cells were measured at 0h, 6h, 16h, 3d, and 7d following irradiation (Figure 2.3A). I found no significant difference in the percentage of cells in S phase in whole bone marrow, Lin- and LSK cells between young B6 and congenic mice under steady-state conditions (no irradiation), as well as at all time points tested following 2 Gy of irradiation (Figure 2.3B). Congenic cells, like B6 cells were able to respond appropriately to stress by increasing cell proliferation in all cell populations. Approximately 4-5% of LSK cells (in both strains) stained positive for BrdU 1 hour following *in vivo* intraperitoneal injection (Figure 2.4A). As expected, almost 90% of LSK cells were in G0/G1 phase of the cell cycle under steady-state conditions, indicated by the lack of BrdU staining as well as by having a diploid content of DNA (Figure 2.4B). Six hours following irradiation, I observed a slight but non-significant decrease in the percent of B6 LSK cells in S phase compared to an increase in congenic LSK cells (Figure 2.4A). Sixteen hours post irradiation the percent of LSK

cells in S phase in both strains increased, with a concurrent decrease in the percent of LSK cells in G0/G1 phase, again demonstrating that the congenic LSK cells do not have a proliferation defect (Figure 2.4A and B). As with B6 cells, congenic LSK cells remained in a high state of proliferation at least one week following irradiation, while the percent of cells in G0/G1 remained low in comparison to steady state levels. This inverse relationship in the percent of LSK cells in S phase and percent of cells in G0/G1 illustrates how the poised LSK cells are activated to enter the cell cycle after stress in order to replace the differentiated cells that were eliminated. Interestingly, six hours post irradiation we observed a large, but non-significant, increase in the percent of B6 cells in G2/M phase of the cell cycle compared to congenic cells (Figure 2.4C). This increase was transient, as by sixteen hours post irradiation the percent of B6 cells in G2/M returned to almost steady-state levels. Three days post irradiation treatment, we saw no significant difference in the percent of LSK cells in G2/M between B6 and congenic mice. However, seven days after treatment, the percent of LSK cells in G2/M in congenic mice returned to steady-state levels, whereas the percent of LSK cells in G2/M in B6 mice remained elevated compared to pre-treatment levels (Figure 2.4C).

Apoptosis Analyses

Numerous studies have shown that apoptosis plays an important role in maintaining HSC homeostasis (Janzen *et al.*, 2006; Opferman *et al.*, 2005; Rodrigues *et al.*, 2005), yet few have addressed the involvement of apoptosis in the aged hematopoietic system. As stem cells age, they accumulate increasing amounts of macromolecular and DNA damage likely due to the replicative stress that occurs over the long lifespan of the cell. Damaged cells in the aged HSC compartment undergo apoptosis in order to rid the pool of dysfunctional cells that would then give rise to damaged progeny or would perhaps become tumorigenic. The same ensues when the cells are exposed to stressors, such as low-dose radiation or chemotherapeutic drugs. Thus, we addressed the question of whether or not congenic HSCs undergo higher levels of apoptosis compared to B6 HSCs, perhaps contributing to the decreased number of stem cells as the mice age. To determine whether D2 alleles in the QTL influence the apoptotic response in irradiated congenic animals, we assessed annexin V staining of whole bone marrow, Lin-, and LSK

cells (Figure 2.5A). Young (2-4 months) B6 and congenic mice were given 0 or 2 Gy of irradiation and after two hours the mice were sacrificed and their bone marrow cells were analyzed for apoptosis. We found no significant differences in annexin V staining in whole bone marrow, Lin-, and LSK cells between B6 (3.9% ± 2.4%, 4.5% ± 1.0%, 4.14 ± 2.1%, respectively) and congenic (2.9% ± 3.1%, 2.9% ± 2.0%, 2.1% ± 2.2%, respectively) mice under steady-state conditions (no radiation) (Figure 2.5B). Similarly, we found no significant variation in annexin V staining in whole bone marrow and LSK cells from B6 (8.7% ± 4.1% and 8.2% ± 6.6%, respectively) and congenic (4.0% ± 2.4% and 13.9% ± 5.0%, respectively) mice 2 hours post 2 Gy of irradiation. However, in the Lin- cell population, we observed a significant (~ 3 fold) increase ($P = 0.01$) in annexin V staining in B6 (34.7% ± 3.2%) mice post irradiation treatment compared to congenic (12.4% ± 8.6%) animals. We also observed increases in Annexin V staining in all cell populations tested except congenic whole bone marrow cells post 2 Gy of irradiation.

Self Renewal Analyses

A signature feature of stem cells is their ability to undergo self-renewing divisions, an attribute that is best tested by the serial transplantation of bone marrow cells through a series of lethally irradiated recipients. We therefore tested the self-renewal capacity of congenic HSCs in serial transplantation experiments in order to determine whether the difference in HSC numbers in old congenic and B6 mice was due to decreased self-renewal capacity of the congenic cells. One or 2 Gy of irradiation was administered to young (2 months) B6 and congenic mice, which we hypothesized would cause significant replicative stress, thus creating an "aged" stem cell population. We chose to irradiate cells with 1 Gy in addition to 2 in hopes of identifying an intermediate phenotype. After 3 days, the mice were sacrificed and their bone marrow cells were harvested and transplanted into lethally (9.0 Gy) irradiated CD45.1 recipients along with and equal number of CD45.1 competitor cells (Figure 2.6). Twenty weeks post transplant, the primary recipients were sacrificed and their bone marrow cells were harvested and injected into lethally irradiated secondary recipients without the addition of competitor cells, and such serial transplantation was continued once more. At each transplant, the peripheral blood and bone marrow of the primary, secondary, and tertiary

recipients were analyzed by staining the cells with antibodies directed against CD45.1, CD45.2, c-kit, sca-1, as well as the Lin- cocktail described above, therefore allowing us to examine the percentage of donor derived cells (CD45.2) in the stem (LSK) and progenitor (Lin-) compartments. Greater self-renewal capacity of HSCs from one strain relative to the other is reflected by the percentage of donor derived cells (either B6 or congeic). In primary, secondary, and tertiary recipients, we saw no significant difference in the percentage of CD45.2+ cells between B6 and congenic mice given no irradiation in peripheral blood as well as in the bone marrow nucleated cells (Figure 2.7). In contrast, in recipient mice injected with cells receiving 2 Gy of irradiation, we observed significantly less CD45.2+ congenic cells in primary recipient bone marrow cells and in secondary and tertiary recipient peripheral blood and bone marrow cells (Figure 2.7). Further, contribution to mature peripheral blood cell lineages (macrophages/granulocytes, B cells, and T cells) from congenic mice subject to 2 Gy of irradiation was compromised compared to similarly treated B6 mice in all three recipient groups (Figure 2.8). Thus, serial transplantation of bone marrow cells demonstrates that 2 Gy of irradiation imparts a stress that severely compromises the self-renewal capacity of congenic cells compared to B6 cells, and that this decline in cellular function is intrinsic to the stem cells.

Surprisingly, in recipient mice injected with cells receiving 1 Gy of irradiation, I observed significantly more CD45.2+ congenic cells in secondary recipient peripheral blood and bone marrow compared to CD45.2+ B6 cells (Figure 2.7). Similarly, I also observed higher contribution to all mature peripheral blood cell lineages in secondary recipients that received cells from congenic mice irradiated with 1 Gy compared to recipients that received cells from B6 mice irradiated with 1 Gy (Figure 2.8). However, despite contributing ~50% to overall CD45.2 chimerism, the percentage of LSK cells derived from congenic mice treated with 1 Gy of irradiation was significantly lower in secondary recipients compared to LSK cells derived from similarly treated B6 mice. This indicates that LSK cells derived from congenic mice treated with 1 Gy of irradiation may differentiate rather than self-renew after the irradiation insult, which is reflected in the large number of differentiated cells but lack of LSK cells in secondary and tertiary recipient mice.

Discussion

HSCs from young congenic mice display no apparent hematopoietic defects compared to HSCs from young B6 mice, whereas old congenic stem cells display overt signs of aging. However, using a surrogate aging assay, we found that we could uncover hematopoietic defects in young congenic mice simply by treating them with irradiation, which causes significant DNA damage in the stem cell pool. However, it remains to be determined why old and irradiated congenic HSCs are functionally compromised compared to age matched or similarly treated B6 HSCs. I therefore measured the proliferative, apoptotic, and self-renewal capabilities of young congenic mice treated with 2 Gy of irradiation. The unavailability of large numbers of old congenic mice prevented the same analyses from being carried out in these animals. Although low dose irradiation does not precisely mimic the aging process, it does elicit a measurable phenotypic response in the congenic mice, which we hypothesize will be recapitulated in old mice as well.

I first showed that while there is no difference in the number of CD150+ CD48- cells between young B6 and congenic mice, old congenic mice have significantly fewer CD150+ CD48- cells compared to old B6 mice. This finding is important for two reasons; (1) it demonstrates that the difference in stem cell number in aged mice can be extended to highly purified primitive stem cell populations, and (2) it excludes the possibility of measuring the frequency of contaminating, non-HSCs in old mice, and ensures that we are analyzing the phenotype in functionally similar cells populations. The fact that I did not detect variation in the frequency of young stem cells demonstrates that D2 alleles at other loci are responsible for this variation. It is not practical to obtain enough CD150+ CD48- cells for use in mechanistic studies due to the fact they make up such a small percentage of the total bone marrow population. Therefore in my studies I rely on the absence of lineage markers and the presence of Sca-1 and c-kit (LSK cells) to denote a population enriched in primitive HSCs. CD150+ CD48- cells and LSK cells display similar levels of long-term multilineage reconstitution potential, therefore I feel that the use of LSK cells represents the best possible cell population in which to study HSC properties.

In order to measure the proportion of HSCs in S phase of the cell cycle at various time points following 2 Gy of irradiation, I performed *in vivo* BrdU labeling studies. Consistent with published data, approximately 4-5% of LSK cells stained positive for BrdU 1 hour following *in vivo* intraperitoneal injection, and no significant differences were observed between B6 and congenic mice under steady-state conditions (no irradiation) (Cheshier *et al.*, 1999; Morrison *et al.*, 1995). As expected almost 90% of LSK cells were in G0/G1 phase of the cell cycle under steady-state conditions, indicated by the lack of BrdU staining as well as by having a diploid content of DNA. Although all HSCs cycle regularly, this method only measures the fraction of HSCs replicating their DNA during a one hour pulse of BrdU, which explains why only a small fraction of HSCs were found to be in S phase. Nonetheless, this finding is consistent with the idea that a small number of HSCs are always dividing in order to maintain steady-state hematopoiesis.

I chose to look at the fraction of cells in S phase at various time points following irradiation in order to determine how hematopoietic cells from B6 and congenic mice respond (in terms of cell cycle kinetics) to stress. At all time points following irradiation, I observed no significant difference in the percentage of WBM, Lin-, and LSK cells in S phase between the two mouse strains, thus presumably excluding the possibility that congenic and B6 hematopoietic cells exhibit differences in cell cycle kinetics following irradiation. In contrast to WBM and Lin- cell populations, the percentage of LSK cells in S phase in both strains remain elevated by 2- to 3- fold at least 7 days following irradiation. Although no significant differences between the percentage of HSCs in S phase were observed, a higher percentage of congenic LSK cells stained positive for BrdU compared to B6 cells prior to and at all time points following irradiation. This slightly higher cycling activity could potentially result in earlier exhaustion of the congenic HSC pool and contribute to the decreased competitiveness seen in transplantation assays. Moreover, the subtle increase in congenic LSK cycling may be indicative of their inability to activate cell cycle checkpoints in response to cellular stressors. In this scenario, congenic LSK cells may accumulate DNA damage, which over time could lead to functional defects in their stem cell population. If cell cycle checkpoint control were compromised throughout the lifespan of an organism, the stem

cell pool would eventually become cluttered with damaged cells, ultimately resulting in an overall decline in function in this cell population with age.

Next, I analyzed the percentage of cells undergoing apoptosis in the bone marrow compartment of B6 and congenic mice exposed to irradiation. Presumably, increased levels of apoptosis in congenic LSK cells could contribute to the decreased numbers of stem cells observed in old mice as well as contribute to the irradiation sensitivity phenotype of congenic LSK cells if they undergo apoptosis after irradiation exposure. Surprisingly, I was unable to detect any profound differences in percentages of WBM and LSK cells undergoing apoptosis between B6 and congenic mice. In both strains, we found that a higher percentage of Lin- cells were undergoing apoptosis compared to LSK cells. This finding is in agreement with other studies, which suggest that progenitor cell populations are more prone to apoptosis compared to more quiescent stem cells (Niedernhofer, 2008; Park and Gerson, 2005; Reya *et al.*, 2001). However, I found that B6 Lin- cells undergo significantly more apoptosis than do congenic Lin- cells after 2 Gy of irradiation. This finding was unexpected, as I hypothesized that increased apoptosis would correlate with decreased HSC functionality. However, the higher percentage of B6 Lin- cells undergoing apoptosis compared to congenic Lin- cells following irradiation may have several causes. First, the higher percentage of apoptosis in the B6 Lin- population supports the idea that B6 cells have a heightened DNA damage response compared to congenic cells. Following cellular damage, B6 progenitor cells may be able to activate checkpoint responses more efficiently than congenic cells, thus enabling them to either repair DNA damage or undergo apoptosis. This in turn would protect the B6 hematopoietic compartment from giving rise to cells with damaged DNA, and thus ultimately protect the long-term function of this cell population. This scenario is consistent with our cell cycle data: congenic cells continue to cycle even when exposed to DNA damaging events instead of activating cell cycle checkpoints or undergoing apoptosis. Inappropriate activation of cell cycle checkpoints and/or induction of apoptosis in response to stress would cause the congenic HSC pool to accumulate damaged cells. These damaged cells may eventually undergo growth arrest and/or become senescent, and thus ultimately contribute to the decreased function the congenic hematopoietic system in aged mice. Indeed, several recent papers indicate that DNA damage accumulation in

HSCs results in a decline in their cellular function (Niedernhofer, 2008; Nijnik *et al.*, 2007; Rossi *et al.*, 2007). I hypothesize that altered cell cycle checkpoint control, resulting in improper cell cycle exit and induction of apoptosis, leads to extensive DNA damage accumulation in congenic Lin- and LSK cells, and thus likely contributes to age associated hematopoietic defects.

Another contributing factor to increased B6 Lin- apoptosis after irradiation is the increased transcript expression of BCL2-like 11, as was later revealed by our microarray analyses. BCL2-like 11, also referred to as Bim, is a BH3-only protein member of the Bcl-2 family of proteins (O'Connor *et al.*, 1998). Bim acts as an apoptosis facilitator and has been shown to play an important role in the apoptosis of early hematopoietic progenitors (Kuribara *et al.*, 2004).

Hematopoietic homeostasis relies on the presence of a functional HSC pool which is maintained throughout the life time of an animal by self renewing stem cell divisions. Given that the primary role of adult stem cells is to replenish aged and functionally impaired tissues, this self-renewal capacity must be maintained during the aging process in order to ensure maximal longevity. In order to determine the effects of the chromosome 2 QTL on HSC self-renewal, I performed serial transplantation experiments, which serve as the most stringent test of HSC potential. I hypothesized that congenic mice exposed to 2 Gy of irradiation (and by extension aged mice as well) would display a stem cell self-renewal defect compared to B6 mice. Defects in self-renewal ability would provide a mechanistic basis as to why the number of congenic HSCs declines during normal aging.

As expected, under steady state conditions (no irradiation), I saw no significant difference in the percentage of CD45.2+ cells from B6 and congenic mice in peripheral blood and bone marrow nucleated cells in all three groups of recipients (Figure 2.7). However, when analyzing the hematopoietic compartment of recipients that received cells irradiated with 2 Gy, I found significantly fewer congenic CD45.2+ cells in the bone marrow of primary, secondary, and tertiary recipients as well as in the peripheral blood of secondary and tertiary recipients 20 weeks post-transplant compared to B6 CD45.2+ cells. Having determined that there is no dramatic difference in the proliferation rate of B6 and congenic HSCs, I next determined whether or not there was a difference in the

number of B6 or congenic derived LSK cells in the recipient mice, thus leading to the competitive repopulation differences that we observed with each serial transplant. Indeed, results from analyzing the bone marrow of recipient mice revealed that significantly fewer LSK cells were derived from congenic cells treated with 2 Gy of irradiation compared to B6 cells in secondary and tertiary recipients. Thus, the competitive disadvantage of the congenic cells treated with 2 Gy of irradiation results from a lower stem cell frequency in recipient mice. Virtually no LSK cells derived from congenic cells treated with 2 Gy of irradiation were observed in secondary and tertiary recipient mice, indicating that the replicative stress imparted after the first transplant resulted in stem cell exhaustion. I hypothesize that the same scenario would ensue in aged congenic LSK cells, in that they would have a severe self-renewal defect when compared to old B6 LSK cells. Indeed, a decreased congenic self-renewal capacity may explain the decrease in stem cell number in these mice during normal aging.

The fact that congenic cells treated with 1 Gy of irradiation contributed significantly more to secondary recipient peripheral blood and bone marrow nucleated cells than did similarly treated B6 cells was not expected. However, it may suggest that B6 differentiated cells are sensitive to irradiation up to some threshold approaching 2 Gy, after which they may activate DNA damage response and repair pathways. Indeed, I observed that B6 Lin- cells undergo significantly more apoptosis after 2 Gy of irradiation compared to congenic cells. Apoptosis may be the mechanism by which they purge their hematopoietic compartment, thus preserving the self-renewal capacity and ensuring maintenance of a high quality HSC pool over time. It may also suggest that congenic LSK cells chose to differentiate rather than self-renew after 1 Gy of irradiation. This scenario would explain why we observed high contribution to overall CD45.2 chimerism and mature blood cells, but a very low percentage of LSK cells in all recipient groups injected with congenic cells. After 2 Gy of irradiation the congenics have already exhausted their LSK pool, thus explaining why I observed virtually no LSK cells as well as differentiated cells in secondary and tertiary recipients. The B6 cells, on the other hand preserve their may LSK pool after 1 Gy at the expense of producing differentiated cells. By the time that they are hit with 2 Gy of irradiation they have enough LSK cells to both maintain the pool as well as differentiate into mature blood cells. Therefore, at

least in B6 and our congenic mice, it is vital to preserve a primitive stem cell pool when faced with cellular stressors, at the expense of differentiated cells, in order to survive the insult. Additionally, the fact that the B6 cells treated with 1 Gy of irradiation reconstituted tertiary recipients as well as congenic cells, suggests that the preservation of their LSK pool in secondary recipients was sufficient to rescue their hematopoietic defect observed in secondary recipients. It is possible however that the LSK cells under these conditions (irradiation as well as serial transplant) have lost fidelity of the markers used to identify them. In this scenario, the LSK cells present in B6 mice after 1 Gy may not be functionally similar to LSK cells under normal conditions. This may explain why both B6 and congenic cells were able to contribute equally to peripheral blood chimerism in tertiary recipients, even though 1 Gy of irradiation apparently eradicated the congenic LSK cells in secondary recipients.

In sum, I demonstrated that D2 alleles in the chromosome 2 QTL reduce the number of CD150+ CD48- cells in old mice compared to B6 alleles. In addition, D2 alleles in the QTL blunt the ability to purge damaged Lin- cells after exposure to 2 Gy of irradiation as well as abolish the repopulating capacity, and hence self-renewal, of congenic cells exposed to 2 Gy of irradiation. I hypothesize that old cells would perform similar to irradiated cells in cell cycle, apoptosis, and self-renewal assays, and am currently testing this hypothesis using aged congenic mice.

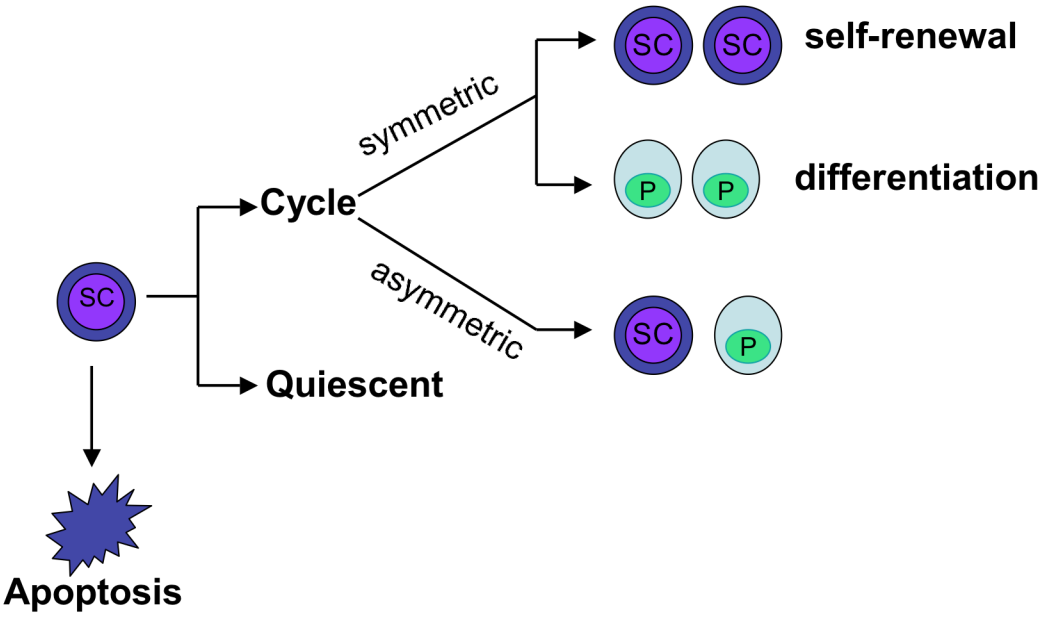


Figure 2.1 Cellular fate decisions of stem cells.

There are four possible cell fate decisions for stem cells: 1) a stem cell may remain quiescent and not divide at all; 2) a stem cell may undergo symmetric cell divisions to generate two new self-renewing stem cells or two new differentiated progeny; 3) a stem cell may undergo asymmetric cell divisions and give rise to one new stem cell and one differentiated cell; and 4) a stem cell may undergo apoptosis and no longer contribute to hematopoiesis. SC = stem cell, P = progenitor cell.

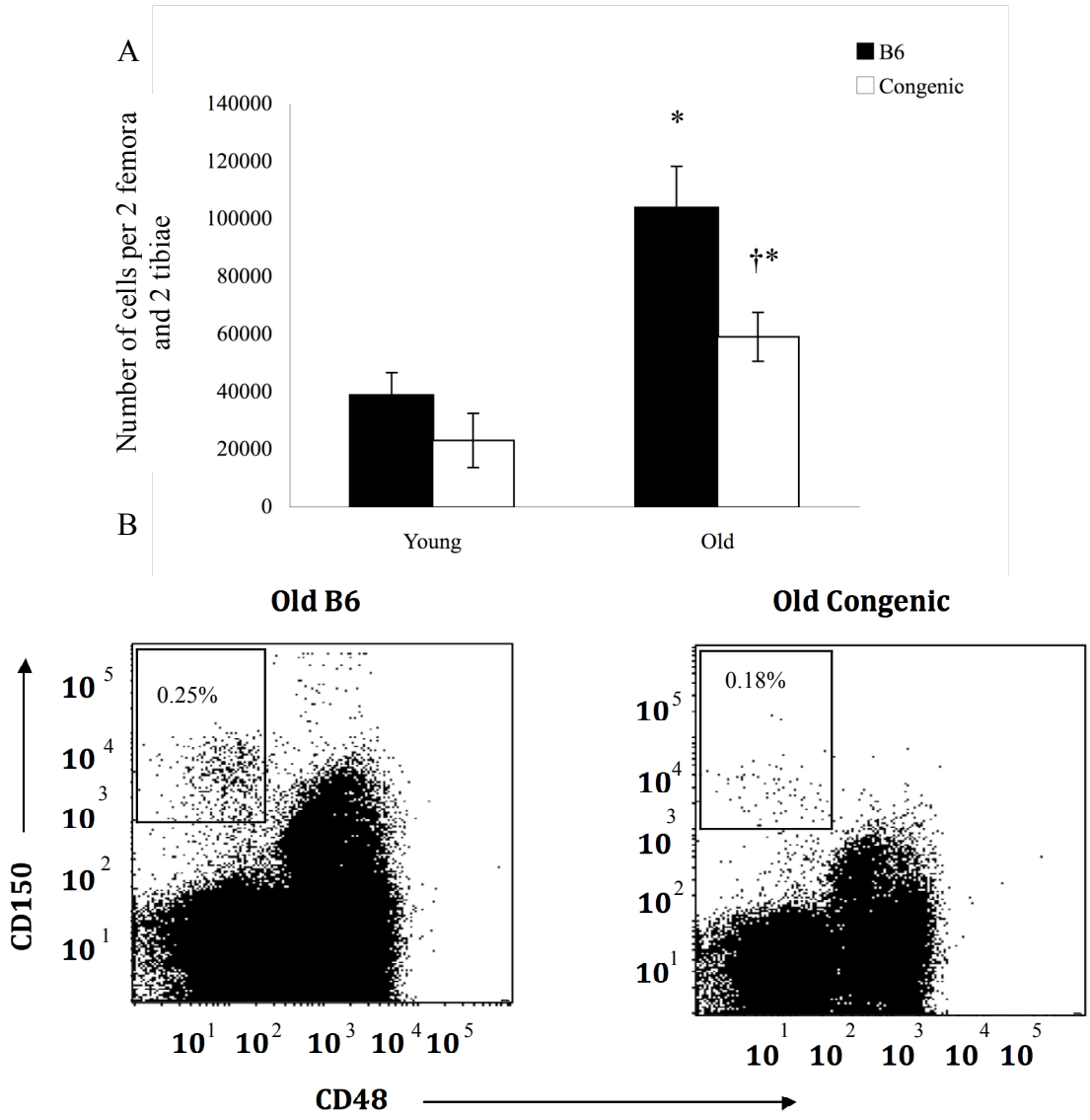


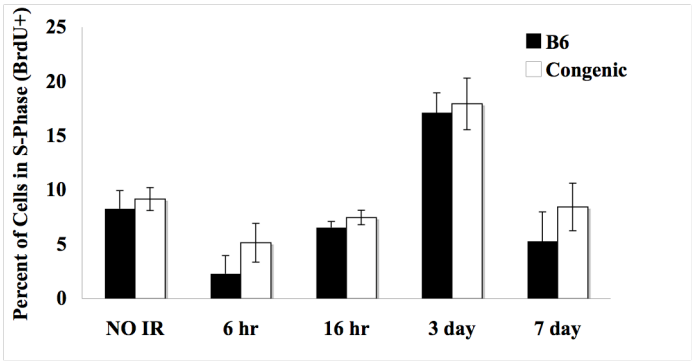
Figure 2.2 The number of CD150+ CD48- cells per 2 femora and 2 tibiae in young and old B6 and congenic mice as determined by flow cytometry. At a young age, there is no significant difference in the number of CD150+ CD48- cells between B6 and congenic mice. As the mice age, B6 mice accumulate significantly more CD150+ CD48- cells per 2 femora and 2 tibiae than do congenic mice (A). Results are given as mean ± SEM (n ≥ 5 mice, three replicate experiments). Representative dot plots of bone marrow cells showing the percentage of CD150+ CD48- cells in old congenic and B6 mice (B). Old congenic mice have significantly

less CD150+ CD48- cells compared to old B6 mice. On average B6 mice have a higher percentage of CD150+ CD48- cells (0.25% ± 0.03%) compared to old congenic mice (0.18% ± 0.01%) ($P \leq 0.01$). The plots represent one experiment where 3-4 mice were pooled. † indicates statistical significance for comparison of old B6 and congenic cells ($P \leq 0.05$). * indicates statistical significance for intra-strain comparison of cells as a function of age ($P \leq 0.05$). Results are given as mean ± SEM.

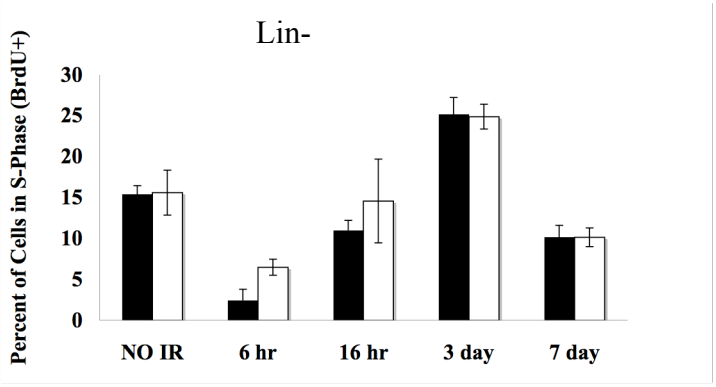
A

B

WBM



Lin-



LSK

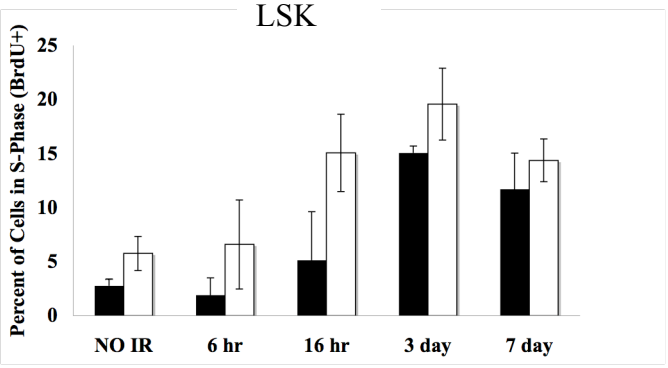


Figure 2.3 Analysis of cell cycling in three hematopoietic cell populations following irradiation treatment. Schematic representing the time scheme used for irradiation treatment (A). Irradiation was administered to B6 and congenic mice 6 hrs, 16 hrs, 3 days**,** and 7 days prior to sacrifice. One hour before the mice were sacrificed, 150 mg per kg of body weight of 5-flurouracil was intraperitoneally injected into the mice. I observed no significant difference in the percentage of cells in S-phase (BrdU+) between B6 and congenic WBM, Lin-, and LSK cells at all time points tested following irradiation treatment ($P > 0.05$) (B). Three replicate experiments were performed using 3-5 mice per strain for experiments. The pooled results are given as mean ± SEM.

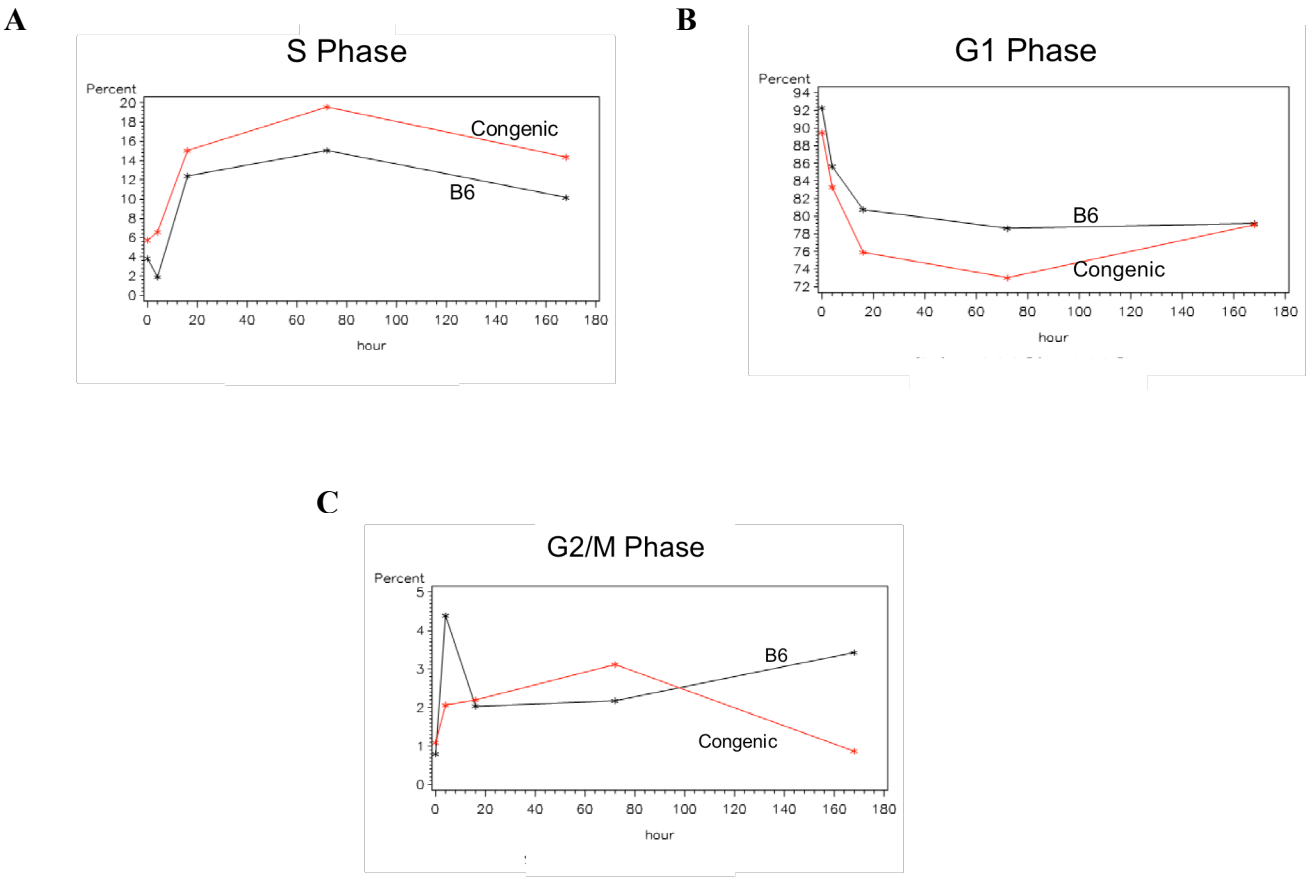
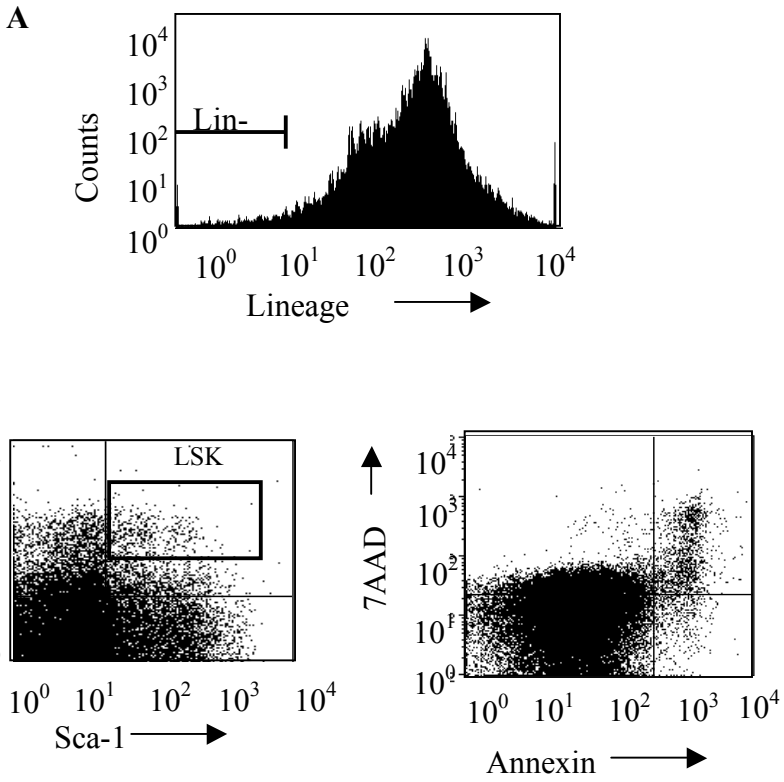


Figure 2.4 Profiles of the percent of LSK cells in various phases of the cell cycle following irradiation treatment. Percent of LSK cells in S phase of the cell cycle prior to (0 hr) and at several time points following irradiation (A). Approximately 4-5% of LSK cells (in both strains) were in S phase under steady-state conditions, however this percentage increased at various timepoints following irradiation. A higher percentage of congenic LSK cells stained positive for BrdU at all time points tested. Approximately 90% of LSK cells were in G0/G1 phase of the cell cycle under steady-state conditions, however this percentage decreased in both strains following irradiation (B). Six hours post irradiation we observed a large, but non-significant, increase in the percent of B6 cells in G2/M phase of the cell cycle compared to congenic cells (C). This increase was transient, as by sixteen hours post irradiation the percent of B6 cells in G2/M returned to

almost steady-state levels. Seven days after treatment, the percent of LSK cells in G2/M in B6 mice remained elevated compared to the percent of LSK cells in G2/M in congenic mice (C).


A
Counts
Lin-
Lineage
LSK
c-kit
Sca-1
7AAD
Annexin

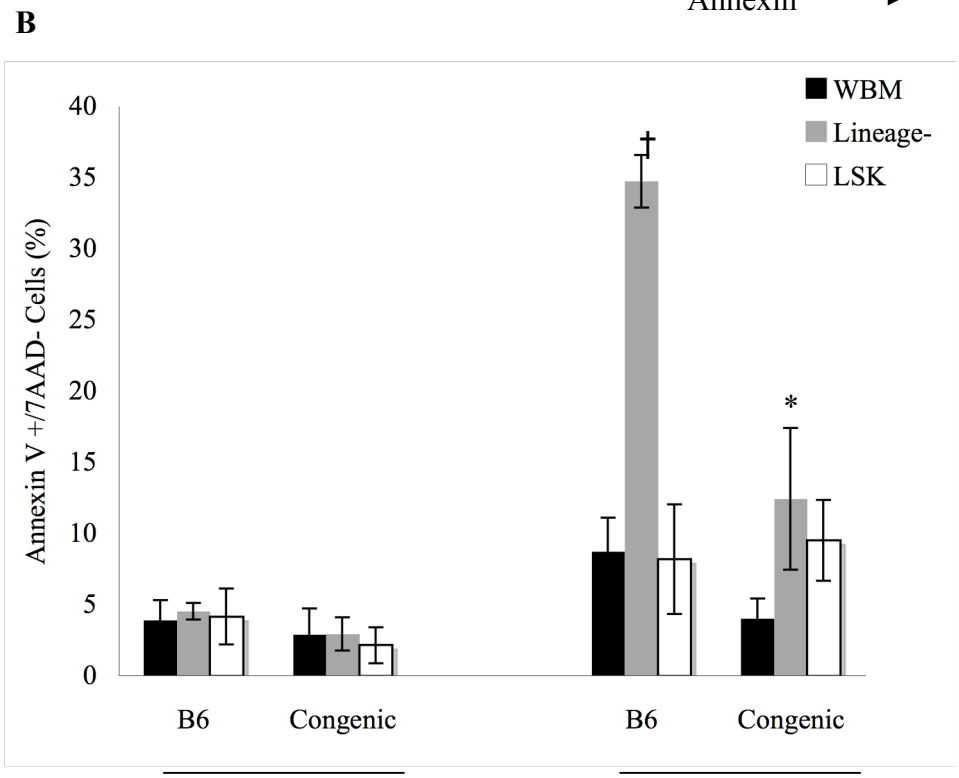

B
WBM
Lineage-
LSK
Annexin V +/7AAD- Cells (%)
B6
Congenic
B6
Congenic
No Irradiation
2 Gy

Figure 2.5 Identification of Annexin V positive/7AAD negative apoptotic HSCs. Cells were stained with a lineage cocktail and a gate was set to identify Lin- cells. Further gates were set to detect sca-1+ and c-kit+ cells. The resulting Lin- sca-1+ c-kit+ (LSK) cells were then analyzed for Annexin V and 7AAD staining. Flow cytometry plots are from one representative animal (A). No differences in the percentage of apoptoic cells between B6 and congenic mice exist under steady state conditions (B). A significant increase in apoptosis was observed in the B6 Lin- population post 2 Gy of irradiation compared to congenics (B). † indicates statistical significance for comparison of B6 Lin- cells before and after irradiation ($P \leq 0.01$). * indicates statistical significance for comparison of B6 and congenic Lin- cells after irradiation ($P = 0.01$). Results are given as mean ± SEM (n ≥ 2 mice, three replicate experiments).

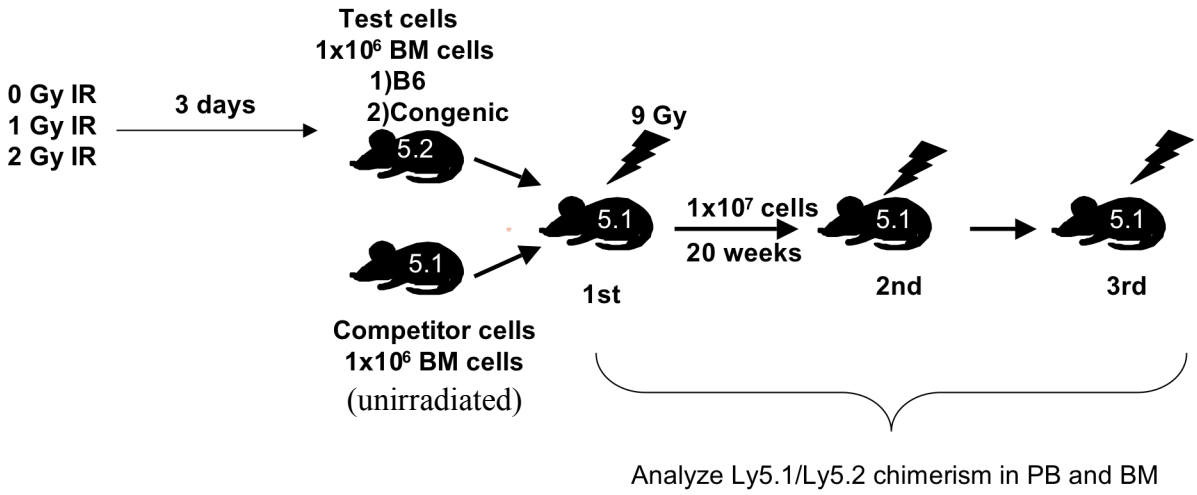


Figure 2.6 Self-renewal analysis of B6 and congenic HSCs pre- and post irradiation treatment. B6 and chromosome 2 congenic mice were subject to 0, 1 or 2 Gy of irradiation and after 3 days bone marrow was harvested and injected in lethally irradiated primary recipients. After 20 weeks, primary recipients were sacrificed and harvested bone marrow was injected to secondary recipients. This regimen was repeated once more. At each transplant, the peripheral blood and bone marrow of the primary, secondary, and tertiary recipients were analyzed. n = 4-5 mice for each recipient group.

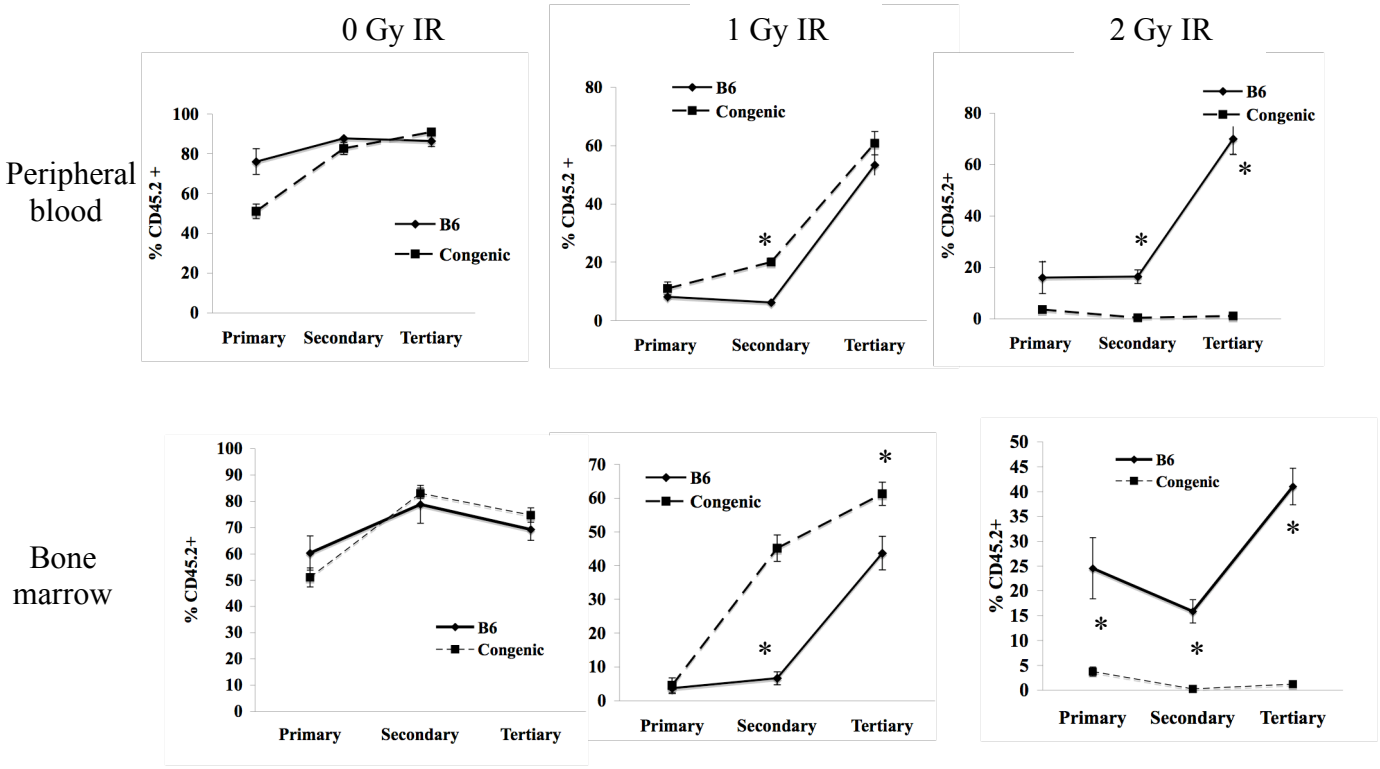


Figure 2.7 Self-renewal analysis of HSC in serial bone marrow transplantation. B6 and congenic derived peripheral blood leukocytes and bone marrow nucleated cells treated with various doses of irradiation were analyzed 20 weeks after they were transplanted into primary, secondary, tertiary recipients. Each data point represents the mean for groups of mice ($P \leq 0.05$).

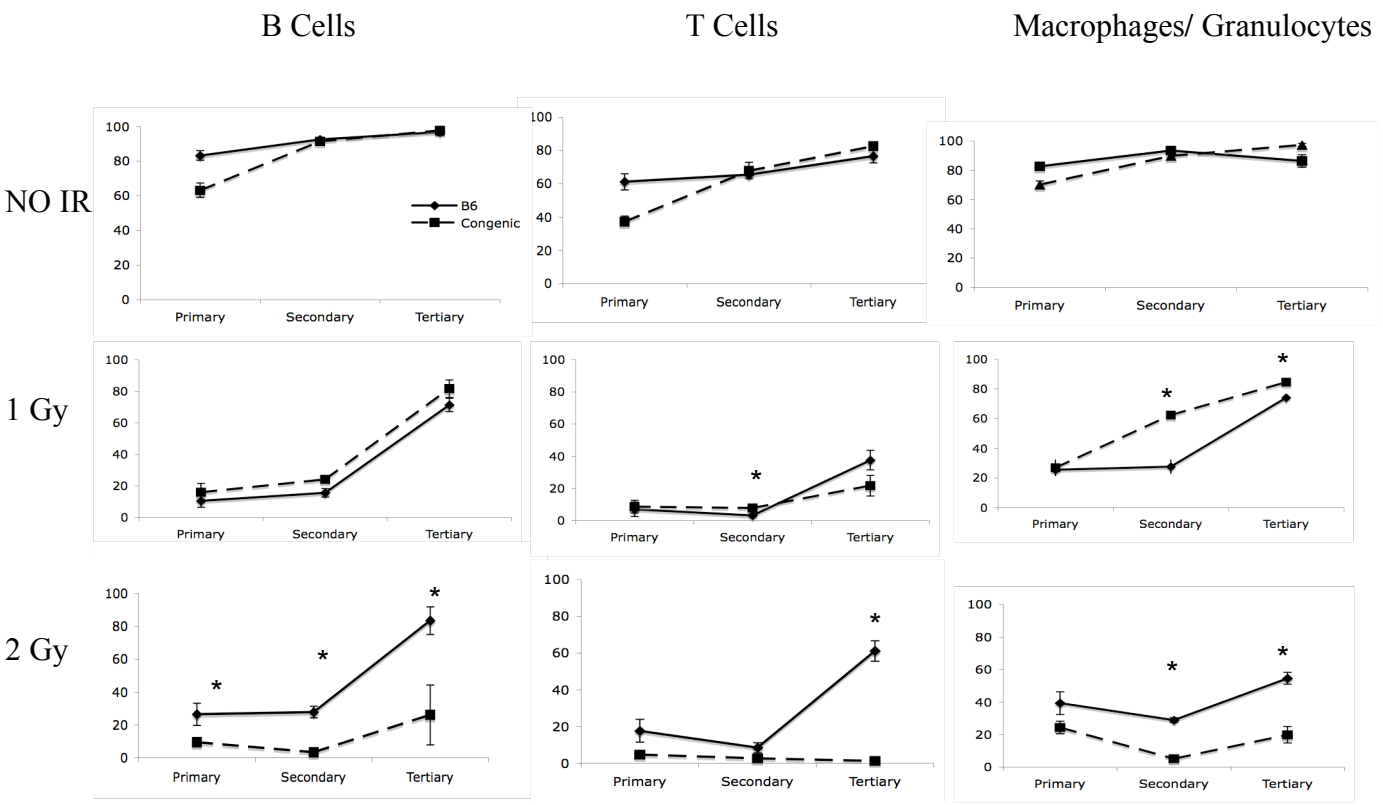


Figure 2.8 B6 and congenic contribution to mature blood cell lineages during serial transplantation. B6 and congenic derived B cells, T cells, and macrophage/granulocyte cells treated with various doses of irradiation were analyzed 20 weeks after they were transplanted into primary, secondary, tertiary recipients. Each data point represents the mean for groups of mice ($P \leq 0.05$).

CHAPTER THREE

Microarray analysis and identification of candidate quantitative trait genes that regulate the HSC aging phenotype

Summary

In order to identify the candidate gene(s) located within the chromosome 2 QTL that regulate the differential HSC aging and/or the differential radiation sensitivity phenotypes of B6 and congenic mice, microarray gene chip studies were performed. I measured the expression profiles of young and old B6 and congenic lineage- (Lin-) cells prior to and post treatment with 2 Gy of total body radiation using Affymetrix M430 microarray chips. Stringent statistical anaylses revealed that a total of 90 genes located within the chromosome 2 QTL were differentially expressed between the B6 and congenic mice. Next, using the WebQTL database, I found that 6 of the 90 differentially expressed genes located within the QTL were *cis*-regulated and therefore served as the best possible candidate genes for regulating the HSC traits. Of the 6 *cis*-regulated genes, I chose to confirm the differential expression of Retinoblastoma like protein 1 (p107) and Sorting nexin 5 (Snx5) due to their previous involvement in hematopoiesis and/or stem cell biology. Real time PCR in stem (LSK) and progenitor (Lin-) cell populations validated the differential expression of both genes. Snx5 mRNA was found to be elevated in young and old Lin- and LSK cells and p107 mRNA was found to be elevated in young and old Lin- cells and in old LSK cells. These results demonstrate that B6 alleles in the chromosome 2 QTL up-regulate the expression of both Snx5 and p107 and suggest that these genes are positive regulators of HSC number and therefore may regulate the HSC aging and/or radiation sensitivity phenotypes.

Introduction

Stem cells are indispensable entities in most multicellular organisms in that they are responsible for forming tissues during early development and maintaining them in the adult. This property places stem cells at the forefront of both developmental biology and regenerative medicine. But what defines a stem cell and provides them with their unique properties? With the development of tools for global gene expression analyses over the past several years, researchers have attempted to answer this question by identifying a universal stem cell gene expression signature. In 2002, two groups independently attempted to identify a so-called "stemness" signature by analyzing the expression profiles of mouse embryonic stem cells (ESCs), HSCs, and neural stem cells (NSCs) (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002). Both studies yielded a list of ~200 stem cell specific genes that mouse ESCs, HSCs, and NSCs commonly expressed, but rather surprisingly, the results of the two studies showed minimal overlap. Approximately a year later, a third expression profiling study comparing ESCs, NSCs, and retinal progenitor/stem cells failed to uncover a unique stem cell fingerprint, with only one gene (*Itga-6*) being commonly identified among the three studies (Fortunel *et al.*, 2003). It is possible that the lack of overlap between the stem cell profiles identified in these studies was due to different experimental approaches. Another possibility is that different stem cell types may use different gene networks to achieve self-renewal or multipotency, thus a universal stemness signature does not exist and cross tissue comparative expression analyses are not useful in identifying stem cell specific genes.

Perhaps a better way to determine a stemness profile is to compare the transcriptional profile of specific stem cells with that of their differentiated progeny. Chambers et al. recently performed this type of analysis by comparing the transcriptional profiles of HSCs, erythroid cells, granulocytes, macrophages, monocytes, natural killer cells, T cells, and B cells (Chambers *et al.*, 2007a). Although this expression database provided lists of genes exclusively expressed in specific hematopoietic cell types, the long list of HSC specific genes still makes it difficult to determine their individual and/or combined roles in stem cell function. Thus it is necessary to combine expression

profiling with other genetic approaches in order to identify important HSC regulatory genes.

As mentioned previously, many hematopoietic traits are genetically controlled since they differ between various strains of inbred laboratory mice. Due to the numerous hematopoietic differences that exist between B6 and D2 mice and the existence of BXD recombinant inbred (RI) mouse strains, we and others have identified numerous genomic regions (QTLs) affecting HSC traits (de Haan *et al.*, 1997; de Haan and Van Zant, 1999; Muller-Sieburg and Riblet, 1996). However, a major limitation of traditional QTL mapping approaches using RI reference panels is that they can only identify genomic *regions* of interest, often containing many genes. It is hypothesized that polymorphic variants, often in the form of single nucleotide polymorphisms (SNPs), underlie phenotypic differences. Since there are thousands upon thousands of SNPs in any one QTL region, and often multiple SNPs in any one gene, it is a daunting task to identify the polymorphism and therefore the gene that underlies a specific trait. Thus it seems that strategies aimed at identifying important stem cell genes using expression profiling or genetic linkage analysis alone have proven to be difficult. However, the combination of gene expression studies with genetic linkage analyses offers a promising approach for identifying specific genes that underlie HSC traits.

In the present study, I performed genome wide gene expression analyses using a cell population enriched in stem and progenitor cells in order to identify genes located in the chromosome 2 QTL that influence HSC aging. DNA microarrays were used to identify genes that are differentially expressed between chromosome 2 congenic and B6 background mice at young and old ages as well as after irradiation treatment. Although this approach makes the assumption that transcriptional regulation underlies the polymorphism, a significant proportion of the QTL that have been experimentally solved with QT gene identification to date have used this approach (Aitman *et al.*, 1999; Flint *et al.*, 2005; Karp *et al.*, 2000; Klein *et al.*, 2004; McBride *et al.*, 2003; Rozzo *et al.*, 2001). The congenic mouse model is a vital component in performing this type of experiment. Because the congenic animals have a genome that is approximately 99% B6 with the exception of the introgressed D2 interval, comparison of the number of differentially expressed genes between the two strains is greatly reduced compared to the number of

differentially expressed genes between D2 and B6 strains. This simplifies the process of identifying potential candidate genes that are responsible for the phenotype of interest.

Another approach that combines genetics with gene expression analyses involves profiling gene expression patterns (using Affymetrix arrays) in recombinant inbred strains of mice (Bystrykh *et al.*, 2005; Chesler *et al.*, 2005). The general idea is to treat mRNA levels as traditional quantitative traits. This in turn allows you to detect and characterize QTLs that are linked to the variation in gene expression of individual transcripts. QTLs that are responsible for variation in expression of transcripts can be classified into two groups: *cis*-acting QTLs which are located at the physical position of the transcript itself, and *trans*-acting QTLs that modulate the expression of large numbers of genes throughout the genome (Bystrykh *et al.*, 2005). This approach has recently been used to identify variation in gene expression patterns in HSCs isolated from the BXD RI strains. This information is in a public database available at www.webqtl.org (Wang *et al.*, 2003). By searching for *cis*-regulated transcripts located in a QTL that controls a particular trait, it is possible to identify potential candidate genes that control the trait. *Cis*-regulated genes within QTLs represent the best candidate genes, as they may contain polymorphisms that affect both their own expression level as well as the trait of interest. Using the WebQTL database, it is possible to determine if differently expressed genes (obtained from microarray analyses) are *cis*-regulated. I used this strategy to identify two novel candidate regulators of HSC aging.

Materials and Methods

Animals

Young (2-4 months) and old (24-25 months) C57BL/6 (B6) ($Ptprc^b$ [CD 45.2]) mice were purchased from the National Institute of Aging (NIA) Aged Mouse Colony at Harlan Sprague Dawley (Indianapolis, IN) and were used as the background strain. Chromosome 2 congenic mice, with a genetic interval from D2 mice spanning 50 Mbp (130Mbp -180 Mbp) on the distal end of chromosome 2, were generated as described previously (Geiger et al, 2005). Young (2-4 months) and aged (24-27 months) congenic animals were used in the experiments. Genotypes of congenic animals were confirmed by PCR screening of genetic markers flanking the congenic interval and located throughout the genome. All mice were female and were housed in the Division of Laboratory Animal Resources (DLAR) at the University of Kentucky Chandler Medical Center and were maintained under pathogen-free conditions according to NIH guidelines for animal welfare.

Immunofluorescent staining of cells and flow cytometry

Mice were euthanized by CO_2 asphyxiation and the femora and tibiae were removed to a Petri dish containing Hanks Balanced Salt Solution (HBSS, GIBCO) supplemented with 2% fetal bovine serum (FBS, Invitrogen). Femora and tibiae were flushed with 4 mL media to obtain a single cell suspension. Mononucleated cells were obtained via Ficoll gradient separation and Fc receptors were subsequently blocked with anti-CD16/32 (clone 2.4G2, Fc Block) to prevent non-specific staining. For Lineage negative (Lin-) isolation, cells were identified by the lack of cell surface antigens characteristic of cells committed to individual lineages of blood cell development. The lineage cocktail consisted of the following antibodies: CD5 (clone 53-7.3) and CD8a (clone 53-6.7) to identify T cells, CD45R/B220 (clone RA3-6B2) to identify B cells, CD11b/Mac-1 (clone M1/70) and Ly-6G/Gr-1 (clone RB6-8C5) to identify macrophages, and Ter119/Ly-76 (clone Ter-119) to identify erythrocytes. All antibodies were biotinylated and were further stained with streptavidin-fluorescein isothiocyanate (SA-

FITC). Dead cells were excluded by propidium iodide (PI) staining. For Lin- Sca+ c-kit+ (LSK) cells, light density bone marrow cells were stained with the above lineage cocktail as well as stem cell specific cell surface markers, phycoerythrin (PE)-conjugated Ly-6A/E (Sca-1; cloneE13-161.7) and allophycocyanin (APC)-conjugated CD117 (c-kit; clone ne2B8). The LSK population is highly enriched in stem cells. Dead cells were excluded by PI staining. All monoclonal antibodies were purchased from BD Pharmingen. Cells were analyzed and sorted on a triple-laser FacsVantage (Becton-Dickinson) flow cytometer.

Total RNA isolation

For microarray analyses, total RNA was extracted from approximately $6x10^5 – 9x10^5$ sorted Lin- cells using RNeasy Kit (Qiagen, Valencia CA). Cells were sorted from the bone marrow of young (2-4 months) and old (24-25 months) B6 and congenic mice prior to and post 2 Gy of irradiation administered from a ^{137}Cs source Mark 1 Irradiator (J.L. Shepherd and Associates) at a dose rate of 1.73 Gy per minute. Bone marrow cells were harvested, labeled, and Lin- cells were isolated by cell sorting. RNA samples consisted of cells pooled from at least 5 mice for each age, strain and treatment group. For quantitative real time PCR analyses, RNA was extracted from either $1x10^5$ or $2x10^5$ LSK and Lin- cells, respectively, using RNeasy kit (Qiagen, Valencia CA). Lin- and LSK cells were sorted as described previously and all samples consisted of pooled cells from at least 5 mice for each stain and age.

Microarray analysis of Lin- cells

RNA samples were hybridized according to Affymetrix protocols onto Murine Genome 430 2.0 arrays at the University of Kentucky Microarray Core Facility. Briefly, RNA samples were reverse transcribed to cDNA, then cRNA was biotin-labeled, fragmented, and hybridized to the array. Three biological samples per experimental group were individually interrogated on separate Affymetrix M430 microarray chips resulting in a total of 24 arrays. A 3-way ANOVA was used to analyze the results obtained from the three replicate chips for each sample group. A statistical cutoff of $P \leq 0.01$ was used and we set no limits on fold-change. Two-way ANOVA's were also performed to compare

expression levels when considering any two of the three variables. Again, a statistical cutoff was set at $P \leq 0.01$ and no limits were set on fold change.

Quantitative real-time PCR

RNA samples from young (2-4 months) and old (24-25 months) B6 and congenic mice were synthesized into cDNA by reverse transcription PCR. The 100 μl reaction mixture consisted of sterile water, RNA, 10X RT buffer, $MgCl_2$, deoxy NTPs, random hexamers, RNase inhibitor, and MultiScribe reverse transcriptase. The cDNA samples were then used for real time PCR to validate the differential expression of specific genes. cDNA synthesis was performed as above using a 20 ul reaction mix. Taqman primers from Applied Biosystems were used according to manufacturer's instructions for each real time PCR reaction. Due to the fact that GAPDH expression changes with age in B6 and the congenic mice, the values obtained for the test gene were not normalized in Lin- and LSK cells. Rather, equivalent numbers of cells were used in each experiment. We initially normalized gene expression to cDNA content, but found that it wasn't necessary since similar values were obtained with non-normalized cDNA. Samples for the derivation of standard curves and no-template controls were run in duplicate and experimental cDNA samples were run in quadruplicate using an ABI PRISM 7700 Sequence Detector (PE Biosystems). Each biological sample consisted of pooled cells from at least 5 mice and three replicate biological experiments were performed for each age and strain.

Results

Identification of differentially expressed genes in chromosome 2 QTL by microarray analysis

I investigated the possibility that transcriptional differences between congenic and B6 Lin- cells could point to candidate genes in the chromosome 2 QTL that contribute to two phenotypic polymorphisms that exist between the strains: HSC aging as well as sensitivity to low dose irradiation. Because the QTL congenic interval spans 50 Mb on chromosome 2, merging our linkage analysis with expressing profiling studies seemed to be a logical scientific approach to identify candidate genes that regulate the HSC phenotypes. Three variables were examined: (a) the effect of strain—congenic vs. background (in which only approximately 1% of the genome was different); (b) the effect of age (2 months vs. 20+ months); and (c) the effects of 2 Gy of irradiation to compare our surrogate assay to natural aging (Figure 3.1). Using a total of 24 Affymetrix 430 2.0 chips in three replicate experiments, a total of 1,630 probes had a significant three-way interaction, i.e. the effect of one variable (strain) was dependent on the remaining two variables (age and treatment). Another 1,922 probes were found to have significant two-way interactions, and finally 3,279 probes were found to have what we refer to as a "significant main effect" (Figure 3.2). That is, the differential expression of the probe was due independently to age, treatment, or stain. Perhaps, not surprisingly, of the 3,279 significant main effect probes, 1,215 had a significant age effect, 2,835 had a significant treatment effect, and only 725 probes had a significant strain effect. In agreement with our hypothesis that irradiation, in this context, mimics aging, we found significant overlap in the age and irradiation treatment probesets, suggesting that irradiation damage causes similar transcriptional changes as does the natural aging process. The smaller number of genes that had a significant strain effect reflects the fact that only approximately 1% of the genome is polymorphic between the background and congenic mice. When 2-way ANOVAs (comparing B6 and congenic cells at young and old ages) were performed, both pre- and post- irradiation treatment, chromosome 2 had the highest number of differentially expressed probesets compared to all other chromosomes. We also found that a large number of probesets located on chromosome 11 were

differentially expressed. This may reflect the finding that a QTL regulating mouse lifespan has been mapped to this chromosome (de Haan et al., 1999). An additional QTL regulating the natural variation in lifespan between B6 and D2 mice was also mapped to the same location on mouse chromosome 2 as the aging QTL, thus suggesting that genes regulating aging and/or the DNA damage response may overlap with, be clustered with, or influence those that affect lifespan. The fact that a total of 6,831 (15% of the total 45,101 probesets on the chip) probes were differentially expressed in the 3 way ANOVA suggests that aging and irradiation, in addition to a small difference in strain, have a profound effect on the transcriptional profiles of stem cells. Moreover, the presence of differentially expressed genes located on chromosomes other than chromosome 2 suggests that genes in the QTL regulate the expression of genes located elsewhere in the genome.

Candidate QT gene identification

Using the congenic mouse model, our search for candidate QT genes was greatly facilitated by allowing us to first focus our attention on genes physically located within the chromosome 2 QTL. I searched for candidate genes that may regulate the different number of LSK cells in aged B6 and D2 mice by first identifying all significant transcripts on the Affymetrix arrays that mapped to the critical congenic interval (130 Mb - 150 Mb) on chromosome 2. From 2-way and 3-way ANOVAs a total of 90 genes located within the chromosome 2 QTL were differentially expressed between the B6 and congenic mice (Table 3.1). I then took advantage of the WebQTL database and searched for genetic loci that are strongly linked to the variation in gene expression of all 90 individual differentially expressed transcripts present in the chromosome 2 QTL. The WebQTL database contains information comparing the strain distribution pattern of individual Affymetrix transcripts with the genetic distribution of B6 and D2 alleles at 779 markers located throughout the genome (Bystrykh *et al.*, 2005). Performing this linkage analysis allowed me to map *cis* and *trans* regions of the genome that regulate the expression of the 90 transcripts located in the chromosome 2 congenic interval. Six genes (Anapc1, Snrbp2, Rrbp1, Snx5, Rbl1, and Ndrg3) located in the interval were found to be *cis*-regulated and were also differentially expressed between B6 and D2

background strains according to other microarray experiments performed by our lab (Table 3.2). Of the six genes, I chose to look more closely at sorting nexin 5 (Snx5) and retinoblastoma like protein-1 (otherwise known as p107) due to their strong *cis* regulation and because they had previously been linked to hematopoiesis and/or stem cell regulation (Vanderluit *et al.*, 2004; Vanderluit *et al.*, 2007). Modifiers of Snx5 expression map to a genomic interval spanning from 139.4 Mb to 154.3 Mb on chromosome 2 (significant LRS linkage), a region containing D2Mit281 and D2Mit 282 (markers most tightly linked to the trait) and Snx5 (Figure 3.3). Modifiers of p107 expression map to a genomic interval spanning from 156.5 Mb to 157.1 Mb on chromosome 2 (suggestive LRS linkage) containing p107 (Figure 3.4). Snx5 (144 Mb) is located upstream from p107 (156.8 Mb), thus there is a possibility that the genes to share a common promoter region (Figure 3.5). However, this scenario is unlikely due to the large distance between the two genes, as well as the fact that both genes have fairly large CpG islands in their 5-prime regulatory regions.

According to the microarray analysis, B6 alleles up-regulate both Snx5 and p107 expression at both young and old ages compared to D2 alleles. I next used real time PCR to confirm the differential expression of the genes in Lin- and LSK stem cell enriched populations. Snx5 transcript levels were elevated in young and old B6 Lin- and LSK cells compared to congenic cells (Figure 3.6 A and B). As expected, LSK cells from DBA mice also express lower levels of Snx5 when compared to B6 cells. Similarly, p107 transcript levels were elevated in young and old B6 Lin- cells compared to congenics, but in LSK cells, p107 expression was significantly higher only in old B6 cells (Figure 3.7 A and B). In young LSK cells, the inter-strain difference approached significance ($P = 0.06$) but was not as pronounced. Again, old D2 LSK cells express lower levels of p107 when compared to old B6 cells, and we found no significant difference in p107 expression between old D2 and congenic LSK cells. Thus, these data confirm the results obtained from the microarray analyses and suggest that Snx5 and p107 are positive regulators of stem cell number. It is important to note that differential Snx5 and p107 expression are apparent in young mice (this is true for both genes in Lin- cells and for Snx5 in LSK cells), but the phenotypic effects entrained by lifelong differential expression do not become apparent until old age.

Discussion

Quantitative trait locus mapping studies have identified thousands of chromosomal regions containing genes that affect a wide variety of complex phenotypes ranging from alcoholism to diabetes and obesity. However, identifying individual QTL genes has been slow and difficult over the past decade. Fortunately, five years ago when I planned my dissertation project, new technologies such as the microarray, which allowed for comparative genomics, and the publication of the mouse and human genomic sequences set the stage for a new generation of QTL gene identification strategies. In my studies, I used a combination of classical genetic linkage and microarray analyses to identify Snx5 and p107 as novel regulators of HSC frequency in mice. Using microaray technology, I found that Snx5 and p107 genes were differentially expressed between young and old B6 and congenic Lin- cells. Quantitative real time PCR was used to confirm these results in Lin- cells, as well as in more primitive LSK cells.

The use of the chromosome 2 congenic mouse model was pivotal in performing this forward genetic approach to identify genes that regulate HSCs. The congenic mice have a genome that is 99% B6 with the exception of the introgressed QTL interval from D2 mice. The remaining 1% difference between the strains greatly reduced the number of differentially expressed genes between D2 and B6 parental strains. A reciprocal congenic model in which the QTL interval was introgressed from B6 mice onto a D2 genome was not available for these studies but would have further reduced the number of potential candidate genes, assuming the genes were reciprocally regulated (i.e. a gene would be upregulated in one congenic and down regulated in the reciprocal). Another unique feature of the study is that cDNA amplification was not performed on any of the samples used in the microarray. Because HSCs are such a rare cell type, other labs that have performed gene expression studies with these cells relied on *in vitro* amplification techniques (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002). Amplification of cDNA by a standard PCR procedure may result in the differential amplification of particular transcripts, since sequences differ in the rate with which they can be amplified by PCR (Lockhart and Winzeler, 2000). We obtained 500,000 Lin- cells from 5-10 mice for each strain, age and treatment group, and three independent biological samples were

hybridized to individual chips. Also, previous microarray studies relied on the use of Affymetrix U74Av2 chips, which contain only 12,488 probesets located throughout the mouse genome. In these studies, I used Affyterix M430 chips that contain 45,000 probesets, thus representing the entire mouse genome.

I used quantitative real time PCR to validate the differential expression of Snx5 and p107 in Lin- as well as in primitive LSK cells. Real time PCR is very sensitive, can distinguish between closely related mRNAs, and yields results that are quantitative in nature. Due to its relative ease and convenience of use, real time PCR is the preferred method for validating microarray results compared to northern blotting and ribonuclease protection assays, which require large amounts of RNA. Real time PCR confirmed that B6 alleles up-regulate Snx5 and p107 expression in young and old Lin- cells. Because Lin- cells contain a mixture of stem and progenitor cells, I also performed real time PCR using LSK cells, which are highly enriched in stem cells. I found that Snx5 expression was higher in young and old B6 LSK cells compared to congenic cells, whereas p107 expression was only increased in old B6 LSK cells. These results suggest that Snx5 and p107 are positive regulators of stem cell number and may regulate the HSC aging trait. The fact that B6 alleles increase the expression of Snx5 and p107 in young *and* old Lin- cells suggests that the rate of HSC aging may be due to lifelong differential expression of the genes. Thus even though the genes are differentially expressed in young cells, the resulting phenotypic effects do not become apparent until old age.

Due to the large number of cells required for Western blot analysis, it was not possible to quantify protein levels of the genes in young and old Lin- or LSK cells. I did however perform Western blots using whole bone marrow cells and found that the differential expression patterns observed above were reproduced for Snx5 but not for p107. Because primitive HSCs comprise only 0.01% of the total bone marrow population, I was not surprised that I could not reproduce the expected expression patterns for p107 and thus did not allow these results to prevent further investigation on the role of this gene in hematopoiesis.

In order to determine the mechanisms that may contribute to the differential expression of p107 and Snx5 between B6 and congenic HSCs, I analyzed the transcripts for single nucleotide polymorphisms (SNPs) that exist between the two strains. The

presence of polymorphisms in coding or regulatory elements of transcripts can have a significant impact on gene expression levels as well as protein function, and thus can potentially effect phenotypic variation in organisms. According to the Ensembl database (www.ensembl.org) B6 and D2 alleles of p107 have four non-synonymous SNPs located in coding regions as well as 16 intronic and seven 3' untranslated region (UTR) SNPs (Figure 3.5). The non-synonomous coding region SNPs are located in exons 9, 14, and 15. One SNP is located in region A of the pocket domain and two are located within the spacer region of the pocket domain. The pocket domain consists of two conserved sequences, A and B, which are separated by a spacer domain. This "pocket" is responsible for mediating interactions with viral-oncoproteins as well as other cellular proteins to carry out the biological function of this protein. Traditionally it is held that the differential expression of a gene is caused by altered transcription factor binding due to the presence of SNPs in regulatory regions (most notably 5'UTR promoter regions) upstream of the gene. Although we did not detect any SNPs in the 5' UTR region of p107 emcompassing 800 base pairs upstream of the start codon, the exonic SNPs located in the functional pocket domain of p107 have the potential to affect transcript stability, which could result in the differential expression of the gene in a cellular context. Further, although not yet studied, the presence of SNPs in the 3' UTR of p107 could also affect its expression. Indeed, Clop et al. identified a QTL on chromosome 2 that effects muscle mass in sheep and showed that a point mutation in the 3' UTR of the myostatin (GDF8) gene (located in the QTL) created a target site for miRNAs. They then demonstrated that differential miRNA binding to the transcript resulted in differential myostatin protein expression (Clop *et al.*, 2006). There are a total of 15 predicted murine microRNA binding sites in the 3' UTR of p107 as identified by PicTar WEB INTERFACE (Krek *et al.*, 2005). Benetti et al. recently showed that the p107-related protein, Rbl2 (p130), is transcriptionally down-regulated by the mir290 cluster of miRNAs (Benetti *et al.*, 2008). It is therefore conceivable that the expression of other members of the retinoblastoma family are regulated in a similar fashion.

In order to identify SNPs between B6 and congenic mice located in the Snx5 transcript, I again used the Ensembl database. I found that there are 8 3'UTR SNPs and 8 intronic SNPs between B6 and D2 mice in Snx5 (Figure 3.5). Although this gene does

not have any SNPs located in its coding region, it is possible that some of the intronic SNPs may modify Snx5 expression. Also, using the PicTar WEB INJTERFACE, I found 7 predicted murine microRNA binding sites in the 3' UTR of Snx5, thus it's possible that SNPs located in the 3' UTR may modulate Snx5 expression.

In order to identify *cis*- and *trans*-acting factors that affect the expression of p107 and Snx5, I used WebQTL software, which contains a quantitative gene expression database in LSK stem cells of all BXD mouse strains. This database allows one to map the regions of the genome that modify the expression of genes of interest (Bystrykh *et al.*, 2005). I found that both Snx5 and p107 are *cis*- regulated, meaning that modifiers of their expression map very close to the actual gene itself. In addition, negative additive effects indicate that B6 alleles increase the trait values (p107 and Snx5 expression), which is consistent with the results obtained from microarray and real time-PCR experiments. These results therefore suggest the existence of regulatory factors in the proximal regions surrounding p107 and Snx5, and further support our hypothesis that these genes are directly or indirectly responsible for the phenotypes conferred by the chromosome 2 QTL.

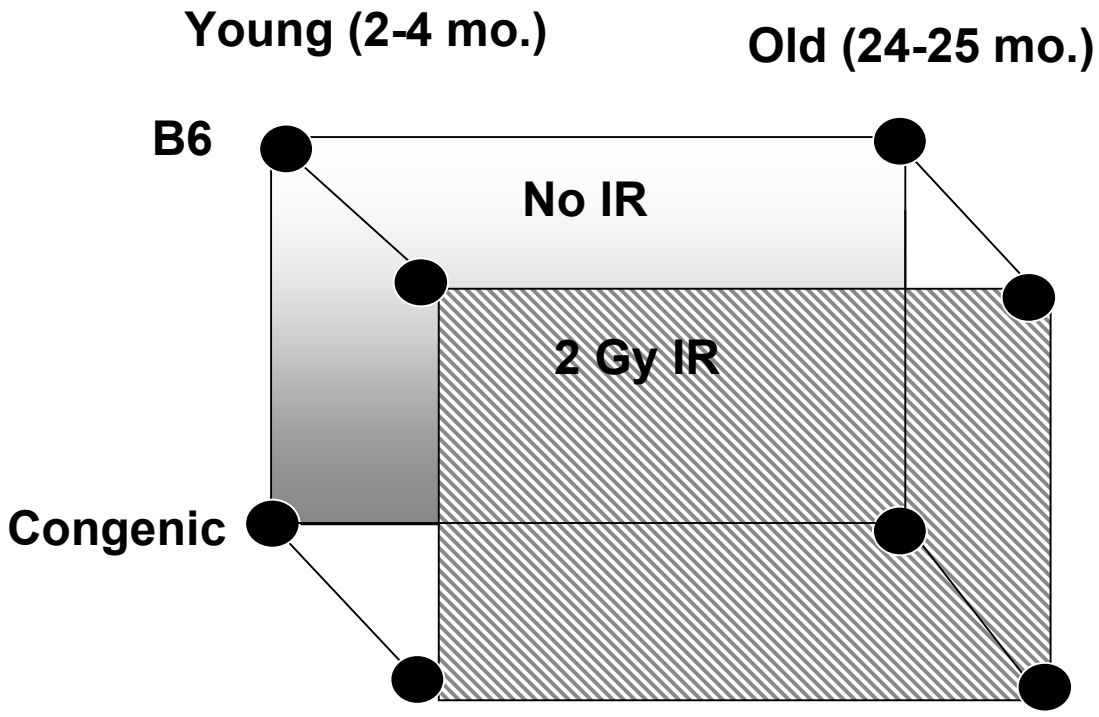


Figure 3.1 Microarray experimental design.

Schematic illustrating the experimental design of the microarray analysis. Using B6 and congenic mice at young and old ages allows for the identification of both inter- and intra-strain changes in gene expression that accompany aging. We chose to use non-irradiated as well as irradiated mice in order to test our surrogate assay to natural aging. A total of 24 arrays (3 for each strain, age, and treatment group) were used.

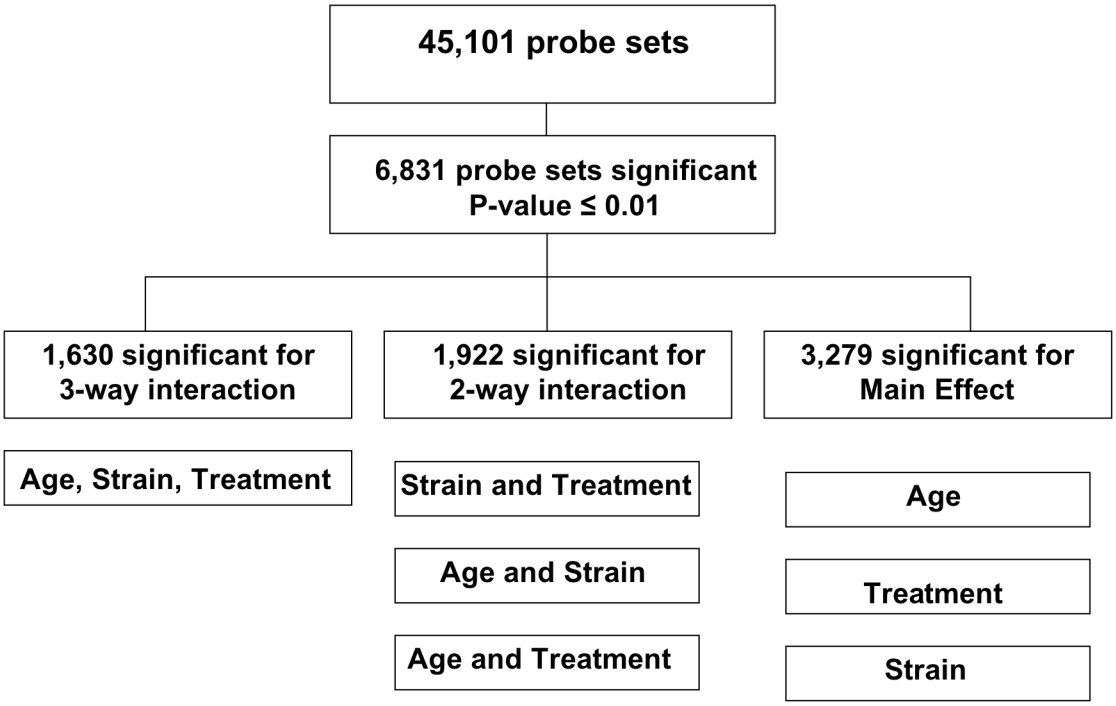


Figure 3.2 Flow Chart of Microarray Data Analysis.

A total of 6,831 probe sets were found to be significant with a p-value of ≤ 0.01. The probesets were further divided into three categories: 1) probesets significant for a 3-way interaction, 2) probesets significant for a 2-way interaction, and 3) probesets significant for a main effect.

Table 3.1. Differentially expressed probesets between B6 and congenic mice located within the chromosome 2 QTL

Probe Set ID	Gene Title	Chromosome	P-value
1450698_at	dual specificity phosphatase 2	2 F1	0.00035
1427496_at	expressed sequence AI851464	2 F1	0.00970
1444524_at	LOC433475	2 F1	0.00035
1449445_x_at	microfibrillar-associated protein 1	2 F1	0.00970
1458358_at	pantothenate kinase 2 (Hallervorden-Spatz syndrome)	2 F1	0.00101
1453055_at	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	2 F1	0.01041
1424268_at	spermine oxidase	2 F1	0.00087
1446807_at	Ubiquitin specific protease 8	2 F1	0.00428
1418495_at	zinc finger CCCH type domain containing 8	2 F1	0.00012
1419270_a_at	deoxyuridine triphosphatase	2 F1|2 70.0 cM	0.00072
1437193_s_at	small nuclear ribonucleoprotein B	2 F1|2 73.0 cM	0.00083
1434197_at	attractin	2 F1|2 73.9 cM	0.00576
1421811_at	thrombospondin 1	2 F1-F3|2 65.0 cM	0.00103
1416800_at	transient receptor potential cation channel, subfamily M, member 7	2 F2	0.00600
1438957_x_at	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2	2 F2|2 73.0 cM	0.00093
1448233_at	prion protein	2 F2|2 75.0 cM	0.00000
1434443_at	Anaphase promoting complex subunit 1	2 F3	0.00038
1451505_at	coiled-coil-helix-coiled-coil-helix domain containing 5	2 F3	0.00160
1426533_at	nucleolar protein 5A	2 F3	0.00015
1428580_at	biliverdin reductase A	2 F3|2 62.0 cM	0.00118
1448534_at	protein tyrosine phosphatase, non-receptor type substrate 1	2 F3|2 73.1 cM	0.00052
1435449_at	BCL2-like 11 (apoptosis facilitator)	2 F3-G1	0.00001
1452767_at	ribosome binding protein 1	2 G1	0.00009
1426613_a_at	U2 small nuclear ribonucleoprotein B	2 G1	0.00409
1419202_at	cystatin F (leukocystatin)	2 G1-G3	0.00083
1433607_at	cerebellin 4 precursor protein	2 G3	0.00096
1448184_at	FK506 binding protein 1a	2 G3	0.00085
1451337_at	proteasome (prosome, macropain) inhibitor subunit 1	2 G3	0.00271
1422842_at	5'-3' exoribonuclease 2	2 H1	0.00088
1419038_a_at	casein kinase II, alpha 1 polypeptide	2 H1	0.00000
1426765_at	COMM domain containing 7	2 H1	0.00001
1441931_x_at	glutathione synthetase	2 H1	0.00289
1417941_at	haloacid dehalogenase-like hydrolase domain containing 4	2 H1	0.00019
1431431_a_at	nitrogen fixation gene 1 (S. cerevisiae)	2 H1	0.00375
1417664_a_at	N-myc downstream regulated gene 3	2 H1	0.00600
1416059_at	SEC23B (S. cerevisiae)	2 H1	0.00340
1424308_at	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	2 H1	0.00550
1434400_at	TGFB-induced factor 2	2 H1	0.01041
1423080_at	translocase of outer mitochondrial membrane 20 homolog (yeast)	2 H1	0.00149
1423204_at	transmembrane 9 superfamily protein member 4	2 H1	0.00039
1428402_at	zinc finger, CCHC domain containing 3	2 H1	0.00477
1417648_s_at	sorting nexin 5	2 H1|2 80.0 cM	0.00000
1417124_at	destrin	2 H1|2 81.4 cM	0.00025
1418244_at	N-acetyltransferase 5 (ARD1 homolog, S. cerevisiae)	2 H1|2 82.0 cM	0.00110
1425895_a_at	inhibitor of DNA binding 1	2 H1|2 84.0 cM	0.00072
1428820_at	microtubule-associated protein, RP/EB family, member 1	2 H1|2 84.0 cM	0.00013
1449455_at	hemopoietic cell kinase	2 H1|2 86.0 cM	0.00064
1417500_a_at	transglutaminase 2, C polypeptide	2 H1|2 89.0 cM	0.00000
1448820_a_at	eukaryotic translation initiation factor 2, subunit 2 (beta)	2 H1|2 90.0 cM	0.00108
1420664_s_at	protein C receptor, endothelial	2 H1-3	0.00464
1422479_at	acetyl-Coenzyme A synthetase 2 (ADP forming)	2 H2	0.00411
1448626_at	CDK5 regulatory subunit associated protein 1	2 H2	0.00077
1455080_at	protein phosphatase 1, regulatory (inhibitor) subunit 16B	2 H2	0.00007

1434438_at	SAM domain and HD domain, 1	2 H2	0.00524
1456867_x_at	serologically defined breast cancer antigen 84	2 H2|2 92.0 cM	0.00499
1448582_at	catenin, beta like 1	2 H2|2 94.0 cM	0.00249
1458039_at	Nuclear receptor coactivator 3	2 H2-H4	0.00309
1424161_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27	2 H3	0.00017
1417068_a_at	protein tyrosine phosphatase, non-receptor type 1	2 H3	0.00029
1436015_s_at	serine/threonine kinase 4	2 H3	0.00371
1420054_s_at	solute carrier family 35, member C2	2 H3	0.00103
1430388_a_at	sulfatase 2	2 H3	0.00519
1448345_at	translocase of outer mitochondrial membrane 34	2 H3	0.00642
1424511_at	aurora kinase A	2 H3|2 100.0 cM	0.00953
1458501_at	vesicle-associated membrane protein, associated protein B and C	2 H3|2 103.0 cM	0.00900
1424411_at	expressed sequence AI840826	2 H3|2 49.6 cM	0.00220
1427844_a_at	CCAAT/enhancer binding protein (C/EBP), beta	2 H3|2 95.5 cM	0.00605
1437414_at	zinc finger protein 217	2 H3|2 99.0 cM	0.00138
1439802_at	Serine/threonine kinase 35 (Stk35), mRNA	chr2 F1	0.00058
1425349_a_at	myelin basic protein expression factor 2, repressor	chr2 F1|2 73.0 cM	0.00058
1452428_a_at	beta-2 microglobulin	chr2 F1-F3|2 69.0 cM	0.00330
1438957_x_at	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2	chr2 F2|2 73.0 cM	0.00977
1417947_at	proliferating cell nuclear antigen	chr2 F2|2 75.0 cM	0.00000
1416985_at	protein tyrosine phosphatase, non-receptor type substrate 1	chr2 F3|2 73.1 cM	0.00004
1416174_at	retinoblastoma binding protein 9	chr2 G1-H1	0.00107
1429328_at	NSFL1 (p97) cofactor (p47)	chr2 G3	0.00000
1422488_at	NTF2-related export protein 1	chr2 H1	0.00730
1433826_at	TSPY-like 3	chr2 H1	0.00047
1425166_at	Retinoblastoma-like 1 (p107)	chr2 H1	0.00003
1420631_a_at	bladder cancer associated protein homolog (human)	chr2 H1|2 88.0 cM	0.00437
1447898_s_at	splicing factor, arginine/serine-rich 6	chr2 H2|2 79.8 cM	0.00636
1424767_at	cadherin 22	chr2 H3	0.00401
1434069_at	cDNA sequence BC067047	chr2 H3	0.00007
1448847_at	tumor differentially expressed 1	chr2 H3	0.00071
1429947_a_at	Z-DNA binding protein 1	chr2 H3	0.00000
1452954_at	ubiquitin-conjugating enzyme E2C	chr2 H3|2 93.0 cM	0.00020
1417976_at	adenosine deaminase	chr2 H3|2 94.0 cM	0.00000
1430288_x_at	ribosomal protein S21	chr2 H4	0.00004
1441923_s_at	endothelin 3	chr2 H4|2 104.0 cM	0.00137
1417786_a_at	regulator of G-protein signaling 19	chr2 H4|2 45.0 cM	0.00019

Table 3.2 Differentially expressed *cis* regulated genes in the chromosome 2 QTL

Probe ID	Gene Name	Gene Symbol	Location (Mb)	Function
1434443_at	Anaphase promoting complex 1	Anapc1	128.4 Mb	regulation of cell cycle
1426613_a_at	U2 small nuclear ribonucleoprotein B	Snrbp2	142.8 Mb	nuclear mRNA splicing, mRNA processing
1452767_at	ribosome binding protein 1	Rrbp1	143.7 Mb	protein targeting and transport
1417648_s_at	Sorting nexin 5	Snx5	144.0 Mb	intracellular signaling, protein transport/trafficking
1425166_at	Retinoblastoma like protein 1	Rbl1	156.8 Mb	cell differentiation
1417663_a_at	N-myc downstream regulated gene 3	Ndrg3	156.9 Mb	negative regulation cell cycle, negative regulation of transcription

A total of six *cis* regulated genes located in the congenic interval were found to be differentially expressed between young and old B6 and congenic mice as well as between B6 and DBA mice. RNA isolated from B6 and chromosome 2 congenic Lin- cells was labeled and hybridized to Affymetrix Mouse Genome 430 chips. Three independent experiments from three treatment groups (strain, age, and irradiation treatment) were used to quantify differential expression patterns.

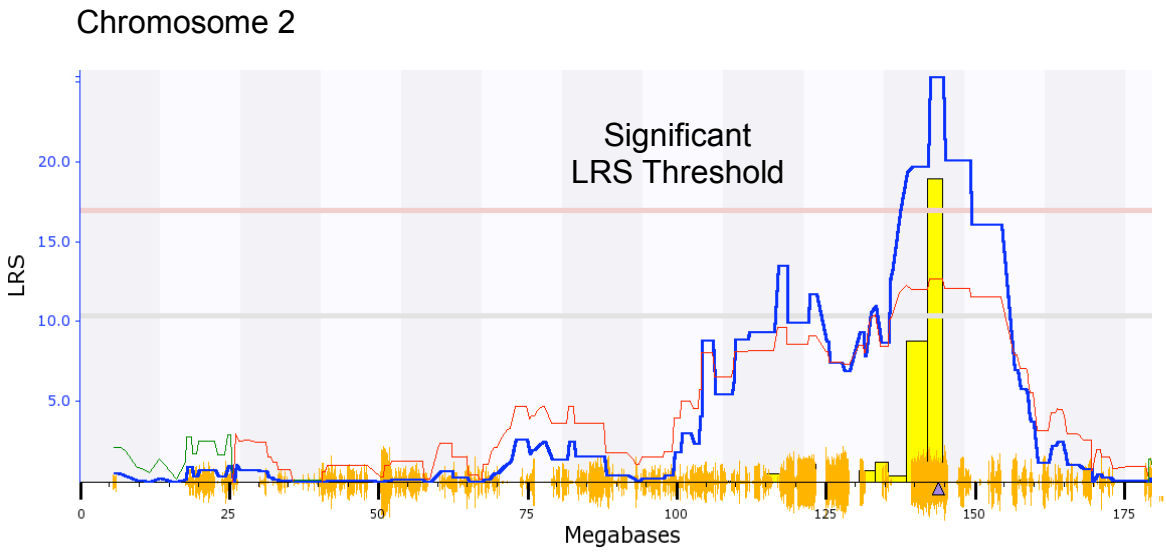


Figure 3.3 *Cis*-acting quantitative trait loci that modulate Snx5 expression. Using WebQTL, we found a QTL on chromosome 2 that influences Snx5 expression. The genomic interval spans from 139.4 Mb to 154.3 Mb on chromosome 2 and reaches significant LRS threshold. The orange tick marks on the bottom of the figure represent SNPs across chromosome 2. The area surrounding Snx5 is highly polymorphic between B6 and DBA mice.

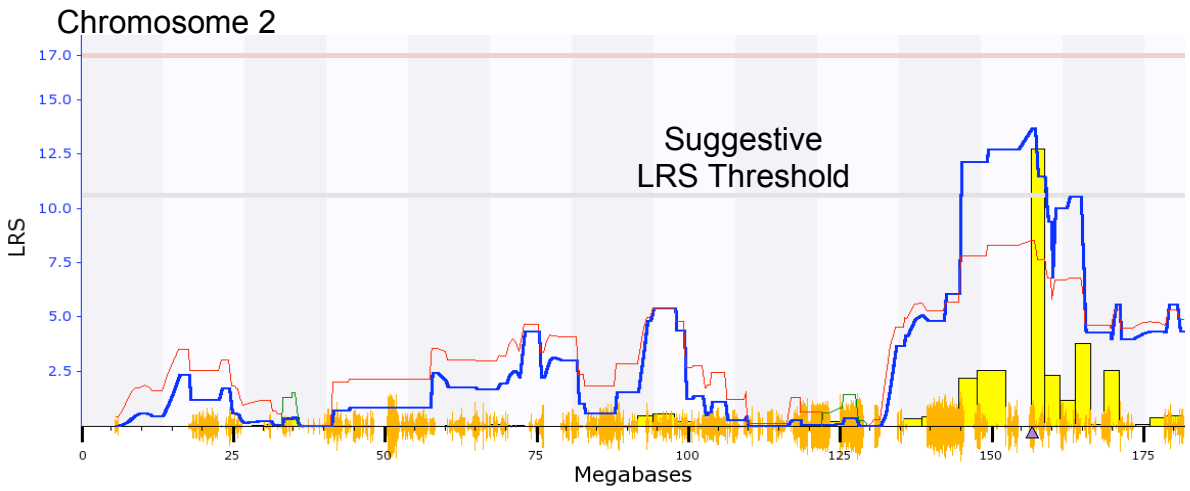


Figure 3.4 ***Cis*-acting quantitative trait loci that modulate p107 expression**. Using WebQTL, we found a QTL on chromosome 2 that influences p107 expression. The genomic interval spans from 156.5 Mb to 157.16 Mb on chromosome 2 and reaches suggestive LRS threshold. The orange tick marks on the bottom of the figure represent SNPs across chromosome 2. Again, the area surrounding p107 is highly polymorphic between B6 and DBA mice.

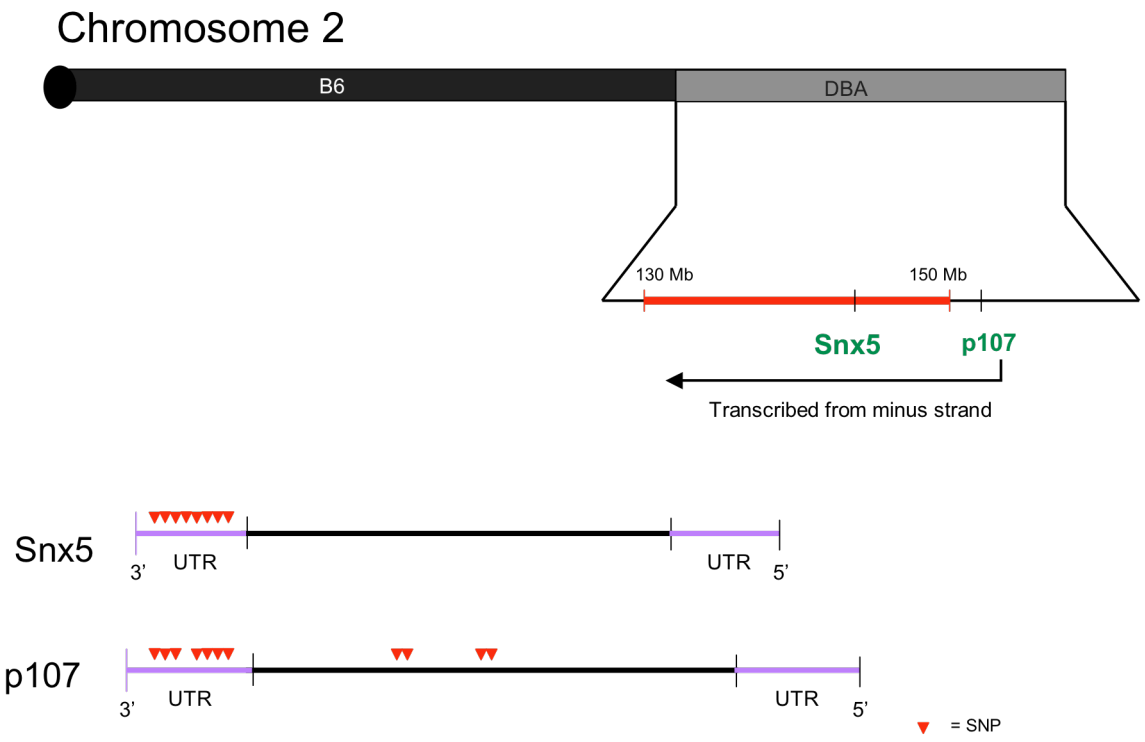
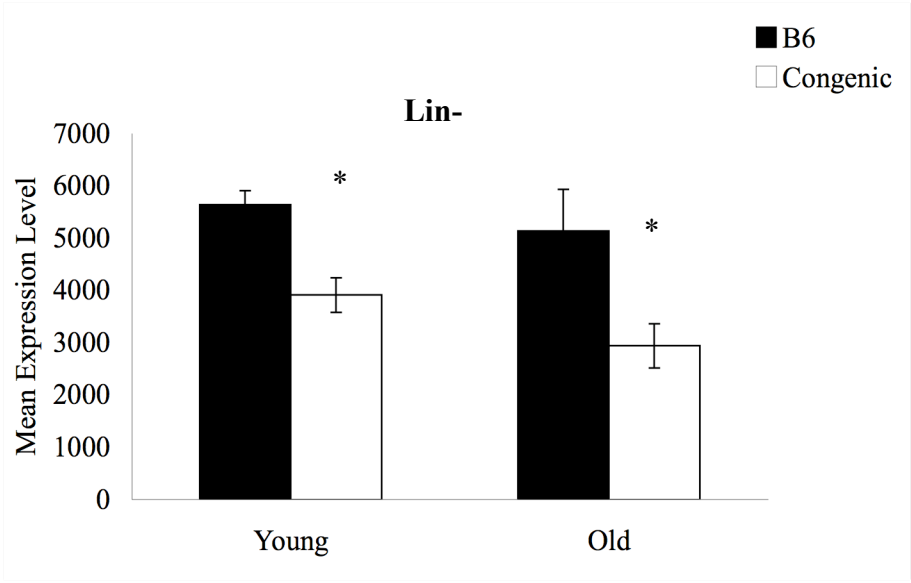


Figure 3.5 Location of Snx5 and p107 within the congenic interval.

p107 and Snx5 are transcribed from the minus strand on chromosome 2. Shown here are 8 SNPs located within the 3' regulatory region of Snx5 and seven SNPs in the 3' UTR of p107 as well as 4 non-synomous coding region SNPs.

A



B

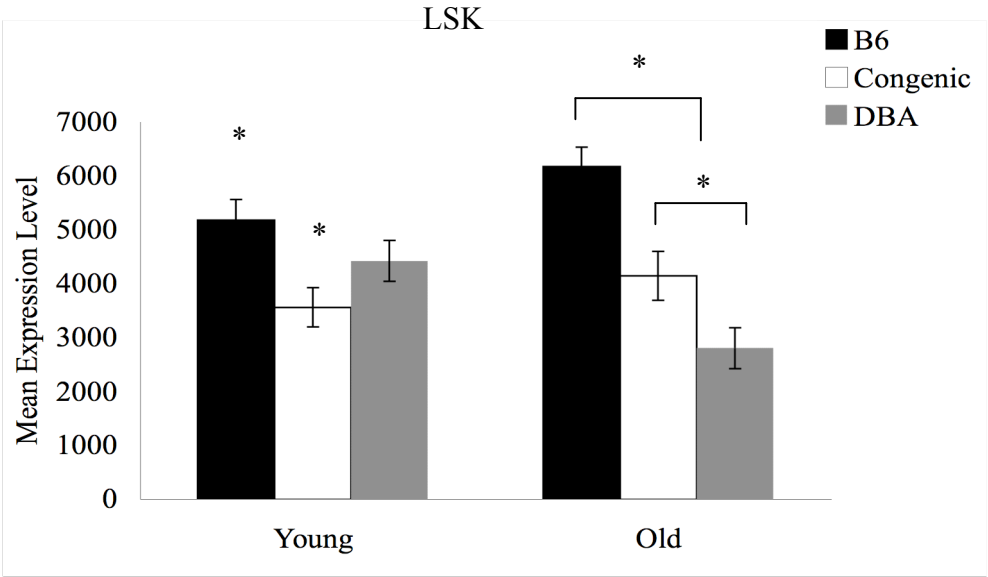
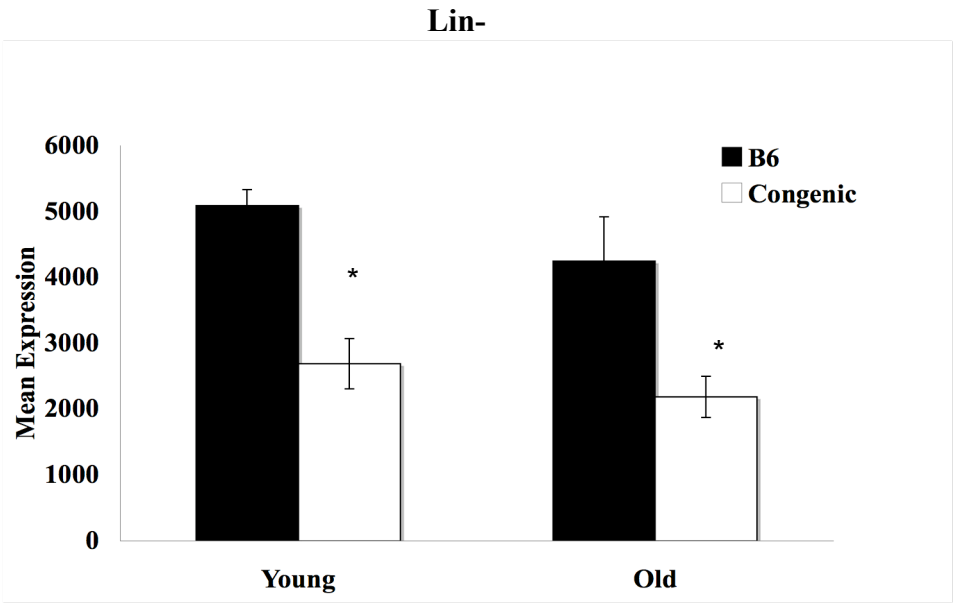


Figure 3.6 Differential expression of Snx5 mRNA in Lin- and LSK cells.
200,000 Lin- cells were sorted from young and old mice and mRNA levels were measured by real time PCR. Snx5 mRNA levels are significantly higher in young and

old B6 Lin- cells compared to congenic cells ($P \leq 0.01$) (A). 100,000 LSK cells were sorted from young and old animals and mRNA levels were measured by real time PCR. Snx5 mRNA levels are significantly higher in young B6 LSK cells compared to congenic cells ($P \leq 0.01$). I detected no significant difference in Snx5 expression in young DBA cells compared to young B6 and congenic cells. Snx5 mRNA expression is significantly higher in old B6 LSK cells compared to old congenic and old DBA LSK cells ($P \leq 0.05$) (B). Results are given as mean ± SEM ($n \geq 5$ mice per experiment, 12 measurements from 3 replicate experiments).

A



B

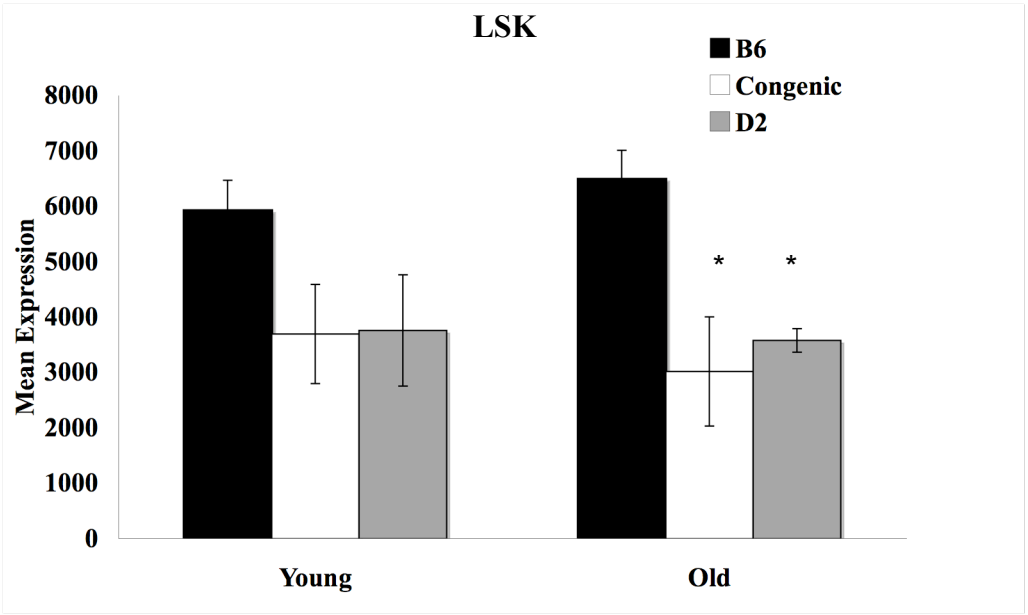


Figure 3.7 Differential expression of p107 mRNA in different HSC compartments. 200,000 Lin- cells were sorted from young and old mice and mRNA levels were measured by real time PCR. p107 mRNA levels are significantly higher in young and old

B6 Lin- cells compared to congenic cells ($P \leq 0.01$) (A). 100,000 LSK cells were sorted from young and old animals and mRNA levels were measured by real time PCR. p107 mRNA levels are not significantly different between the three strains in young LSK cells. However, p107 mRNA expression is significantly higher in old B6 LSK cells compared to old congenic and DBA LSK cells ($P \leq 0.05$) (B). Results are given as mean ± SEM (n ≥ 5 mice per experiment, 12 measurements from 3 replicate experiments).

CHAPTER FOUR

Functional effect of retroviral overexpression of p107 and Snx5 in murine HSCs

Summary

In order to determine whether p107 and Snx5 expression influence HSC frequency, I used retroviral vectors to overexpress both genes in congenic HSCs. I constructed individual vectors containing p107 and Snx5 cDNA and transfected these as well as an empty vector control into young congenic bone marrow cells. Using GFP to identify transduced cells, p107, Snx5, and empty vector control cells were sorted using flow cytometry and subsequently used for *in vitro* cobblestone area forming cell assays CAFC). Upon performing CAFC assays, I showed that congenic HSCs overexpressing p107 increased the frequency of day21, day28, and day35 CAFCs compared to cells expressing the empty vector control. Similarly, congenic HSCs overexpressing Snx5 increased the frequency of day14, day21, and day35 CAFCs compared to cells expressing the control vector. These results provide strong evidence that p107 and Snx5 are positive regulators of HSC number and suggest that life-long elevated expression of the genes (such as that seen in B6 HSCs) may protect the stem cell compartment and delay the signs of aging in this vital cell population. Although not available at the time when the dissertation was under preparation, functional CRU assays in which I measure the *in vivo* frequency of congenic HSCs transduced with p107 or Snx5, are being performed to investigate the role of each gene's expression on HSC number. As HSC expansion techniques have not proven very effective, the data presented here may be useful for the development of new strategies to expand HSCs in the clinic.

Introduction

The murine hematopoietic system is the best-characterized mammalian stem cell model despite the fact that HSCs are inherently rare cells types. This is due in part to the widespread use of gene transfer techniques in which the gene of interest is delivered into target cells under the control of a retroviral promoter. Retroviral-mediated gene transfer techniques have been used extensively in HSC biology, and have identified numerous genes that are vital for the proliferation, differentiation and self-renewal of HSCs. This methodology can be used to reconstitute a defective function or to analyze protein function during hematopoiesis by overexpression. In my studies I employ the latter and determine the effect of p107 and Snx5 overexpression on the number of HSCs in B6 and congenic mice.

Retroviral vectors are appealing vehicles for gene transfer for several reasons: 1) they stably integrate into host chromosomal DNA, 2) they deliver genes to target cells at high efficiency and allow for long-term, stable expression of introduced genetic elements, and 3) the retroviral promoter can direct high-level, efficient gene expression. The main advantage of using retroviruses when manipulating hematopoietic cells is that they integrate into the host genome and therefore are transmitted to progeny through cell division. This ensures that the retrovirus construct remains present and continues to express the gene of interest in differentiated progeny of HSCs that were originally infected. In this regard, bone marrow cells can be harvested, manipulated *in vitro*, and either be used for *in vitro* HSC assays or transplanted back into conditioned recipient mice (where they will reconstitute the entire blood system of the recipient), thus making it possible to study how the overexpression of a gene affects the entire hematopoietic system.

In our lab, we use Phoenix helper-free retrovirus producer lines to package retroviral particles. The Phoenix packaging cell lines were created by placing cell constructs capable of producing gag-pol and envelope protein for ecotrophic and amphotrophic viruses into the 293T cell line. The 293T cell line is an easily transfectable human embryonic kidney line transformed with adenovirus E1a and carrying a temperature-sensitive T antigen co-selected with neomycin (Graham *et al.*, 1977).

Packaging cells provide the structural and regulatory proteins required in *trans* for assembling a viral particle. Retroviral vectors carry only *cis* elements necessary to facilitate packaging, reverse transcription, and integration. This is important because once transduced, the retroviral vector is unable to replicate.

Infection of HSCs by retroviral vectors is dependent on numerous factors, including cycling status of the target cell population, the specific infection protocol used, and pre-stimulation of the cells to growth stimulatory factors before infection. It is vital to preserve the functional qualities of HSCs during the period of *in vitro* manipulation since down stream assays require intact functional capabilities of HSCs. In order to achieve successful retroviral transduction, the target cells must be actively replicating at the time of infection (Miller *et al.*, 1990). Since HSCs are a relatively quiescent population, it is necessary to pre-treat donor animals with 5-fluorouracil (5-FU), a cytotoxic drug that acts as a pyrimidine antagonist. 5-FU functions to inhibit DNA synthesis both by blocking the formation of normal pyrimidine nucleotides via enzyme inhibition and by interfering with DNA synthesis after incorporation into a growing DNA molecule, thus it selectively kills hematopoietic progenitor cells that are actively cycling while sparing the primitive HSC population. In response to the elimination of progenitor cells, the HSCs begin to cycle, thus allowing retroviral gene transfer studies to be performed in this cell population. Additional studies with hematopoietic cells show that gene transfer efficiency is improved when the HSCs are pre-stimulated with growth factors (Lim *et al.*, 1989). Particularly, pretreatment *in vitro* of donor cells from 5-FU treated mice with the combination of interleukin 3 (IL-3), IL-6, and stem cell factor (SCF) greatly improves gene transfer efficiency (Luskey *et al.*, 1992). In my hands, I usually achieve 8-10% infection efficiency of primary HSCs, thus even with enhanced transduction protocols, it is still very difficult to obtain large numbers of transduced cells.

The efficiency of gene transfer varies considerably and not all target cells become infected. This results in a mixed population of transduced and non-transduced cells, therefore it is necessary to tag your vector with a selectable marker that allows for the distinction between infected and non-infected cells. For my experiments, I used a retroviral vector (Sfbeta 91) that contained green fluorescent protein (GFP) placed under internal ribosome entry sequence (IRES) translation. The gene of interest and GFP are

co-transcribed into a single mRNA encoding both genes from the same promoter. Protein synthesis is achieved by cap driven translation of the first gene and IRES driven translation from the second cistron (Haviernik *et al.*, 2008). Cells that express the gene will also express GFP, thus making it possible to isolate the cells of interest for use in functional experiments. In my experiments, I individually cloned the coding sequences of p107 and Snx5 into the Sfbeta 91 vector. Primary HSCs transduced with these vectors were then used for *in vitro* CAFC experiments.

p107 is a member of the reintoblastoma (Rb) family of proteins, which include pRb and p130 (Classon *et al.*, 2000). pRb is the best known member of this pocket protein family, and was the first tumor suppressor gene to be identified and cloned (Lee *et al.*, 1987; Sparkes *et al.*, 1983). Members of the pRb family are structurally similar, with greatest homology lying in a pocket domain that mediates interaction of the proteins with the E2F family of transcription factors as well as with viral oncoproteins (Classon and Dyson, 2001). Studies examining mice with targeted mutations in pRb genes suggest that the three proteins have overlapping functions (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1994), the most prominent of which is to regulate cell proliferation by negatively regulating the activity of E2F transcription factors. Hypophosphorylated pRb proteins bind to members of the E2F family of transcriptional regulators and block E2F proteins from activating genes involved in cell cycle progression (Nevins, 1992). However, unlike pRB, mutations in p107 and p130 are not frequently observed in human cancers, thus suggesting that the proteins have at least some non-redundant functions. Vanderluit et al. looked specifically at the role of p107 in neural stem cells, and consistent with its role as a growth suppressor, found that it negatively regulates the number of neural stem cells in both the developing and adult brain (Vanderluit *et al.*, 2004). Additional studies by Kondo et al. used a non-phosphorylatable and thus constitutively active p107 to demonstrate that it can inhibit S phase progression a BaF3-derived mouse pro-B cell line (6-1 cells) (Kondo *et al.*, 2001). They also show that the exposure of cells to cisplatin, a DNA-damaging agent, causes S phase arrest, which is associated with the accumulation of hypophosphorylated p107, and thus conclude that p107 participates in inhibition of S phase progression in response to DNA damage (Kondo *et al.*, 2001). I hypothesize that overexpression of p107 in bone

marrow cells exposed to irradiation (i.e. cellular stressor) will force them to arrest at G1, thus allowing cells time to repair damage, enter a senescent state, or undergo apoptosis. Over the lifetime of the animal, this intact cell cycle checkpoint would prevent DNA damage accumulation in the stem cell compartment and thus would ensure long-term function of the hematopoietic system. Several labs have studied the functional role of pRB in the HSC compartment, but limited data exists on the role of p107 in hematopoiesis (Daria *et al.*, 2008; Spike *et al.*, 2004; Spike and Macleod, 2005; Walkley and Orkin, 2006). In order to determine the effect of p107 overexpression on the regulation of HSC numbers, I constructed the aforementioned retroviral vector containing p107 and overexpressed this gene in congenic bone marrow cells enriched in HSCs.

The other candidate gene uncovered in the microarray analysis, Snx5, is a member of a large family of sorting nexin proteins that are involved in various aspects of endocytosis and protein trafficking (Worby and Dixon, 2002). The Snx5 protein has been reported to bind the Fanconi anemia complementation group A (FANCA) protein, and increased expression of Snx5 in cells increases FANCA protein levels (Otsuki *et al.*, 1999). Eleven Fanconi anemia (FA) genes have been identified, and collectively the proteins function to regulate how cells recognize and repair DNA damage (Mathew, 2006). It has been shown that FA genes are highly expressed in primitive hematopoietic cells, suggesting the genes may be involved in maintenance of the stem cell pool and/or differentiation (Aube *et al.*, 2003). The Fanconi anemia syndrome is a rare recessive genetic disorder characterized by chromosomal fragility, bone marrow failure, and predisposition to cancer. The altered DNA repair and damage response phenotype of this syndrome is shared by several accelerated aging syndromes including Bloom,Werner, and ataxia telangiectasia (Zhang *et al.*, 2007). I hypothesize that Snx5 controls cellular levels of FANCA, thereby facilitating the cells ability to respond to and/or repair DNA damage. Reduced levels of Snx5, and possibly FANCA, in aged congenic stem cells (and young cells exposed to radiation) may dampen DNA response/repair pathways, thus leading to senescence, cellular aging, and stem cell depletion.

In addition to binding the FANCA protein, Snx5 has also been shown to translocate to the plasma membrane in response to Epidermal growth factor (EGF) stimulation and inhibit EGF receptor (EGFR) degradation (Liu *et al.*, 2006; Merino-Trigo

et al., 2004). Recently it has been shown that EGFR is phosphorylated and activated after cellular stressors such as irradiation (Dittmann *et al.*, 2005). Upon activation, EGFR translocates to the nucleus where it binds and activates DNA-dependent protein kinase (DNA-PK), which mediates non-homologous end joining (NHED) to repair DNA double strand breaks (Bandyopadhyay *et al.*, 1998; Dittmann *et al.*, 2005; Lin *et al.*, 2001). It is possible that increased Snx5 expression aids in the stabilization of EGFR, which in turn results in enhanced DNA repair capacity in the face of damage. Thus, decreased Snx5 expression in congenic HSCs may result in reduced DNA repair efficiency, which explains why they are extremely sensitive to 2 Gy of irradiation.

In order to determine the effect of Snx5 overexpression on HSC number, I constructed a second retroviral vector encoding Snx5 and overexpressed this gene in congenic bone marrow cells enriched in HSCs. It was not possible to overexpress p107 and Snx5 in old congenic cells due to large number animals that this task would require. It takes approximately 12 young mice to yield enough cells (2-4 x 10^6 cells) to be injected into one recipient mouse. Since the experiments were performed in triplicate and quadruplicate, it would require nearly 100 old congenic mice to perform these experiments. I maintain an active congenic breeding colony in the DLAR at the University of Kentucky, therefore making it possible for me to obtain enough young mice for the completion of these studies.

Materials and Methods

Animals

Young (2-4 months) C57BL/6 (B6) (Ptprc^{b} [CD 45.2]) mice were purchased from the National Institute of Aging (NIA) Aged Mouse Colony at Harlan Sprague Dawley (Indianapolis, IN) and were used as recipients of GFP+ cells. The mice served as *in vivo* "incubators" to expand infected cells for use in CAFC assays. Chromosome 2 congenic mice were generated as described previously (Geiger *et al.*, 2005). The congenic interval spans 50 Mbp (130Mbp -180 Mbp) on the distal end of chromosome 2. All mice were female and were housed in the Division of Laboratory Animal Resources (DLAR) at the University of Kentucky Chandler Medical Center and were maintained under pathogen-free conditions according to NIH guidelines for animal welfare.

Retroviral vectors

The coding regions of the genes were subcloned into the multi-cloning site of the retroviral vector Sfbeta 91. The Sfbeta 91 backbone served as a control. Sfebta 91 contained a myeloproliferative sarcoma virus (MSFV)-derived 5' long terminal repeat (LTR) and a spleen focus forming virus (SFFV)-derived 3'LTR. It also contained an internal ribosome entry site (IRES) derived from the encephalomyocarditis virus which ensured simultaneous translation of the gene insert and the enhanced green fluorescent protein (GFP) reporter gene. The gene cDNA sequence was cloned upstream of the IRES in Sfbeta 91, to yield the recombinant the vectors, MSFV-p107-IRES-GFP-SFFV (p107GFP+) and MSFV-Snx5-IRES-GFP-SFFV (Snx5/GFP+). The recombinant vectors were transfected into Phoenix helper-free packaging cells using a calcium phosphate precipitation method. Production of high-titer retrovirus was carried out according to standard procedures in the Phoenix packaging cells.

Infection of primary bone marrow cells

Primary bone marrow cells were harvested as described above from young congenic mice that had been injected intraperitoneally with 150 mg per kg of body weight of 5-fluorouracil (5-FU) (Sigma-Aldrich) 4 days prior to harvest. Cells were then cultured

over night in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS, 1% penicillin/streptomycin, 50 ng/mL recombinant mouse stem cell factor (mSCF), 10 ng/mL mouse interleukin 6 (mIL-6), and 10 ng/ml mouse interleukin 3 (mIL-3) (R&D Systems). Cells were subsequently harvested and placed onto transwell membranes (Corning Incorporated Life Sciences) at a density of $2x10^6$ cells per well. Viral supernatants plus 50 ng/mL mSCF, 10 ng/mL mIL-6, 10 ng/mL mIL-3, and 4 ug/mL polybrene were added to the cells. Fresh supernatant (plus cytokines and polybrene) was added to the cells 4 times over a period of 48 hours. The infected cells were then harvested and transplanted into sub-lethally irradiated (6 Gy) B6 mice at a dose of $2x10^6$-$4x10^6$ cells per mouse to repopulate the myeloablated tissues.

Isolation of GFP+ cells

Twelve weeks post transplant, mice injected with infected cells were euthanized by CO_2 asphyxiation and the femora, tibiae, and humeri were removed to a Petri dish with Hanks Balanced Salt Solution (HBSS, GIBCO) supplemented with 2% fetal bovine serum (FBS, Invitrogen) (media). Humeri were used in order to isolate the maximum amount of bone marrow from the mice. Bones were flushed with 4 mL media to obtain a single cell suspension. Erythrocytes in the bone marrow were eliminated by hypotonic lysis and the cells were washed with media. GFP+ (empty vector, p107/GFP+, Snx5/GFP+) cells were sorted from bone marrow on a FACS Vantage cell sorter (Becton-Dickinson) and were used for cobblestone area forming cell (CAFC) assays. Frequencies of CAFCs were calculated using maximum likelihood analysis with L-Calc software (Stem Cell Technologies). Frequencies equal one divided by the number of cells yielding 37% negative wells.

CAFC Assay for functional analysis of retrovirally transduced cells

Equal numbers of GFP+ retrovirally transduced cells (empty vector, p107/GFP+, Snx5/GFP+) were sorted from primary recipient mice 12 weeks after initial transplant. The CAFC assay was carried out as described previously (de Haan *et al.*, 1997; Geiger *et al.*, 2001) with slight modifications described below. FBMD-1 stromal cells were plated in a 96-well tissue culture plate 7-10 days before the start of the assay to form a confluent

layer. After 7-10 days, 20 replicate wells were seeded with GFP+ cells at cell doses of 81,000, 27, 000, 9000, 3000, 1000, or 333 cells per well. In circumstances where it was not possible to sort enough cells for six dilutions, cells were seeded at doses of 27, 000, 9000, 3000, 1000, or 333 cells per well. The cell culture media contained IMDM (GIBCO) plus 20% horse serum, 80 U/ml penicillin, 80 mg/ml streptomycin (Life Technologies), and 0.1 mM each of B-mercaptoethanol and hydrocortisone (Sigma-Aldrich). Individual wells were screened at days 7, 14, 21, 28, and 35 for the presence of a cobblestone area, defined as colonies of at least five non-refractile cells growing beneath the stromal layer.

Results

Over-expression of p107 in congenic cells

In order to assess whether the overexpression of p107 affects HSC numbers, I first overexpressed the gene in young unmanipulated congenic mice . Vectors (empty vector control/GFP+ and p107/GFP+) were transfected into Phoenix cells and viral supernatant was harvested after 24 and 48 hours. Once the viral supernatant was obtained, bone marrow cells were harvested from congenic mice that received 5-FU four days prior to sacrifice. Bone marrow cells were placed on a transwell and the viral supernatant was applied to the cells for 48 hours. New supernatant was added approximately every 12 hours, and after 48 hours the cells were injected into sub-lethally irradiated recipients (Figure 4.1). Based on results obtained in neural stem cells, we hypothesized that over-expression of p107 would decrease stem cell numbers. However, since p107 has been reported to regulate cell proliferation in response to stress (Kondo *et al.*, 2001; Voorhoeve *et al.*, 1999), I hypothesized that overexpression of p107 in cells exposed to radiation would cause the cells to undergo cell cycle exit, thus facilitating the repair of DNA damage, and ultimately preserving the long-term function of the cells. Proper cell cycle control and exit in the HSC compartment in times of stress and throughout the lifespan of the animal would ensure preservation of the HSC pool and contribute to decreased aging of this population in B6 mice. Thus, a subtle increase in p107 expression throughout the lifetime of an individual may help to preserve the primitive HSC compartment. I first made sure that the sorted p107/GFP+ cells were in fact overexpressing p107 compared to the empty vector control at both transcript and protein levels. I found that p107 transcript and protein expression was higher in cells transduced with p107/GFP+ compared to those transduced with the empty vector control (Figure 4.2). After verifying that the congenic cells overexpress p107 compared to empty vector control cells, p107/GFP+ cells were sorted and used for *in vitro* CAFC assays. In this assay, primitive stem cells migrate beneath a pre-extablished stromal layer and form colonies (cobblestones) at various time-points over a period of a month or more. Cobblestones are defined as a cluster of at least five non-refractive cells growing beneath the stromal layer. The longer the latency for a cobblestone to appear, the more primitive

in nature the cell. Thus the frequency of long term repopulating stem cells correlates with the frequency of CAFC day35 cells whereas more committed progenitor cells correspond to CAFC day21 and day28 cells (Figure 4.3). To our surprise, we observed a statistically significant two-fold increase in the frequency of CAFC day35 cells in cells overexpressing p107 (1.79 ± 0.31 per 100, 000 GFP+ cells) compared with cells that expressed the empty vector control (0.8 ± 0.14 per 100, 000 GFP+ cells) (P = 0.001) (Figure 4.4). We also observed a statistically significant increase in day21 (6.44 ± 0.75) and day28 (2.93 ± 0.42) CAFCs in cells overexpressing p107 compared to those overexpressing the empty vector control (3.82 ± 0.43 and 1.44 ± 0.20, respectively) ($P \leq 0.001$). The increase in CAFC frequency in progenitor (day21 and day28 CAFC) as well as stem (day35 CAFC) cells, suggests that p107 plays an important role in regulating cell proliferation throughout various stages of hematopoiesis.

Overexpression of Snx5 in congenic cells

Similar to experiments with p107, I first overexpressed Snx5 in young unmanipulated congenic mice. According to microarray experiments and RT-PCR, Snx5 expression is elevated in old B6 stem cells compared to old congenic cells. Since old B6 mice have higher numbers of HSCs compared to old congenic mice, I hypothesized that the overexpression of Snx5 in congenic cells would increase HSC frequency in these mice. Vectors (empty vector control/GFP+ and Snx5/GFP+) were transfected into Phoenix cells and viral supernatant was harvested after 24 and 48 hours. Viral supernatant was added to bone marrow cells harvested from congenic mice that received 5-FU four days prior to sacrifice. The bone marrow cells were placed on a transwell and the viral supernatant was applied to the cells for 48 hours and new supernatant was added approximately every 12 hours. Finally, infected cells were injected into sub-lethally irradiated mice, which served as in vivo "incubators" to expand the GFP+ cell population. After 12 weeks, the recipient mice were sacrificed and GFP+ cells (empty vector control and Snx5/GFP+) were sorted and used for the *in vitro* CAFC assay. A period of 12 weeks post transplant allows time for the establishment of stem and progenitor cell populations *in vivo*. After this length of time, donor contributions to all hematopoitic compartments is maximized and derived from stem cells originally present in the donor marrow. Before performing

CAFC assays, I first measured Snx5 mRNA and protein levels in sorted empty vector control/GFP+ and Snx5/GFP+ cells. I found that Snx5 mRNA and protein expression were higher in Snx5/GFP+ cells compared to the control vector (Figure 4.5) and proceeded to perform CAFC assays. In agreement with my hypothesis, I found a significant increase in the frequency of day35 CAFCs in cells overexpressing Snx5 (1.17 ± 0.13 per 100,000 GFP+ cells) compared to cells expressing the empty vector (0.77 ± 0.13 per 100,000 GFP+ cells) ($P \leq 0.05$) (Figure 4.5). I also observed a significant increase in the number of day14 (20.8 ± 2.07) and day21 (5.26 ± 0.50) CAFCs in cells overexpressing Snx5 compared to cells expressing the empty vector (13.59 ± 1.91 and 3.86 ± 0.39, respectively) ($P \leq 0.05$) (Figure 4.6). The fact that I did not detect a difference in the number of day28 CAFCs can probably be explained by the fact that the cumulative results are from 4 individual experiments, two of which involved sorting Snx5/GFP+ cells and empty vector control/GFP+ cells on separate days. It is best to sort the test gene cells and the control cells on the same day so that they are exposed to the exact same stromal cell layers and so that they can be counted on the same day. However, due to flow cytometer sorting schedules and time constraints this was not always possible, and thus likely influenced the CAFC results when the experiments were combined. For the two experiments in which Snx5/GFP+ and empty vector control/GFP+ cells were sorted together, I observed a significant increase in day14, day21, day28, and day35 CAFCs in cells overexpressing Snx5 compared to those overexpressing the empty vector. Thus it is likely that Snx5 increases cell number exclusively in stem and progenitor populations, but not in committed cell types, such as day7 CAFCs.

Discussion

Upon confirming the differential expression of p107 and Snx5 between young and old B6 and congenic Lin- and LSK cells, I next used recombinant retroviruses containing p107 and Snx5 cDNA to overexpress the genes in congenic bone marrow cells to further validate the genes as candidate regulators of HSC aging and/or sensitivity to irradiation. It must be noted that the radiation sensitivity phenotype was discovered serendipitously, thus the gene(s) that regulates this trait may or may not be the same gene(s) that regulates the stem cell aging phenotype. Upon performing *in vitro* CAFC experiments, I demonstrated that p107 and Snx5 do in fact regulate the number of HSCs in chromosome 2 congenic mice. I found that the individual overexpression of p107 and Snx5 in young congenic bone marrow cells resulted in a statistically significant increase in the number of day35 CAFC cells compared to cells overexpressing the control vector. These findings provide strong evidence that p107 and Snx5 positively regulate HSC number and suggest that elevated expression of these genes in HSCs throughout the lifespan of mice may affect the rate at which their HSCs age as well as affect HSC sensitivity to cellular stressors. For these experiments I singly overexpressed p107 and Snx5 in congenic bone marrow cells. It will be interesting in future experiments to determine if the dual overexpression of p107 and Snx5 in the same cells leads to a further increase in stem cell number compared to the present studies where the genes were individually expressed. Additionally, although the data is not available at the time of dissertation writing, I am performing CRU assays *in vivo* in which graded doses of donor Snx5 and p107 overexpressing cells are injected into lethally irradiated, but genetically distinguishable, recipient mice along with a set number of competitor cells. These experiments will allow me to calculate the absolute frequency of Snx5 and p107 overexpressing cells compared to cells expressing the empty vector in recipient mice, and thus will undoubtedly reveal whether the genes regulate HSC number in an *in vivo* setting.

Because HSCs are extremely rare, it is not possible to obtain them in sufficient numbers for classical biochemical characterization. Therefore, in order to facilitate functional studies of the hematopoietic system, retroviral gene transfer techniques serve as useful tools. Enhanced gene transfer techniques such as pre-treatment of donor

animals with 5-FU, the use of cytokines, and the use of selectable markers that allow for the distinction between transduced and non-transduced cells have greatly improved the efficiency of this technique in HSCs. Treatment of donor animals with 5-FU prior to bone marrow harvest forces primitive cells into cycle and thus facilitates retroviral gene transfer into HSCs. Additionally, pre-stimulation of donor marrow cells from 5-FU treated mice with IL-3, IL-6, and SCF further enhances gene transfer into HSCs. Studies have shown that SCF synergizes with other cytokines to promote the survival of primitive cells during *in vitro* manipulations associated with retroviral infection (Luskey *et al.*, 1992). Even though the use of these techniques enhances retroviral gene transfer into HSCs, the efficiency of gene transfer is never 100% and thus it is necessary to have a way to assess infection efficiency in HSCs. In my studies, GFP served as a selectable marker to track transduced cells which were then isolated by flow cytometry and used in subsequent CAFC functional assays.

In the present study, I overexpressed p107 in primary congenic hematopoietic bone marrow cells and performed *in vitro* CAFC assays to assess the role of p107 in regulating HSC numbers. Surprisingly, I found that the overexpression of p107 resulted in a significant increase in the frequency of CAFC day21, day28, and day35 cells compared to empty vector controls. Based on studies performed by Vanderluit et al., I expected to see a decrease in the frequency of CAFC day35 cells, especially since the cells used in the assay had not been exposed to any overt replicative stress. I originally hypothesized that overexpression of p107 in bone marrow cells exposed to irradiation (i.e. cellular stressor) would force them to arrest at G1, thus allowing cells time to repair damage, enter a senescent state, or undergo apoptosis. Over the lifetime of the animal, this intact cell cycle checkpoint would prevent DNA damage accumulation in the stem cell compartment and thus would ensure long-term function of the hematopoietic system. In contrast, congenic cells may proceed through the cell cycle, thus resulting in the accumulation of DNA damage in the congenic HSC pool. The accretion of damaged cells may ultimately result in impaired function of congenic HSCs in old mice (Figure 4.6).

Daria et al. recently investigated the function of pRb in hematopoiesis by creating an animal model in which floxed pRb alleles were deleted in the hematopoietic

compartment by Cre recombinase driven by the Vav1 promoter (Daria *et al.*, 2008). In the absensce of pRB there was a significant decrease in the frequency of CAFC day35 cells. They went on to perform competitive transplant experiments with Vav-Cre Rb KO cells, and observed a 8-fold reduced contribution to chimerism in peripheral blood compared with control cells. Since they found identical numbers of HPCs and HSCs in Vav-Cre Rb KO bone marrow and control bone marrow, they reasoned that pRb was a regulator of hematopoietic stem and progenitor cells under conditions of stress, such as transplantation and 5-FU treatment. Based on our observations with p107 in hematopoietic cells, we propose that the cell sorting procedure and the CAFC assay itself may serve as efficient stressors to trigger retinoblastoma family protein-mediated cell cycle exit and arrest. Indeed, it is well established that primitive HSCs in the bone marrow reside in a hypoxic microenvironment and that low oxygen levels play a role in the maintenance of normal stem cell function (Cipolleschi *et al.*, 1993; Danet *et al.*, 2003; Parmar *et al.*, 2007). The CAFC assay was performed in 5% humidified incubators in normoxic conditions (20% O_2), whereas *in vivo* HSCs are maintained in hypoxic (< 3% O_2) conditions. This oxygen imbalance likely imparts additional stress on primitive HSCs.

To my knowledge, extensive analysis of the function of the closely related pRb gene, p107, has never been performed in the HSC compartment. LeCouter et al. did however report a myeloproliferative disorder as well as extensive extramedullary hematopoiesis in the spleen and liver (which was predominantly myeloid in composition) in germ line mutated p107-/- BALB/cJ mice (LeCouter *et al.*, 1998). They also observed increased numbers of myeloid progenitor cells in the femurs of p107-/- mice, consistent with results of Daria et al. (Daria *et al.*, 2008). Although LeCouter et al. reported a less severe phenotype when p107-/- mice were backcrossed to a B6 background, they did not look closely at individual organ systems and certainly did not assess the phenotype in response to conditions of stress. I propose that in addition to pRB, p107 plays an important role in regulating HSCs. I further hypothesize that hematopoietic stem cells are very sensitive to situations in which they are removed from their natural environment and p107 may play a role in sensing this stress. Perhaps this is a protective mechanism that HSCs have acquired in order to preserve the function and integrity of this vital cell

population. Further, the elevated expression of p107 in B6 LSK cells may serve to protect these cells from the damaging effects of low dose irradiation. The cells elicit proper cell cycle checkpoints and are able to preserve the integrity of their population over time. Cogenic LSK cells, on the other hand, may continue to cycle and therefore accumulate cells with damaged DNA. Ultimately this scenario may result in impaired self-renewal capacity and diminished numbers of HSCs over the life span of an organism.

Numerous studies have assessed the role of the closely related protein, pRB, in the hematopoietic compartment (Daria *et al.*, 2008; Lee *et al.*, 1992; Spike *et al.*, 2004; Spike and Macleod, 2005). Spike et al. demonstrated cell intrinsic defective erythroid maturation under stress conditions in Rb-/- cells as well as the loss of hematopoietic stem and progenitor cells from the bone marrow and spleen of mice transplanted with Rb-/- fetal liver cells. An additional study conducted by Spike et al., showed that Rb null erythroblasts contained increased levels of reactive oxygen species (ROS), greater levels DNA damage, as well as altered chromosome structure compared to wild-type cells (Spike and Macleod, 2005). These data suggests that the Rb, and perhaps additional pocket protein members, is required for hematopoietic homeostasis. Moreover, my studies with p107 suggest that increased levels of these proteins may increase stem cell number, although the mechanism of this function is not yet known. I am currently performing an extensive *in vivo* analysis of the role of p107 in the HSC compartment. CRU experiments with cells overexpressing p107 are being conducted in order to determine if elevated expression of the gene corrects the early stem cell aging phenotype observed in D2 and congenic mice.

The fact that p107 contains four SNPs resulting in amino acid substitutions provides additional strength supporting our hypothesis that p107 is a QT gene that regulates HSC aging because the exonic SNPs may explain differential p107 expresssion in B6 and congenic mice. Rb family members contain a common region know as a "pocket" which consists of two conserved domains, A and B, and a non-conserved spacer region (Chow *et al.*, 1996). Starostik et al. have shown that the central pocket in p107, including domains A and B, but not the spacer region, is sufficient for p107 to act as a general cellular repressor as well as its ability to inactivate E2F (Starostik *et al.*, 1996). Additional studies have shown that the spacer region in p107 mediates an interaction with

the cell cycle regulatory protein cyclin A, thus providing p107 with dual interaction domains (Ewen *et al.*, 1992; Faha *et al.*, 1992; Smith and Nevins, 1995; Zhu *et al.*, 1995). Mutations in these regions critical for p107 function, although not causing differential expression of the gene, could interfere with the normal functioning of the protein and thus render cells more vulnerable to cellular stressors.

Snx5, the other candidate gene in these studies, has been shown to bind to the FANCA protein and its knock down in zebrafish was shown to lead to an 80% reduction in circulating blood cell production, thus making it an attractive potential regulator of HSC function. Similar to studies performed with p107, I overexpressed Snx5 in congenic bone marrow cells and performed *in vitro* CAFC assays to determine whether Snx5 affected the frequency of HSCs. According to microarray, real time PCR and western blot data, Snx5 expression is elevated in young and old B6 HSCs compared to young and old congenic HSCs. Because the number of primitive HSCs increases in B6 mice as they age, I hypothesized that Snx5 was a positive regulator of HSC number and that its overexpression in congenic bone marrow cells would increase HSC frequency. Upon performing CAFC assays, I found a significant increase in the frequency of day14, day21, and day35 CAFCs in congenic HSCs overexpressing Snx5 compared to cells expressing the control vector. As explained previously, the reason I did not see an increase in the number of day28 CAFCs in cells overexpressing Snx5 is likely due to the fact that in two of the replicate assays, Snx5 overexpressing cells and empty vector control cells were not isolated on the same day. This added technical variability to these studies, which could have been avoided if the test cells were sorted at the same time. In two of the four individual CAFC experiments, Snx5/GFP+ and empty vector control/GFP+ cells were sorted on the same day. Upon combining these data, I observed a significant increase in day14, day21, day28, and day35 CAFCs in cells overexpressing Snx5 compared to those overexpressing the empty vector. These data suggest that elevated Snx5 expression affects HSC frequency specifically in stem and progenitor cells and not in differentiated cells and thus further validates our hypothesis that Snx5 may regulate the HSC aging trait.

The CAFC results reinforce our hypothesis that overexpression of Snx5 increases HSC number and suggest that the life-long elevated expression of Snx5 in B6 HSCs may

help the cells respond to and repair DNA damage more efficiently than congenic HSCs. This enhanced DNA repair capacity, possibly due to Snx5's interaction with FANCA and/or EGFR, may slow the aging of the cells and possibly increase their self-renewal capacity which leads to an increase in HSC number with advancing age (Figure 4.7).

Unlike p107, Snx5 does not have any non-synomous coding region SNPs between B6 and D2 mice. According the Ensembl genome browser, the gene does however have 8 3'UTR and 8 intronic SNPs. It is possible that the intronic SNPs could affect transcript stability of thus expression of Snx5 mRNA. Also, due to the presence of 7 predicted murine microRNA binding sites in the 3' UTR of Snx5, it is possible that SNPs located in the 3' UTR may modulate Snx5 expression.

The fact that Snx5 has been shown to interact with two proteins that are involved in mediating DNA repair processes is intriguing. The overexpression of Snx5 in 293 cells was shown to increase levels of the FANCA protein in a dose dependent manner (Otsuki *et al.*, 1999). Similarly, upon overexpressing Snx5 in a NR6/EGFR cell line, EGFR expression was induced and maintained thus suggesting reduced degradation of the receptor during EGFR signaling (Liu *et al.*, 2006). As mentioned previously, FANCA is one of eleven Fanconi anemia genes that collectively function to regulate how cells recognize and repair DNA damage (Mathew, 2006). Cells (such as B6 HSCs) that express high levels of Snx5 may also express high levels of FANCA, thus increasing the DNA repair capacity and enhancing the longevity of these cells. Like FANCA, EGFR has also been implicated in DNA repair pathways. EGFR is a cell surface receptor that mediates cell proliferation, migration and survival. EGFR is frequently expressed in cancers of epithelial origin, and its expression and activity correlate with tumor resistance to radiotherapy (Chen and Nirodi, 2007). Recent studies show that EGFR plays a radioprotective role which involves translocating to the nucleus and binding to and activating DNA-PK, which then mediates NHEJ to repair DNA double stranded breaks (Bandyopadhyay *et al.*, 1998; Dittmann *et al.*, 2005; Lin *et al.*, 2001). Since Snx5 has been shown to stabilize and prevent the degradation of EGFR, it is possible that increased Snx5 expression enhances DNA repair capacity through the NHEJ repair pathway as well. Snx5 may be working through both the FANC and the DNA-PK/NHEJ DNA repair pathways to help protect the accumulation of damage in B6 HSCs. Reduced expression

of Snx5 in congenic HSCs throughout the lifespan of the animal may reduce the ability of the cells to respond to and repair DNA damage, which ultimately leads to a qualitative and quantitative decline the in their hematopoietic system with age.

In summary, I have identified p107 and Snx5 as quantitative trait genes that regulate the number of HSCs in B6 and congenic mice. CAFC assays confirmed that increased expression of both genes increases HSC number in an *in vitro* setting. Although the increased expression of both Snx5 and p107 resulted in small increases in HSC number, the changes are biologically significant given the extensive proliferative potential of primitive stem cells. Extemely small fluctuations in the size of the HSC pool can result in dramatic differences in mature blood cell production, and thus immune function, due to the fact that HSCs are the sole source of all differentiated blood cells. *In vivo* CRU assays will be performed in future experiments to unequivocally demonstrate that these genes regulate HSC numbers. I hypothesize that long term elevated expression of the genes protects the HSC pool and thus ultimately slows the aging of the cells and possibly the entire individual.

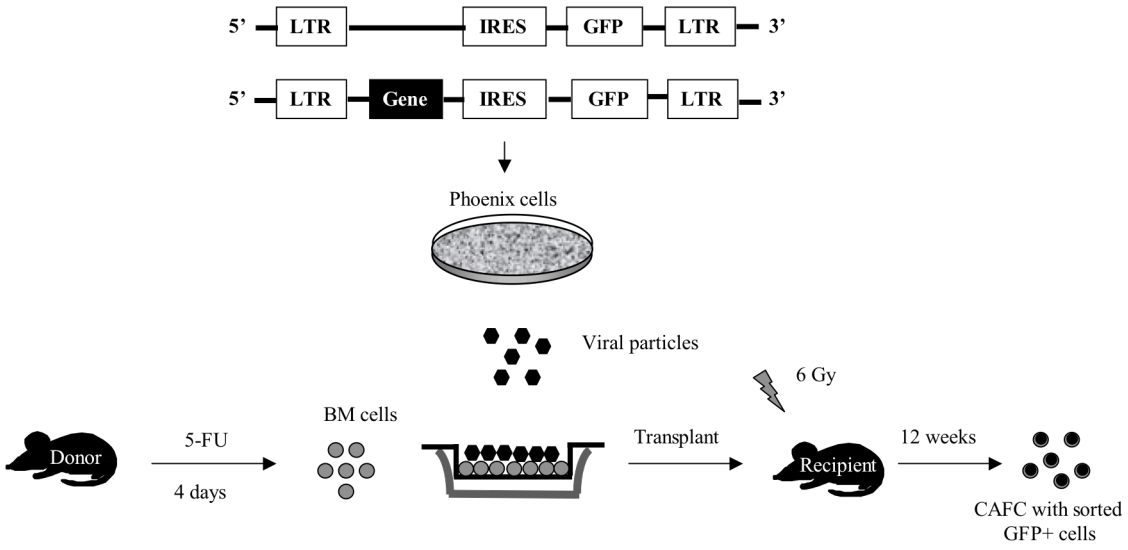


Figure 4.1 Schematic illustrating the experimental design used for retroviral transduction of HSCs. The Sfbeta 91 retroviral vector served as the backbone for p107 and Snx5 cDNA-containing vectors as well as an empty vector control. Phoenix packaging cells were transfected with the vectors and high titer viral supernatant was collected after 24 and 48 hours. Bone marrow cells were harvested from young congenic mice that had been injected intraperitoneally with 150 mg per kg of body weight of 5-FU 4 days prior to harvest. The bone marrow cells were placed on a transwell insert (in a six well plate) and were exposed to the viral supernatant for 2 days. Infected cells were then injected into sub-lethally irradiated B6 recipient mice. Twelve weeks post transplantation, GFP+ cells were sorted and used for *in vitro* CAFC assays.

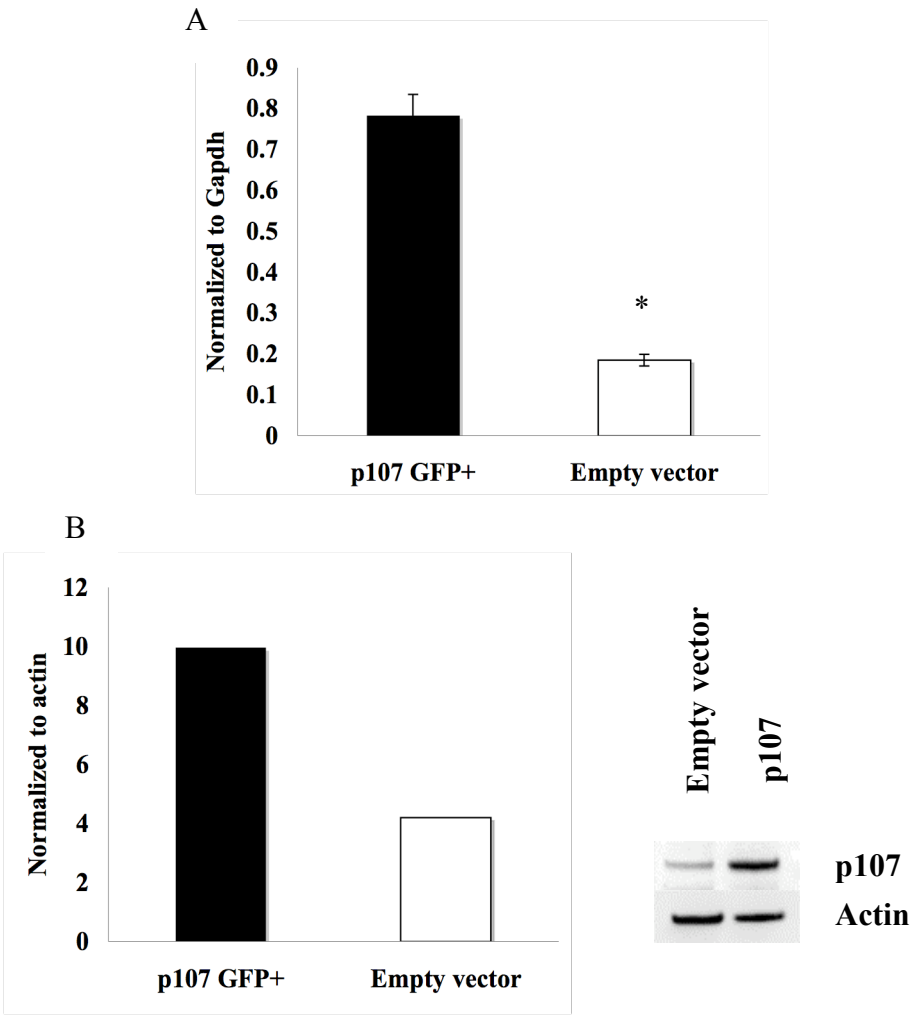


Figure 4.2 Overexpression of p107 in transduced HSCs. Real time PCR data showing that p107 mRNA levels are elevated in primary GFP+ cells compared to cells infected with the empty vector control ($P \leq 0.01$) Results are given as mean ± SEM, n = 8 measurements from 2 individual experiments. Representative western blot showing overexpression of p107 protein in sorted GFP+ bone marrow cells (B).

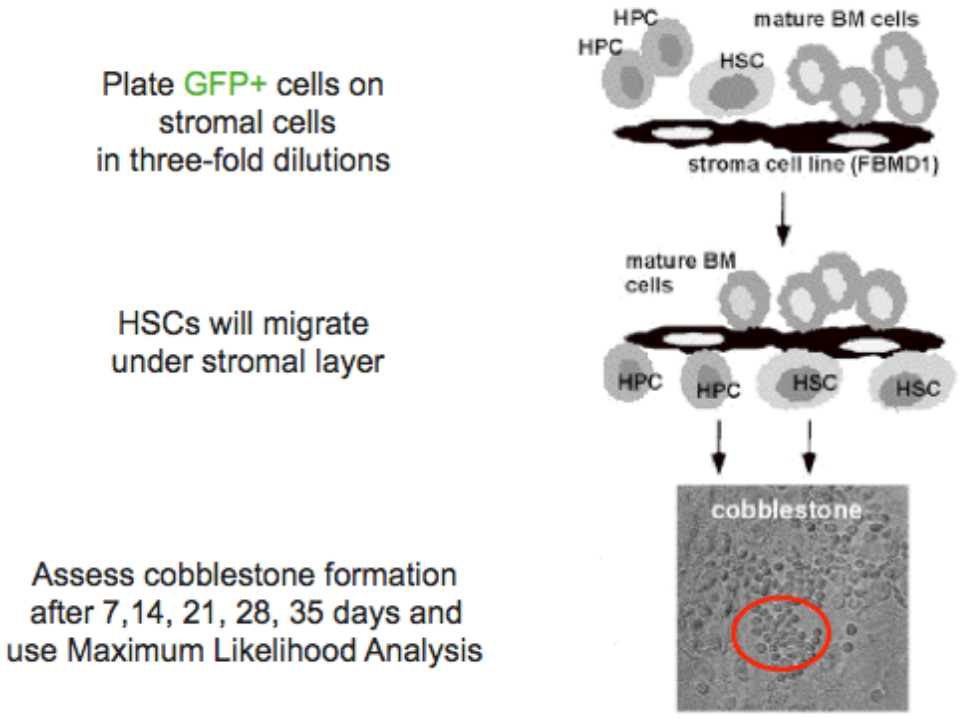


Figure 4.3 Diagram of the cobblestone area forming cell (CAFC) assay.

The CAFC assay is in an *in-vitro* limiting dilution assay to measure HSC number. Stem and progenitor cells of a particular developmental stage migrate beneath a stromal cell layer and form cobblestone-like colonies. Cobblestone formation is assessed at various days after plating and the maximum likelihood analysis is used to determine stem cell number. The longer the latency before a cell proliferates and forms a cobblestone area, the more primitive in nature the cell.

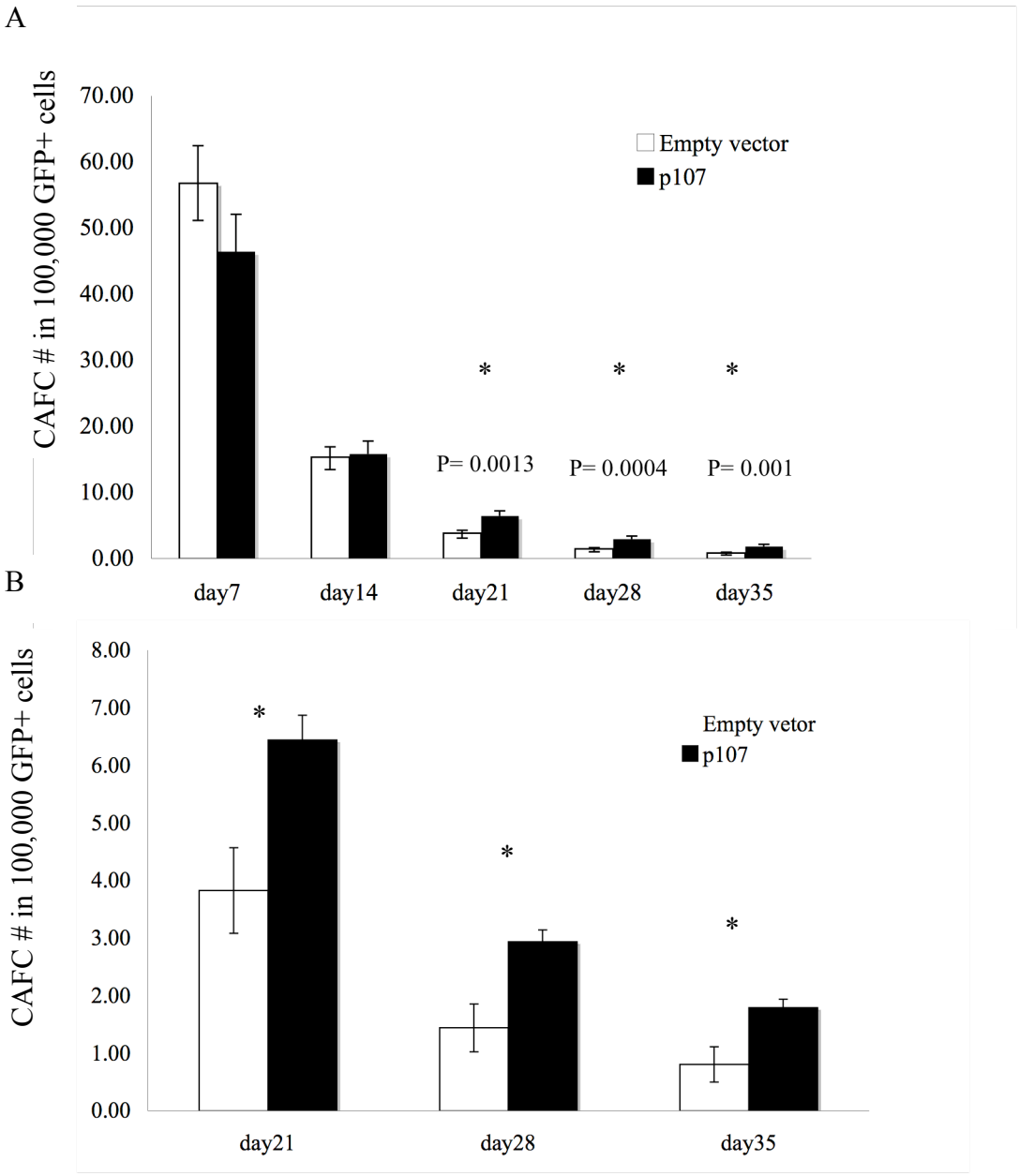


Figure 4.4 CAFC numbers in congenic cells overexpressing p107 and the empty vector control. I observed a statistically significant two-fold increase in the frequency of day35 CAFCs in cells overexpressing p107 compared with cells that expressed the empty vector as well as a significant increase in day21 and day28 CAFCs in cells overexpressing p107 compared to those overexpressing the empty vector control ($P \leq$

0.001) (A). Closer view of day 21, day 28, and day 35 CAFC numbers in young congenic bone marrow cells overexpressing p107 or empty vector control (B). Results are given as mean ± SEM, n = 4 replicate experiments.

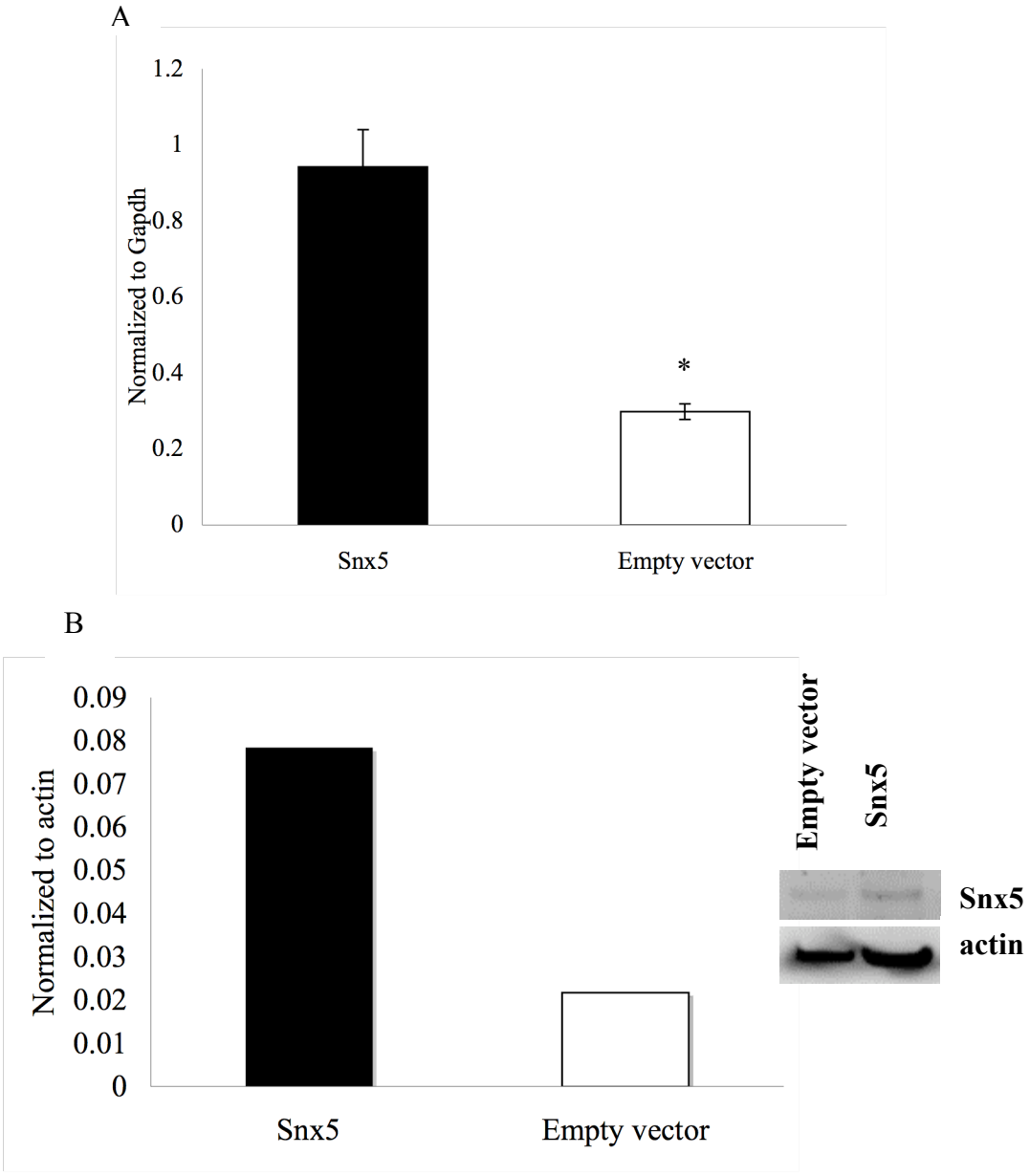


Figure 4.5 Overexpression of Snx5 in transduced HSCs. Real time PCR data showing that Snx5 mRNA levels are elevated in primary GFP+ cells compared to cells infected with the empty vector control ($P \leq 0.01$) Results are given as mean ± SEM, n = 12 measurements from 3 individual experiments (A). Representative western blot showing overexpression of Snx5 protein in sorted GFP+ bone marrow cells (B).

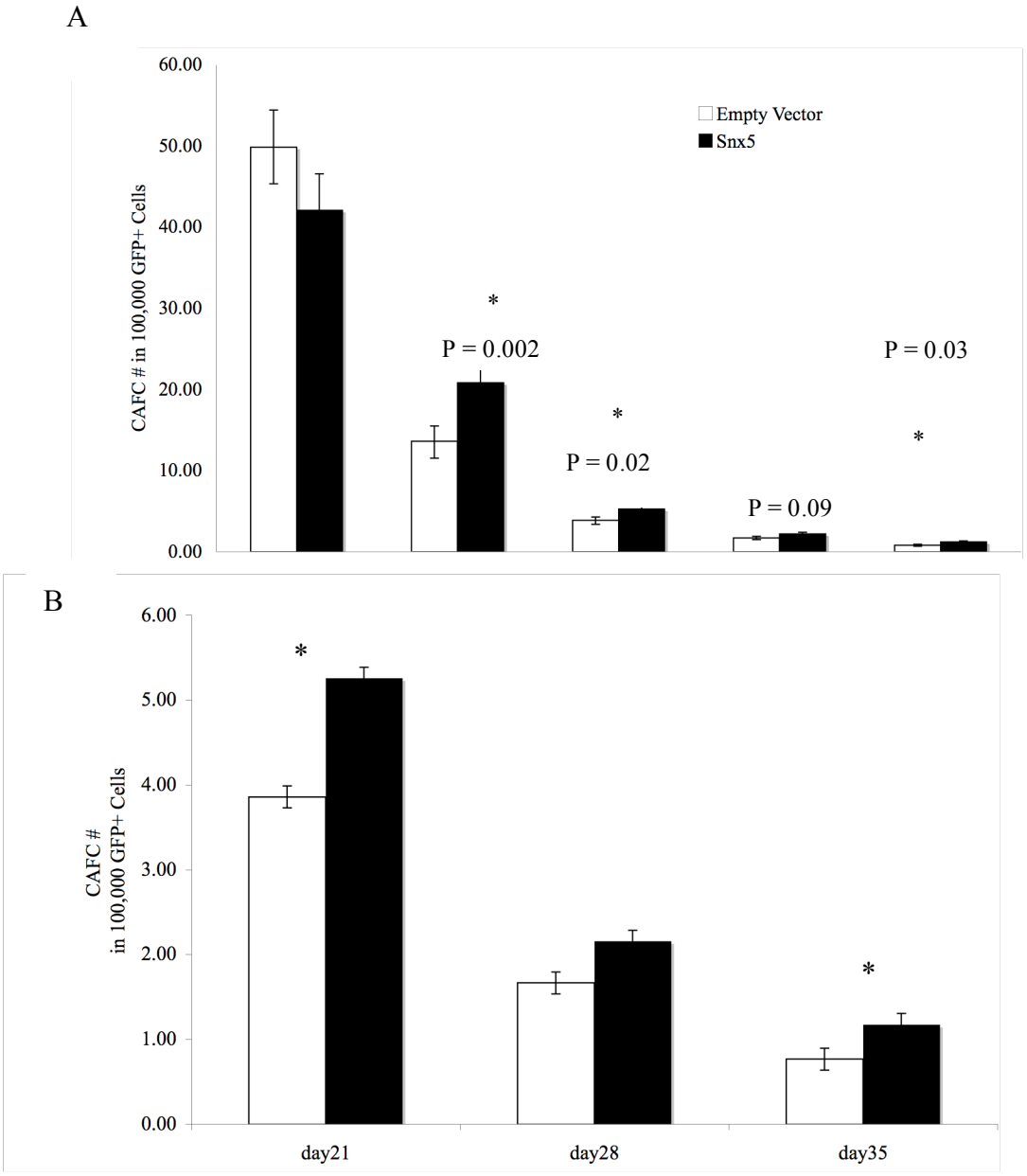


Figure 4.6 CAFC numbers in congenic cells overexpressing Snx5 and the empty vector control. I observed a statistically significant increase in the number of day14, day21 and day35 CAFCs in cells overexpressing Snx5 compared to those overexpressing the empty vector conrol ($P \leq 0.05$) (A). Closer view of day21, day28 and day35 CAFC numbers in congenic cells overexpressing Snx5 and the empty vector (B). Results are given as mean ± SEM, n = 4 replicate experiments.

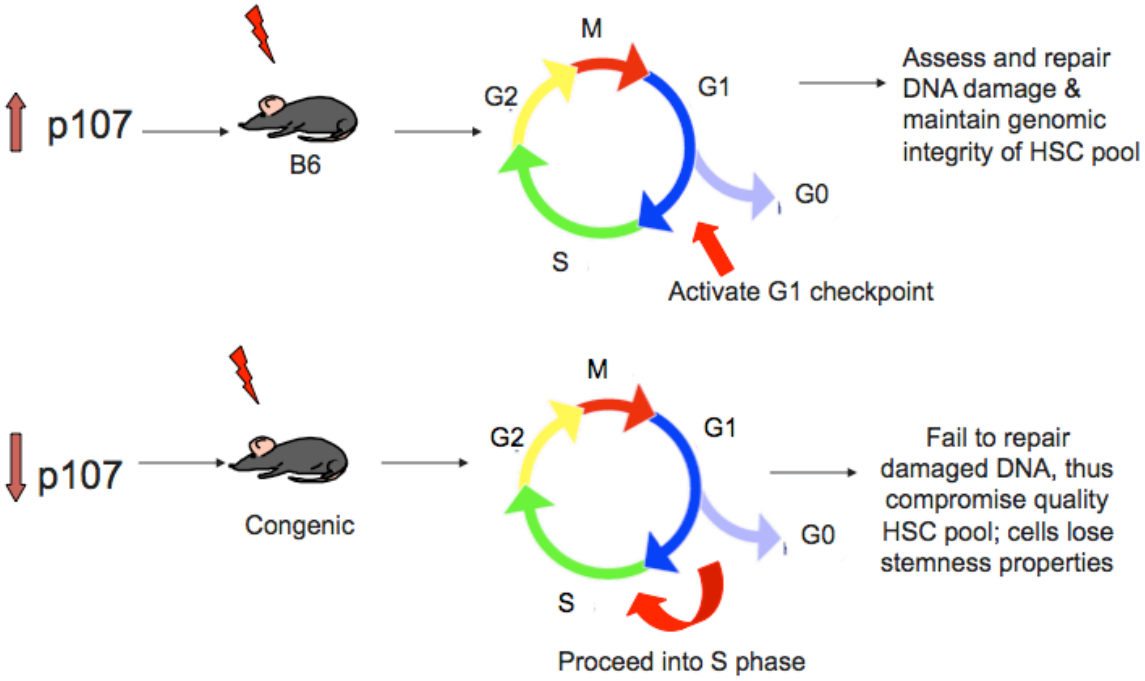


Figure 4.7 Mechanism by which p107 may regulate HSC number

B6 bone marrow cells, which express high levels of p107, may undergo cell cycle arrest after exposure to irradiation, thus allowing cells time to repair damage, enter a senescent state, or undergo apoptosis. Over the lifetime of the animal, this intact cell cycle checkpoint would prevent DNA damage accumulation in the B6 stem cell compartment. In contrast, the congenic cells, which express low levels of p107, may proceed through the cell cycle after the insult, thus resulting in the accumulation of DNA damage in the congenic HSC pool. The accretion of damaged cells may ultimately result in impaired function of congenic HSCs in old mice.

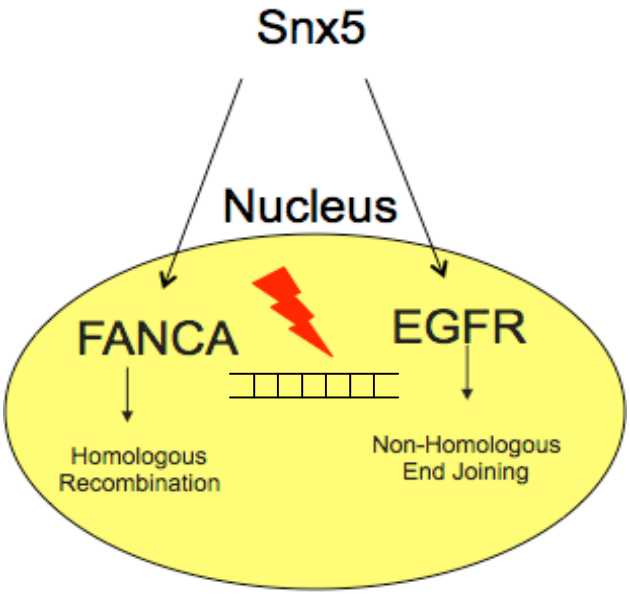


Figure 4.8 Mechanisms by which Snx5 may regulate stem cell number.
Snx5 interacts with both FANCA and EGFR, two genes involved in DNA repair pathways. Snx5 may serve to increase or stabilize FANCA and EGFR expression, thereby facilitating enhanced DNA repair capacity in stem cells. Enhanced DNA repair capacity throughout the lifespan of an organism may protect HSCs from accumulating DNA damage and thus slow the aging process in these cells.

CHAPTER FIVE

General Discussion and Conclusions

Despite their ability to self-renew and maintain essentially normal levels of hematopoietic function throughout the lifetime of an organism, it has become increasingly evident that hematopoietic stem cells (HSCs) are subject to qualitative and quantitative changes and that these changes may affect both the rate of aging and longevity of organisms. The main goal of my dissertation study was to evaluate the hypothesis that one or more genes located in a QTL on murine chromosome 2 contributes to the strain specific difference in the frequency of HSCs in aged C57BL/6 (B6) and DBA/2 (D2) mice. However, in a broader sense, the ultimate goal of my research was to identify genes in a murine mouse model that could one day be extended to human HSCs, thus contributing to the understanding of the complex biology of HSC aging.

Following a classical forward genetic approach, I proposed three specific aims to explore the aforementioned hypothesis: 1) to better understand the mechanistic basis for the different number of HSCs in aged B6 and congeic mice by determining the cell cycle, apoptotic, and self-renewal status of HSCs using a surrogate assay to mimic aging; 2) to identify differentially expressed genes between B6 and congenic mice that may contribute to the HSC aging phenotype using microarray technology; 3) to overexpress candidate gene(s) uncovered in the microarray analysis and determine their effect on HSC number and function.

Novel findings in this dissertation include the discovery that D2 alleles in the chromosome 2 QTL affect the level of apoptosis and the self-renewal capacity of HSCs. Specifically, D2 alleles decreased the percentage of lineage negative (Lin-) cells undergoing apoptosis following 2 Gy of irradiation and drastically decreased the self-renewal capacity of HSCs exposed to 2 Gy of irradiation as revealed by serial transplantation experiments. In addition, microarray studies identified two novel candidate genes that may regulate HSC aging, Retinoblastoma like protein 1 (p107) and Sorting nexin 5 (Snx5). B6 alleles are associated with upregulated p107 and Snx5 expression as well as increased HSC number in aged mice, therefore I hypothesized that

both genes are positive regulators of HSC number. Further, cobblestone area forming cell assays (CAFC) revealed that p107 as well as Snx5 increased the frequency of day35 CAFCs, which correspond to primitive HSCs. Therefore these studies not only reveal the power of combining classic QTL studies with modern microarray technologies to identify genes underlying complex quantitative traits, but also provide strong evidence that p107 and Snx5 may regulate some aspect of HSC aging.

Unfortunately these studies cannot definitely determine whether p107 and Snx5 control the HSC aging phenotype or the sensitivity to low dose irradiation. It is possible that the genes control both of the traits and that our surrogate assay to measure aging activates the same molecular pathways that are at work in aged stem cell populations. Indeed, my microarray studies showed significant overlap in the age and irradiation treatment probesets, suggesting that irradiation damage causes similar transcriptional changes as does the natural aging process. I am in the process of expanding my aged congenic colony, and future experiments will be performed in which p107 and Snx5 will be overexpressed in aged congenic cells. If I obtain similar results as observed in young congenic cells, I will conclude that the genes do in fact regulate the number of HSCs in old mice. In addition, performing self-renewal assays with old B6 and congenic cells will allow for me to determine whether on not increased self-renewal capacity is the mechanism by which B6 HSCs increase in number with age. Also, performing CAFC experiments with transduced congenic cells that have been irradiated will determine whether the genes affect HSC response to cellular stressors. Nonetheless, these current studies do in fact indicate that elevated expression of p107 and Snx5 increase the number of day35 CAFC numbers, which correspond to primitive stem cells.

In light of recent data suggesting that DNA damage accumulation throughout the long lifespan of HSCs contributes to their decline in cellular function with age, the fact that p107 and Snx5 may regulate the response to and/or the repair of DNA damage makes them great candidates for regulating the complex biology of HSC aging. p107 participates in mediating cell cycle arrest in response to stress, which in turn allows cells time to repair damage and proceed through the cell cycle or alternatively, undergo apoptosis. Indeed, p107 may help prevent the accumulation of DNA damage in HSCs, therefore preserving their function in old age. Moreover, the mechanistic phenotypes of

the congenic cells, including reduced apoptosis in Lin- cells and a subtle but non-significant increase in LSK cell cycling may result from the decreased expression of p107 in these mice. Reduced levels of p107 expression are consistent with increased cycling as well as the inability to exit cycle and undergo apoptosis in response to DNA damage in the form of irradiation. Like p107, Snx5 may also be involved, through FANCA or EGFR, in the regulation of HSC response to and repair of DNA damage, and thus may prevent the accumulation of DNA damage in HSCs. Reduced levels of Snx5 in congenic HSCs may compromise their ability to repair DNA damage, which may contribute to the profound decrease in self-renewal capacity of these cells in response to irradiation. To my knowledge there is no available data linking the cellular pathways of p107 and Snx5, however it is possible that the two genes work in the same pathway to regulate HSC number. The underlying mechanisms by which p107 and Snx5 control HSC pool size must be resolved in order to determine whether the genes may act together to effect stem cell number.

In conclusion, although the clinical applications of these findings may be far away, my studies have identified two novel regulators of HSC number and thus bring us one step closer toward understanding the genes and molecular pathways that regulate stem cell number as well as stem cell aging and response to stress. However, as mentioned previously, more experiments need to be performed in order to determine the exact context in which p107 and Snx5 regulate HSC number. In addition to the aforementioned experiments, I will examine the effects of the knock-down of p107 and Snx5 using morpholino technology to help validate them as candidate genes. I hypothesize that performing CAFC assays with cells in which p107 and Snx5 have been knocked-down will decrease the frequency of day35 CAFCs. Ultimately, I would like to expand these findings to human HSC biology in order to determine whether the expression of p107 and Snx5 regulate human stem cells. Hopefully, these studies will aid in the development of HSC amplification strategies, which will expand the use of these cells in transplantation procedures for the treatment of hematological diseases. For example, the overexpression of p107 and Snx5 may be able to expand a cancer patient's normal HSCs for use in autologous stem cell transplants. Overexression of the genes in normal stem cells may be able to increase the number of the patient's HSCs which could

then be reintroduced to the patient after high dose chemotherapy and/or radiation treatment. The reintroduced cells would function to restore or rescue hematologic and immunologic function following high dose therapy. This procedure would not only reduce the number of cells necessary for the initial harvest, but would also eliminate the need to locate allogenic donors which are often difficult to find. Stem cell therapies to treat various cancers have advanced greatly in past several years. It is my hope that the research performed in this dissertation will aid in the advancement and use of such therapies.

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Abstracts and Presentations

Oakley, E. J., and G. Van Zant. Identification of a Novel Candidate Regulator of HSC Aging. 48th Annual American Society of Hematology Meeting and Exposition, December 2006, Orlando, FL (poster)

Oakley, E. **J**., and G. Van Zant. Identification of a Novel Candidate Regulator of HSC Aging. Molecular Genetics of Aging Meeting, Cold Spring Harbor Laboratory, August 2006, Cold Spring Harbor, NY (poster)

Oakley, E. **J**., and G. Van Zant. Identification of Candidate Regulators of Hematopoietic Stem Cell Aging Using a Combined QTL Mapping and Microarray Analysis Approach. 4th Annual Midwest Blood Club Symposium, April 2006, Lexington, KY (oral)

Oakley, E. **J**., and G. Van Zant. Identification of Candidate Regulators of Hematopoietic Stem Cell Aging Using a Combined QTL Mapping and Microarray Analysis Approach. 3rd Annual International Conference on Functional Genomics of Aging, March 2006, Palermo, Sicily (oral and poster)

Oakley, E. **J**., A. Miller, and G. Van Zant. Identification of Candidate Regulators of Hematopoietic Stem Cell Aging Using a Combined QTL Mapping and Microarray Analysis Approach. 47th Annual American Society of Hematology Meeting and Exposition, December 2005, Atlanta, GA (poster)

Bell, D., **E. J. Oakley**, C.T. Jordan, and G. Van Zant. Stem Cell Analysis in a Mouse Model of Leukemia. 3rd Annual Midwest Blood Club Meeting, April 2005, Louisville, KY

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Publications and Book Chapters

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