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ABSTRACT OF DISSERTATION

Ying Liang

The Graduate School

University of Kentucky

2005

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GENETIC REGULATION OF HEMATOPOIETIC STEM CELL NUMBERS IN MICE

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
YING LIANG

Lexington, Kentucky

Co-Directors: Dr. Gary Van Zant, Professor of Medicine and Physiology
Dr. Lu-Yuan Lee, Professor of Physiology

Lexington, Kentucky
2005

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ABSTRACT OF DISSERTATION

GENETIC REGULATION OF HEMATOPOIETIC STEM CELL NUMBERS IN MICE

Hematopoietic stem cells (HSCs) transplantations are widely used for the treatment of hematological and non-hematological disorders in clinic. Successful transplantation requires sufficient number and efficient homing of HSCs. Many studies have focused on developing an effective strategy to expand functional HSC population. Some regulatory molecules have been recently shown great promise for controlling the amplification of HSCs.

In these dissertation studies, I first aim to identify gene(s) and their allelic variants contributing to strain-specific difference in HSC numbers between C57BL/6 (B6, low) and DBA/2 (D2, high) mice by using a classic forward genetic approach. Firstly, 3 quantitative trait loci (QTL) on chromosome (Chr) 3,5 and 18 were mapped by linkage analyses and confirmed in congenic mice. Secondly, Chr.3 QTL affected several HSC number-related biological processes. The D2 allele increased cycling and self-renewal whereas it decreased apoptotic rates of HSCs. Both actions conspired to increase HSC population size. Lastly, a small number of differentially-expressed genes was identified in Chr.3 congenic HSCs

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by a microarray-based candidate gene method, and the differential expression of one candidate, latexin, was found to relate to HSC number variations. Our studies report the strong evidence for the potential functions of latexin in HSC number regulation, and they are important for understanding molecular mechanisms of stem cell regulation and developing effective stem cell expansion strategies for clinical applications.

In the second part of my studies, I studied homing and engraftment capabilities of HSCs. By using functional assays for progenitor and stem cells, I first reported the absolute homing efficiencies of murine young or old donor cells into young or old recipient mice. The results indicated that homing of primitive hematopoietic cells was not efficient and significantly decreased by aging of donors and recipients. The proliferation and differentiation states of HSCs were also impaired by homing itself, as well as by donors' and recipients' age. Moreover, the hematopoietic reconstitution dynamics following transplantation were also affected by aging. Together, these findings will provide useful information for clinical applications especially when older individuals increasing serve as stem cell donors for elderly patients.

KEYWORDS: Hematopoietic Stem Cells, Hematopoietic Stem Cell Number, Genetic Regulation, Homing and Engraftment, Aging

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GENETIC REGULATION OF HEMATOPOIETIC STEM CELL NUMBERS IN MICE

By

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For Rendong Bai and my family, Mom, Dad

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TABLE OF CONTENTS

ACKNOWLEDGMENTS……………………………………………………………..iii

LIST OF FIGURES……………………………………………………………vii

LIST OF TABLES……………………………………………………………..ix

CHAPTER ONE: INTRODUCTION…………………………………………...1
Hematopoietic Stem Cells and Hematopoietic Hierarchy………………………1
Identification of Hematopoietic Stem Cells………………………………………2
In vivo long-term reconstitution assay to identify HSCs…………………2
In vitro long-term cell culture assay to identify HSCs……………………4
Flow cytometric analysis to identify HSCs………………………………..5
Genetic Approach to Study Stem Cell-Specific Genes…………………………7
HSC Frequency (Number) and Its Regulation…………………………………10
Murine HSC frequency as measured by different methods…………...10
Factors involved in the regulation of HSCs and their numbers……….11
Cell cycle, apoptosis and HSC number…………………………………14
Self-renewal and HSC number…………………………………………...16
Hematopoietic Stem Cell Expansion and Clinical Application………………..17
Aging Effects on Hematopoietic Stem Cells and Bone Marrow Niche………18
Experimental Goals and Significance…………………………………………...20

CHAPTER TWO: MAPPING AND CONFIRMATION OF QUANTITATIVE TRAIT LOCI LINKED TO STRAIN-SPECIFIC DIFFERENCE IN HEMATOPOIETIC STEM CELL NUMBER…………………………………………………………..28
Summary……………………………………………………………………….. 28
Introduction……………………………………………………………………...30
Materials and Methods…………………………………………………………34
Results…………………………………………………………………………...39
Discussion………………………………………………………………………43

CHAPTER THREE: CHANGES IN CYCLING, APOPTOSIS AND SELF-RENEWAL CAPABILITIES OF HSCs IN CONGENIC MOUSE MODELS……...58
Summary………………………………………………………………………...58
Introduction……………………………………………………………………...60
Materials and Methods…………………………………………………………65
Results…………………………………………………………………………...71
Discussion………………………………………………………………….…78

CHAPTER FOUR: IDENTIFICATION OF QUANTITATIVE TRAIT GENE(S) RESPONSIBLE FOR HSC NUMBER REGULATION……………………………..95
Summary………………………………………………………………………...95
Introduction……………………………………………………………………...97
Materials and Methods………………………………………………………100

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Results……………………………………………………………………………108
Discussion…………………………………………………………………..114

CHAPTER FIVE: EFFECTS OF AGING ON THE HOMING AND ENGRAFTMENT OF MURINE HEMATOPOIETIC STEM AND PROGENITOR CELLS………………………………………………………………………………132
Summary…………………………………………………………………………132
Introduction………………………………………………………………………134
Materials and Methods…………………………………………………………137
Results……………………………………………………………………………141
Discussion…………………………………………………………………..149

CHAPTER SIX: GENERAL DISCUSSION AND CONCLUSION……………….172

APPENDIX…………………………………………………………………………180

REFERENCES……………………………………………………………………..183

VITA………………………………………………………………………………220

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LIST OF FIGURES

Figure 1.1 The hematopoietic system is hierarchical in nature…………………...21

Figure 1.2 Schematic illustration of forward and reverse genetics………………22

Figure 1.3 Intrinsic and extrinsic regulation of hematopoietic stem cells………..25

Figure 1.4 HSCs fate outcomes……………………………………………………...26

Figure 1.5 Diminished functional capacity of hematopoietic stem cells during aging...27

Figure 2.1 Schematic illustration of derivation of BXD recombinant inbred mouse strains……………………………………………………………………..49

Figure 2.2 Scheme depicting generation of congenic animals…………………..50

Figure 2.3 Illustration of cobblestone area under microscope……………………51

Figure 2.4 Experimental design for Competitive Repopulation Unit (CRU) assay……………………………………………………………………….52

Figure 2.5 Genome-wide quantitative trait loci (QTL) mapping…………………..53

Figure 2.6 CAFC day35 analyses in chromosome 3, 5 congenic and background mice……………………………………………………………………….54

Figure 3.1 Identification of Lin-, Sca-1+, c-Kit+ (LSK) HSCs by flow cytometry..86

Figure 3.2 Schematic illustration of serial transplantation of bone marrow cells to measure self-renewal of HSCs……………………………………….…..87

Figure 3.3 Cell cycle analysis of unseparated bone marrow cells and LSK stem cells by DAPI staining…………………………………………………….88

Figure 3.4 Cell cycle kinetics of HSCs measured by BrdU incorporation……….89

Figure 3.5 Measurement of apoptotic LSK cells by Annexin V and 7-AAD……..90

Figure 3.6 Serial transplant of B6 and B.D Chr3 congenic bone marrow cells to measure HSC self-renewal capability……. ……………………………91

Figure 4.1 Schematic illustration of chromosome 3 congenic map and genomic location of candidate quantitative genes………………………………122

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Figure 4.2 Development–dependent differential expression of latexin mRNA in chromosome 3 congenic mice……………………………………….123

Figure 4.3 Latexin protein expression in mouse and human cells……………...124

Figure 4.4 Development-dependent differential expression of latexin protein in chromosome 3 congenic mice……………………………………….125

Figure 4.5 Experimental design for latexin overexpression model……………..127

Figure 4.6 Identification of QTL regulating latexin expression…………………..128

Figure 5.1 Reduced homing capacity of HPCs with donor and recipient age…157

Figure 5.2 Experimental design for competitive repopulation studies…………158

Figure 5.3 Age-related decline in the homing efficiency of murine HSCs……..159

Figure 5.4 Effects of aging and prior transplantation on the proliferation potential of CRU…………………………………………………………………161

Figure 5.5 Effect of aging and transplantation on the differentiation potential of CRU…………………………………………………………………….163

Figure 5.6 Differential contribution of single BM CRU to hematopoietic engraftment……………………………………………………………165

Figure 5.7 Differential engraftment kinetics of young or old BM cells in old or young recipients………………………………………………………167

Figure 6.1 Models of potential latexin-involved biological processes…………..179

المنارة للاستشارات

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LIST OF TABLES

Table 2.1 Determination of long-term competitive repopulating units (CRUs) frequency in B6 and B.D Chr3 mice……………………………………55

Table 2.2 Comparison of hematopoietic progenitor cells (HPCs) and peripheral blood cell numbers among congenic and background mouse strains…………...57

Table 3.1 Cell cycle analysis of LSK HSCs by DAPI staining in chromosome 3 congenic and their background mouse strains……………………..…………93

Table 3.2 Apoptotic analysis of LSK HSCs by Annexin V and 7-AAD staining in chromosome 3 congenic and their background mouse strains…………………..94

Table 4.1 Differentially expressed genes in both chromosome 3 congenic and their respective background mice..129

Table 5.1 Determination of the number of intravenously transplanted young CRU that home to the BM of old irradiated mice……………………………169

Table 5.2 Determination of the number of intravenously transplanted old CRU that home to the BM of young irradiated mice………………………..171

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CHAPTER ONE

Introduction

Hematopoietic Stem Cells and Hematopoietic Hierarchy

Hematopoiesis is the process by which blood cells of multiple and distinct lineages are produced throughout life. The ultimate burden of the life-long production of mature blood cells is carried out by a small population of primitive cells, termed hematopoeitic stem cells (HSCs), which reside predominantly in the bone marrow in adults. The distinguishing functional capacities of HSCs are self-renewal to maintain their population throughout life, and multilineage differentiation to produce all mature blood cell types (Morrison et al. 1995; Spangrude et al. 1991). The balance between self-generation and differentiation, at individual cell and population levels, is critical for maintenance of steady-state hematopoiesis(Lessard, 2004).

The hematopoietic system is organized as a hierarchy of stem and progenitor cells along a developmental continuum. The HSC population at the apex of this hierarchy is heterogenous and composed of long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs). LT-HSCs can self-renew and function as stem cells throughout the life, whereas ST-HSCs can do this only for limited periods of time (typically less than 8 weeks in mouse) before they are fully differentiated (Capel et al. 1990; Rosendaal et al. 1979; Spangrude and Johnson 1990). The hematopoietic progenitor cells (HPCs), which are progeny of HSCs,

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have been traditionally recognized as clonogenic cells that give rise to cells of a single lineage, or a subset of the hematopoietic lineages (Abramson et al. 1977). Common lymphoid progenitors (CLP) produce T, B lymphocytes and natural killer cells (NK); common myeloid progenitors (CMP) generate other lineage blood cells, including erythrocytes, macrophages, granulocytes and platelets. All these mature blood cells are circulating in the peripheral blood and responsible for constant maintenance and immune protection of the body.

Identification of Hematopoietic Stem cells

In vivo long-term reconstitution assay to identify HSCs

The hallmark of the HSCs is their capacity for the lifetime generation of hematopoietic cells. Therefore, assays designed to identify this rare population must enable their distinctive properties to be measured. That is, a functional HSC is one that demonstrates complete and sustained (at least 12 weeks in mouse) regeneration of the lymphohematopoietic system following transplantation. "Stem" cells were first functionally defined by Till and Maculloch over 40 years ago (Till and McCulloch 1961). In this assay, bone marrow-derived cells formed colonies in the spleen of myelo-ablated recipients after intravenous injection, termed colony forming unit–spleen (CFU-S). Further experiments revealed that CFU-S cells were capable of forming additional spleen clonies when serially transplanted, indicating they could self-renew. Also, this population was heterogeneous with respect to their latency before forming colonies, the more primitive cells were, the later they produced colonies (Becker et al. 1963;

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Siminovitch et al. 1963; Wu et al. 1967). Although further studies showed most CFU-S were not HSCs, but rather early progenitors (Jones et al. 1990), this assay underpinned all contemporary methods for the functional identification and quantitation of HSCs *in vivo and in vitro*.

The most stringent and reliable method to assay HSCs is one demonstrating long-term multi-lineage reconstitution capability of HSCs in vivo. Based on this concept, a competitive repopulation assay (CR assay) was designed in which the cells being evaluated ("test" cells) are co-injected with genetically or phenotypically distinguishable "competitor" cells into lethally irradiated recipient mouse (Harrison 1980). The roles of "competitor" cells are to exert selective pressure on "test" cells and to ensure the survival rate of recipients following lethal irradiation. Two different types of competitive repopulation assays are used in the studies of HSCs. The standard method is to inject equal numbers of "test" and "competitor" cells into lethally-irradiated recipients; theoretically 50% of hematopoietic cells will be regenerated by each population. The skewing of chimerism in favor of either donor indicates its competitive advantage, which may be due to increased HSC numbers, proliferation or both (Harrison et al. 1993).

To distinguish these two different mechanisms, the competitive repopulation assay was modified to incorporate a limiting-dilution design in which groups of recipient mice are co-injected with graded number of "test" cells together with a certain number of "competitor" cells. Several months later, the proportion of recipients injected with each cell dose group that had been

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engrafted with “test” stem cells is determined, and the absolute frequency (number) of HSC (operationally defined here competitive repopulating unit, CRU) can be calculated by applying maximum likelihood and Poisson statistics (Szilvassy et al. 1990). Moreover, another advantage of the CRU assay is that it enables the retrospective identification of recipient mice that were repopulated by a single HSC using two criteria: (i) they were determined by limiting-dilution analysis to have been transplanted with $\leq$ 0.3 CRU (“0.3” comes from the p_0 value in Poisson equation applied in limiting dilution design: $-\ln p_0 = m$ when m equals 1), and (ii), they contained >5% peripheral blood (PB) cells detectable among B, T and myeloid lineages that had been derived from “test” cells. By analyzing the percentage of “test” cell-derived PB leukocytes in such recipient mice, the proliferation and differentiation state of a single HSC may be determined in terms of clone size and lineage distribution of this single clone (Szilvassy et al. 2001b; Szilvassy et al. 2003).

In vitro long-term cell culture assay to identify HSCs

As described previously, single HSC can proliferate to generate a colony containing all stages of hematopoietic cells (Osawa et al. 1996), the late colony-forming cells are more primitive than those generating colony at earlier times. This temporal hierarchy reflects the developmental hierarchy of the hematopoietic system (Rosendaal et al. 1979). Therefore, surrogate in vitro culture assays have been developed to measure HSCs and HPCs. Cobblestone area forming cell (CAFC) assay is one of such assays which is routinely used in

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our lab. A stromal cell-derived cell line, such as FBMD-1, is used to mimic the bone marrow microenvironment to support the growth of bone marrow cells in the *in vitro* culture system. Via unknown mechanisms, the primitive cells can migrate through the stromal layer and start proliferating to form a transient colony underneath it at characteristic time-points for a period spanning 35 days. The more primitive a cell, the later it will proliferate to form a cobblestone. Cells generating cobblestone at day 28 and 35 are considered long-term HSCs and CAFC day 7 to 21 as HPCs. Combined with a limiting dilution design, this assay can also be used to determine HSC frequency (Breems et al. 1994; Ploemacher et al. 1989). The major advantages of this long-term limiting dilution *in vitro* assay are that: (i) it measures the entire spectrum of HSCs and HPCs in a single assay system and avoids separate analysis by using different assays to measure HSC and HPC activity, and (ii), CAFC frequency correlates very well with stem cell frequency obtained by in vivo assays, such as the previously mentioned CFU-S and CRU assays (Ploemacher et al. 1992; Ploemacher et al. 1991). Other *in vitro* methods, including the long-term culture-initiating cell (LTC-IC) assay, colony-forming cells (CFC) assay (Hao et al. 1996; Sutherland et al. 1989; Sutherland et al. 1990), are also commonly used to measure HSC and HPC activity and operated by similar procedures.

Flow cytometric analysis to identify HSCs

Previously discussed *in vitro* methods for identification of HSCs rely on functional detection of stem cell progeny. The stem and progenitor cells

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themselves are lost in the assay procedure, and their existence is deduced retrospectively. Fortunately, development of flow cytometry has revolutionized hematopoietic stem cell study in the last 20 years. In this technique, fluorescence, which is attached to cells through either antigen-antibody interactions or direct binding, is excited when single cells pass one at a time through a laser beam. The subsequent detection and measurement of fluorescent intensity per cell enables not only identification and quantitation of HSCs, but also procurement of this rare population prospectively for further studies.

The more commonly used strategy for HSC separation relies on the presence or absence of cell surface markers. It is widely accepted that stem cells are not committed to any lineage of differentiated blood cells, therefore, they do not express many lineage markers that are characteristic of terminally differentiating hematopoietic cells. Thus, negative selection removes such lineage-positive cells and leaves predominantly immature cells. In order to enrich HSCs to higher purity, positive selection of stem cells is subsequently performed through stem cell specific markers. At least 2 surface markers are commonly used for sorting murine bone marrow HSCs. Sca-1 (Ly6a/e) is more or less restricted to stem cells and has formed the keystone for stem cell purification ever since its practical use was introduced (Spangrude et al. 1988). C-kit is a second murine stem cell marker that is widely used and is the tyrosine kinase receptor for stem cell factor (SCF) or kit ligand (Ikuta et al. 1991). In addition to these "classical" phenotypes, other markers or strategies to purify murine HSCs

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have been developed over the past several years. For example, lack of expression of Flk-2/Flt3 receptor tyrosine kinase and CD34 characterizes a population of primitive hematopoietic cells with long-term repopulating activity (Christensen and Weissman 2001). Recent studies have identified a small population of cells in bone marrow that efficiently efflux a family of fluorescent dyes, typified by Hoechst 33342, that contains essentially all of the long-term repopulating cells. Thus cells dimly stained by Hoechst 33342, which is also named the “side population”, may be analyzed and sorted by flow cytometry as an enriched source of HSCs (Goodell et al. 1996; Goodell et al. 1997; Wolf et al. 1993). Irrespective of the methods used, an important point in the isolation of HSCs is the one-to-one correspondence between physically purified cells and their potential ability to function as stem cells; that is, to have long-term repopulating activity.

Genetic Approach to Study Stem Cell–Specific Genes

With the development of various of technologies and methods, there have been significant advances in our understanding of the genes that regulate stem cells. Both forward and reverse genetics approaches have contributed to the broadening of our knowledge of this crucial cell population. Figure 1.2 depicts the similarities and differences between these two approaches. The fundamental difference is the starting point and, as the name implies, the direction followed by the experimental pathway. Whereas a forward genetics approach begins with a natural variation in a stem-cell phenotype and proceeds to the cause of the

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phenotypic difference at the level of the genome (phenotype to genotype) (Biola et al. 2003; Glazier et al. 2002), a reverse genetics approach begins with the discovery of polymorphisms at the genetic level and proceeds to the phenotypic effects on stem cells (genotype to phenotype).

Until recently, comprehensive studies concerning stem cell specific genes have been reported using reverse genetics approach. Briefly, two such studies generated profiles of genes expressed in embryonic stem cells (ESCs), neural stem cells (NSCs) and hematopoietic stem cells (HSCs) by using microarray analysis (Ivanova et al. 2002; Ramalho-Santos et al. 2002). Genes common to all three types of stem cells (200 to 300 genes) were identified and fell into several functional categories, including those encoding transcription factors, membrane proteins, nucleic acids binding proteins, and proteins involved in cell-cycle control and apoptosis. Although these "stemness" genes are theoretically surveyed and screened using such an approach, the considerable task remains of unraveling the mechanistic role of the gene in stem cell functioning. This can be especially difficult because it must be remembered that stem cell genes are always uncovered in the context of a discrete genetic background that may enlist, silence, or modify the expression of genes that in another genetic context may go undetected, be uncovered, or be perceived variously as minor or major determinants.

Forward genetics does not have the problem of determining phenotype because a measurable difference in stem cell function is the starting point of analysis. Because phenotypic variation is usually compared between two genetic

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models, for example, mouse strains, the potential genetic causes are limited to the genetic polymorphisms that exist between the chosen strains. Because of extensively documented differences between the hematopoietic systems of C57BL/6 (B6) and DBA/2(D2) mice, and the powerful genetic tools afforded by the recombinant inbred strains that have been derived by crossing them, several genetic studies have begun to shed light on loci in this genetic context that account for the interstrain variation in stem-cell properties (Liang and Van Zant 2003). Geiger *et al.* have reported the mapping of quantitative trait loci (QTL) on chromosomes 1, 3, 5 and 18 regulating natural variation in HSC numbers in bone marrow. More importantly, their studies have also confirmed the mapping by phenotypically characterizing congenic strains generated by introgressing the genomic interval surrounding QTL onto a non-native background (Figure1.2) (Geiger et al. 2001). In addition, other researchers have identified QTL governing other phenotypic variations among different inbred mouse strains, including change in HSC and hematopoietic progenitor cells (HPCs) during aging (Chen et al. 2000; de Haan and Van Zant 1999), responses of HSCs and HPCs to early-acting cytokines (Henckaerts et al. 2002), the sensitivity of HSCs and HPCs to the genotoxic effects of the cell cycle-specific drug hydroxyurea (de Haan et al. 1997) and mobilization of HPCs (Geiger et al. 2004). Furthermore, Bystrykh and de Haan et.al have recently analyzed gene expression profile in purified HSCs isolated from BXD recombinant inbred mouse strains and measured the variations in expression of HSC-specific transcripts among these strains. By combining QTL mapping, they not only identified genes and loci that are

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responsible for these variations, but also found that a large number of genes are regulated through cis-acting QTL, and several key trans-acting QTL are involved in controlling the expression of HSC-specific genes (Chesler et al. 2005).

Altogether, both forward and reverse genetics approach are designed to reveal genotypic and phenotypic differences. The two methods can be parallel and even complementary in the study of complex biological traits. For example, using a forward genetics approach, Morrison *et al.* have found that a QTL on chromosome 17 is responsible for the difference in HSC number between ARK/J and C57BL/Ka-Thy-1.1 mice (Morrison et al. 2002). Similarly, a reverse genetics study by Ramalho-Santos *et al.* have found a relatively large proportion of genes characterizing “stemness” are distributed on chromosome 17. Therefore, the possibility exists that the locus identified by Morrison et al. maps to one of the core stem-cell genes present in embryonic and adult stem cells (Ramalho-Santos et al. 2002). The complementary results represent the potential genetic power of integrating the two approaches to search for conceptual and mechanistic relationships among the limited information that we can get so far.

HSC Frequency (Number) and its Regulation

Murine HSC frequency as measured by different methods.

Different methods of enumeration of HSCs have provided estimate of the exact numbers of HSCs, although all methods reveal that they are exceedingly rare cells. In addition there are clear strain and age differences with respect to stem cell frequencies. Using *in vivo* limiting dilution competitive repopulation

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assays, murine long-term repopulating cells represent ~1 per 10,000~20,000 adult bone marrow cells (Rebel et al. 1994; Rebel et al. 1996; Szilvassy et al. 1990; Szilvassy et al. 2003). *In vitro* long-term culture assay, for example CAFC assay, around 100,000 bone marrow cells in the C57BL/6 mouse contain 1 CAFC day35, a cell considered to be equivalent to HSCs (de Haan et al. 2000; de Haan and Van Zant 1997; Ploemacher et al. 1989). In addition, using immunophenotypic staining of HSCs, typical frequencies of Lin- Sca-1+ c-kit+ cells is 0.01% to 0.02% of total bone marrow cells (Morrison and Weissman 1994). Assuming there are $3x10^8$ bone marrow cells in an adult mouse, this means that there are approximately 3 to $6x10^4$ HSCs are in the bone marrow. Mounting evidence has shown that HSC frequency changes with age and also this age effect is strain-dependent. We and others have found that C57BL/6 mice contain less HSCs than do DBA/2 mice at a young age. However, HSC number increase steadily as C57BL/6 mice age, whereas in DBA/2 mice HSC numbers decline with age (Chen et al. 2000; de Haan and Van Zant 1999; Geiger et al. 2001). The strain- and age- specific differences have been experimentally suggested to be regulated by cell-intrinsic mechanisms (Van Zant, 1990 #480) and affected by several quantitative trait loci (QTL) in the murine genome.

Factors involved in the regulation of HSCs and their number

What genes and signaling pathways are involved in the regulation of stem cell? And how are stem cell numbers maintained? As shown in Figure 1.3, HSCs are regulated by both cell intrinsic (cell autonomous) and cell extrinsic

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mechanisms. Recent studies have shown that some genes, such as *HoxB4* (Antonchuk et al. 2002; Beslu et al. 2004), *Runx1* (North et al. 2002), *Tel/Etv6* (Hock et al. 2004b) and *Gfi-1* (Zeng et al. 2004), encode transcription factors that activate transcription of specific genes which in turn dictate self-renewal or differentiation decisions by HSCs. Other genes that are involved in DNA repair, such as *Rad50* and *Ercc-1*, have also been reported to affect primitive hematopoietic cell population (Bender et al. 2002; Prasher et al. 2005). Some intrinsic factors, such as membrane transporters (Bcrp1/Mdr1a/1b) (Uchida et al. 2002; Zhou et al. 2002; Zhou et al. 2001) and integral membrane receptors (Flk-2) (Christensen and Weissman 2001), confer HSC-specific phenotypes and can be used in stem cell isolation. Because HSCs reside in bone marrow intimately associated with stroma, signals from this microenvironment also exert their effects on HSCs. Wnt/ß-Catenin and Notch-Delta signaling pathways have been recently found to extrinsically regulate HSCs through cell-cell interactions (Sorrentino 2004). The binding of stroma-derived Wnt proteins to their receptors Frizzled was found to protect ß-catenin from degradation. The intact ß-catenin subsequently translocates into the nucleus and activates downstream target genes. Some members in this signaling pathway are expressed in HSCs . Co-culture of Wnt proteins with HSCs and HPCs results in increased proliferation and expansion of these primitive cells (Austin et al. 1997; Van Den Berg et al. 1996; Willert et al. 2003). Moreover, overexpression of ß-catenin in HSCs showed a 5-50 fold expansion of HSCs in vivo, suggesting that Wnt signaling is important in regulating self-renewal of HSCs (Reya et al. 2003). The role of

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Notch-Delta interaction is similar to that of Wnt-Frizzled pathway. Upon stroma-derived delta ligand binding, Notch receptors on the surface of HSC are activated and translocated into the nucleus where it functions as a transcription factor and regulates self-renewal and differentiation of HSCs (Stier et al. 2002). Most interestingly, upregulated expression of *HoxB4* and *Notch-1* was observed in *ß-catenin*-transduced cells, indicating that different signaling pathways may interact with each other and form a complex regulatory network to control HSC fate (Duncan et al. 2005; Reya et al. 2003)). In addition, molecules from the extracellular matrix, such as MMp-9, have also been shown to be responsible for the translocation of HSCs between different functional niches, thus affecting their fate decisions (Heissig et al. 2002).

Under the influences of intrinsic and extrinsic mechanisms, HSC may face multiple choices. As shown in figure 1.4, some may undergo apoptosis and be lost from the population. Some may remain viable and progress through the cell cycle and yet others may remain quiescent or become senescent. If and when they divide, HSC replication may be symmetric to give rise to two identical daughter cells—HSC or progeny destined for differentiation—or the division may be asymmetric to give rise to one daughter HSC and one differentiated progeny. An asymmetric division preserves the HSC population and at the same time provides for the replenishment of mature blood cells (Ema and Nakauchi 2003; Faubert et al. 2004; Muller-Sieburg et al. 2002; Yan et al. 2003). Therefore, cell cycling, apoptosis and self-renewal are the major processes by which HSC pool

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size is regulated. Intrinsic and extrinsic signals involved in these processes thus have an effect on HSC number regulation.

Cell cycle, apoptosis and HSC number

Cell cycle is commonly thought to consist of four orderly phases: G1 (gap1), S (DNA synthesis), G2 (gap2) and M (mitosis) phases. Progression through these phases is controlled by positive and negative regulators. Positive contributors include the cyclins and the cyclin-dependent kinases (CDK). Negative regulators include the cyclin-dependent kinase inhibitors (CDKI) and the retinoblastoma protein (pRb) (Dao and Nolta 1999). It was originally thought that the majority of HSCs remained quiescent (G0 phase; a sub-state of G1) over a long period of time (Lajtha 1979), with only one or a few HSC at any one time actively cycling and contributing to the mature hematopoietic cell populations. Once a HSC clone is exhausted a new clone would be activated in a process called clonal succession (Lemischka et al. 1986). More recent BrdU labeling data have pointed to a more dynamic and active system, in which 75% of HSCs were in G0 phase, 25% were in S, G2, and M phase of the cell cycle at any given time, and 8% completed a cell division each day (Cheshier et al. 1999). Whatever model is correct, the critical point during the cell cycle is the G1-S transitional checkpoint, where external and internal factors can exert negative or positive influences on and thus regulate the entry of HSCs into the cell cycle (Dao and Nolta 1999).

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Apoptosis is caused by the activation of the caspase cascade, which is initiated by two signaling routes (stress-induced death and death-domain receptor-induced death) (Domen 2001). This process can be prevented by anti-apoptotic molecules, such as Bcl-2 (Domen and Weissman 2000). Direct evidence for the involvement of apoptosis in HSC number regulation came from the findings that overexpression of the anti-apoptotic gene *bcl-2* led to increased numbers of Thy-1.1^{low}, Sca-1+, c-kit+, Lin- cells, a population with long-term multi-lineage repopulation potential (Domen et al. 2000).

Considerable evidence has shown that factors affecting cell cycling and apoptotic processes can exert great effects on the HSC population. For example, p21, one type of CDKI, was found to be highly expressed in HSC and to be required for stem cell quiescence. Absence of p21 increased proliferation and therefore absolute numbers of HSCs, but resulted in impaired self-renewal and radioprotection capabilities of HSCs upon serial transplantation (Cheng et al. 2000b). Presumably the p21 checkpoint is overcome by cytokines and transcription factors such as Hoxb4 (Steinman 2002). In addition, p21 overexpression protects cells from apoptosis (Steinman and Johnson 2000). Another good example involved in cell cycle progression and apoptosis is the IL-6-initiated JAK-STAT intracellular pathways. They stimulate G1 to S transition by activating cyclin and preventing apoptosis through upregulation of bcl-2, all of which resulted in expansion of HSCs (Hirano et al. 2000).

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Self-renewal and HSC number

In addition to the above two regulators, enhanced self-renewal capability also results in HSC expansion. For example, *Hoxb4*, has been shown to induce higher in vitro or ex vivo expansions of HSCs by functioning as transcriptional factor when overexpressed in bone marrow cells. Mice transplanted with these genetically modulated HSCs showed a greatly enhanced regeneration in the hematopoietic stem cell compartment, but with normal bone marrow and spleen cellularity, and normal numbers and distribution of peripheral blood cells (Antonchuk et al. 2001; Antonchuk et al. 2002). In addition, the recent finding that *Bmi-1*-deficient HSCs fail to contribute to hematopoiesis in lethally-irradiated recipients over the long term suggests that *Bmi-1* is another important molecule regulating self-renewal. In particular, the effects of *Bmi-1* are suggested to be mediated through its repression of the genes encoding $p16^{Ink4a}$ and $P19^{ARF}$, which respectively inhibit cell proliferation and enhance cell death, so that in Bmi-1 null mice one would predict premature exhaustion of the stem/progenitor cell pool (Park et al. 2003). Other signaling pathways, such as Notch-Delta interaction and Wnt pathway, are also revealed to be important to self-renewal of HSCs (Sorrentino 2004).

Taken together, interactions among the proteins that govern cell cycle progression and those involved in regulating apoptosis and self-renewal play critical roles in regulating the HSC pool size. It is a complicated network controlled by both intrinsic and extrinsic signals. So, a greater understanding of underlying mechanisms could provide important insights into achieving enhanced

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or even selective HSC expansion, which will be important in clinical transplantation (Kondo et al. 2003).

Hematopoietic Stem Cell Expansion and Clinical Applications

Since HSCs have tremendous potential for reconstituting the hematopoietic system by producing all mature blood cells, HSC transplantation has been developed as a clinical strategy to treat patients undergoing chemo- and/or radio-therapy, or patients with hematological malignancies, such as leukemia, lymphoma and myeloma (Appelbaum et al. 1995; Brown and Weissman 2004; Verfaillie 2002). However, the limited success to expand HSCs ex vivo imposes major limitations on the current use of HSC transplantation. This is especially true in cases where the number of available stem cells is low (e.g., cord blood-derived stem cells for transplantation into adults) (Amos and Gordon 1995). Furthermore, most culture conditions employing cytokine combinations nonetheless result in net HSC losses due to the fact that differentiation is favored over self-renewal (Bhatia et al. 1997; Glimm and Eaves 1999). Some recent attention has been focused on cell intrinsic pathways, whose activation has caused some HSC expansion ex vivo (Bernstein, 2004 #5424; Lessard, 2004 #5404). Therefore, understanding the underlying mechanisms and identifying gene that regulate self-renewal and differentiation of HSCs has been a long-standing goal in both basic and clinic science (Weissman 2000). The discovery of these molecules will provide useful information for designing effective HSC expansion protocols that can be used for stem cell and current gene therapy.

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Aging Effects on Hematopoietic Stem Cells and Bone Marrow Niche

As discussed above, HSC expansion and transplantation is clinically important to treat patients with hematological and non-hematological disorders. It is also well known that cancer risk increases in older people (Balducci and Extermann FEB 2000). Therefore, understanding aging effects on hematopoietic system, especially on HSCs and their bone marrow microenvironment ("niche"), may not only help to prevent malignant transformation, but also to determine efficacy of aging stem cells for transplantation (Pinto et al. 2003; Van Zant and Liang 2003) .

In experimental animal models, accumulating evidence indicates that both the number and functional properties of HSCs are altered with age (figure 1.5). For example, marrow from old C57BL/6 mice contains more HSCs (measured by cobblestone area formation, primitive phenotype, and competitive repopulating ability) than bone marrow from young mice (Chen et al. 2000; de Haan et al. 1997; Morrison et al. 1996). In contrast, the stem cell pool from DBA/2 and all other mouse strains studied contracts in size during aging. This strain-specific variation in HSC number and other stem/progenitor cell parameters is regulated by cell-intrinsic mechanisms, and affected by several quantitative trait loci (QTL) (de Haan and Van Zant 1999; Geiger et al. 2001). In serial transplantation experiments, marrow from old animals was less able to engraft later passage recipients than young BM cells (Chen et al. 1999; Harrison 1983). Moreover, old HSCs exhibit a differentiation pattern skewed toward the myeloid lineage at the

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expense of lymphopoiesis, particularly the production of B cells (Linton and Dorshkind 2004; Miller and Allman 2003; Offner et al. 1999; Sudo et al. 2000)). Further evidence of age-related changes in stem cells include the finding that a higher proportion of Thy-1^{lo}Sca-1^{+}Lin^{-}Mac-1^{-}CD4^{-}c-kit^{+} cells from old mice are in S/G_2/M phases of the cell cycle (Morrison, 1996), and the results of Henckaerts et al., who showed that the proliferative response of Lin^{-}Sca-1^{+}c-kit^{+} marrow cells to the early-acting cytokines KL, Flt3L and TPO, decreased dramatically with age (Henckaerts et al. 2002).

As mentioned previously, the bone marrow niche is the optimal microenvironment for the growth and functional maintenance of HSCs (Moore 2004; Nilsson et al. 2001). In all experimental and clinical stem cell transplants, the critical first step leading to successful engraftment is "homing" of stem cells to the bone marrow, which involves adhesion molecules and cytokines (Ema and Nakauchi 2004; Spradling et al. 2001; Whetton and Graham 1999). Although more and more evidence demonstrates that HSCs undergo age-related quantitative and functional changes, little is known about the effects of aging on bone marrow niche and HSC homing (Albright and Makinodan 1976; Boggs et al. 1984; Globerson 1999; Liang and Van Zant 2003; Morrison et al. 1996; Van Zant and Liang 2003). As more and older patients become candidates for transplantation in the treatment of hematological malignancies and non-hematological diseases, these studies are obviously clinically relevant, yet remain largely unexplored.

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Experimental Goals and Significance

The first experimental goal described in this dissertation is to study the genetic control of murine hematopoietic stem cell numbers using a forward genetic approach. Three specific aims are formulated to address this question: (i) to confirm the quantitative trait locus (QTL) mapping in congenic mouse model, (ii) to study stem cell number-related phenotypes, including cell cycle, apoptosis and self-renewal of HSCs, and (iii) to identify gene(s) responsible for HSC number regulation. A second goal is to investigate the effects of donor and recipient age on the bone marrow homing efficiency and engraftment properties of HSCs and HPCs in mice.

These studies are important because they may lead to the discovery of novel genetic and molecular pathways that control HSC numbers. Clinically, the findings can potentially help to develop effective strategies for stem cell expansion and gene therapy using HSCs as target cells.

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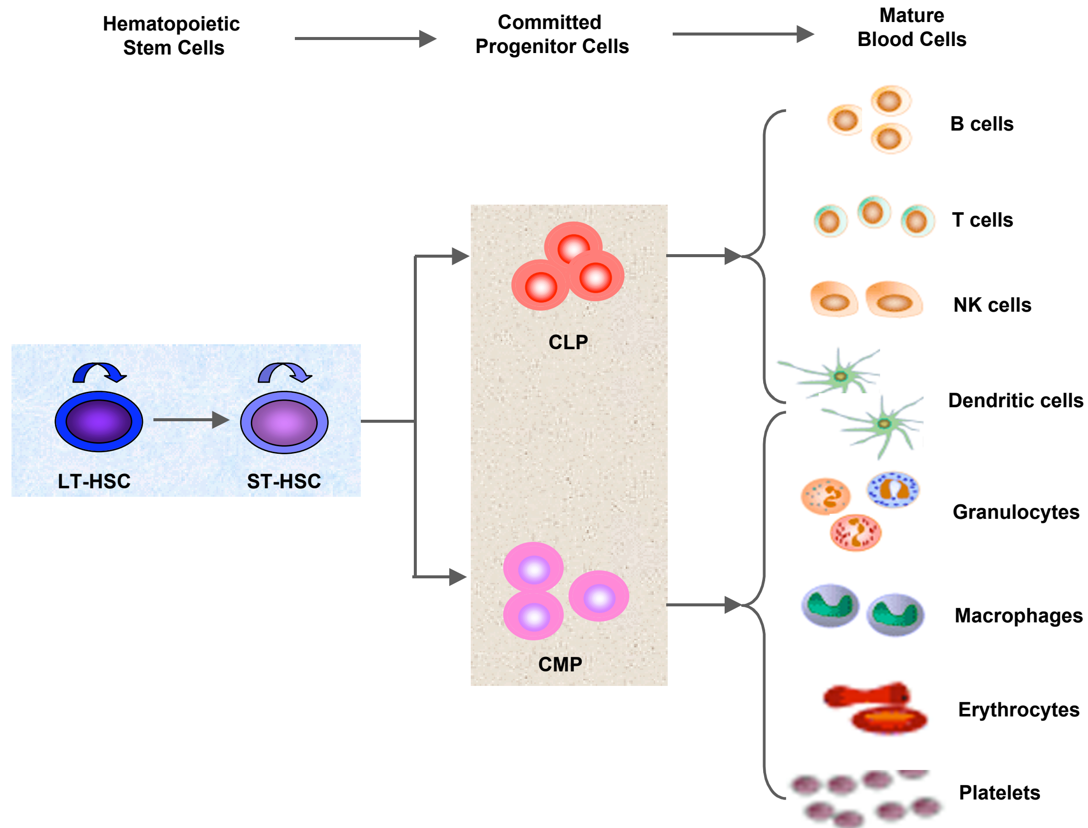

Figure 1.1. The hematopoietic system is hierarchical in nature. A small number of pluripotent stem cells, especially hematopoietic stem cells (HSCs), give rise to committed progenitor cells, which can generate all lineages of mature blood cells. HSCs are a heterogeneous population structured in a hierarchy, including long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs) with different self-renewal and differentiation capabilities. The progeny of HSCs are common lymphoid progenitors (CLP) and common myeloid progenitors (CMP), which generate B, T lymphocytes, nature killer (NK) cells, dendritic cells, granulocytes, macrophages, erythrocytes and platelets.

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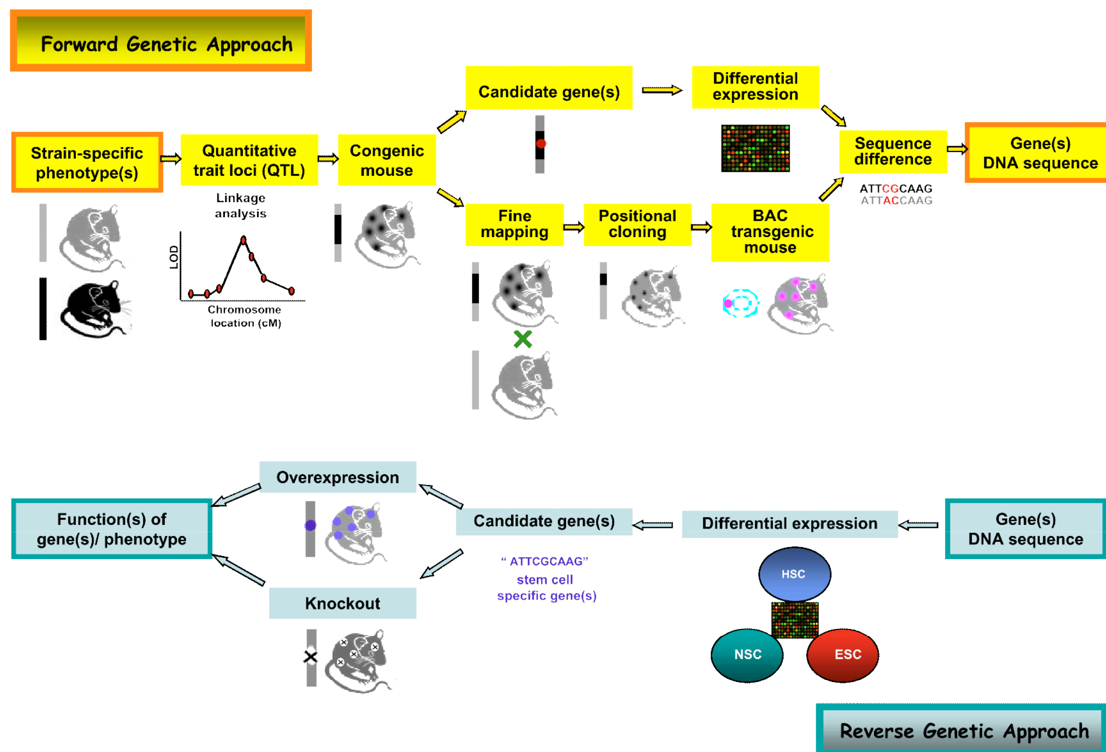

Figure 1.2. Schematic illustration of forward and reverse genetics. Generally speaking, two strategies are utilized to lead to gene discovery: forward and reverse genetic approaches. A forward genetic approach starts with strain-specific phenotypes; subsequent linkage analysis maps the location of quantitative trait loci (QTL) in the genome that contribute to the phenotype. Generation of congenic mice is the main tool to confirm the existence, function and location of these QTL. The purpose is to transfer and isolate a specific interval surrounding the QTL from one strain to another stain to determine whether phenotype results from introgression of the opposite strain alleles. Congenic strains can then serve as model systems for gene discovery using a candidate gene approach and by using positional cloning. Candidate gene in (or

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close to) the QTL may obviously be related to the phenotype; differential expression between the congenic and recipient (background) strains supports the candidacy of the gene. Another feasible way to investigate the underlying genes is fine mapping through backcrossing congenic mice to background strain animals to narrow down the interval around the QTL. Characterization of phenotype in subcongenics is carried out to confirm effects of the QTL. Once the QTL is mapped to a sufficiently small region (≤1cM), bacterial artificial chromosome (BAC) clones spanning the interval may be used to generate transgenic mice. BACs conferring the expected phenotype contain sufficiently small fragments of mouse genomic DNA (50-100kb) to be sequenced. Sequence differences in the open reading frame of the candidate gene, or more likely, in the promoter region, between two parental alleles will be taken as proof of identity between the candidate and the QTL.

In summary, the forward genetic approach is based on the strain-specific phenotype and proceeds toward the underlying genes. Conversely, reverse genetic approaches adopt an opposite direction, proceeding from polymorphism in DNA sequence of a gene to its functional analysis usually through gene overexpression and/or a gene knockout animal model. For example, in recent studies of stem cell-specific genes, candidates have been selected by differential expression analysis using microarrays. Gene expression profiles of embryonic stem cells (ESC), neural stem cells (NSC), and HSC have been compared to arrive at candidates that specifically confer stem cell attributes. Their functions

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must then be identified through gene overexpression in transgenic mice and/or loss of function mutations in gene knockout animals.

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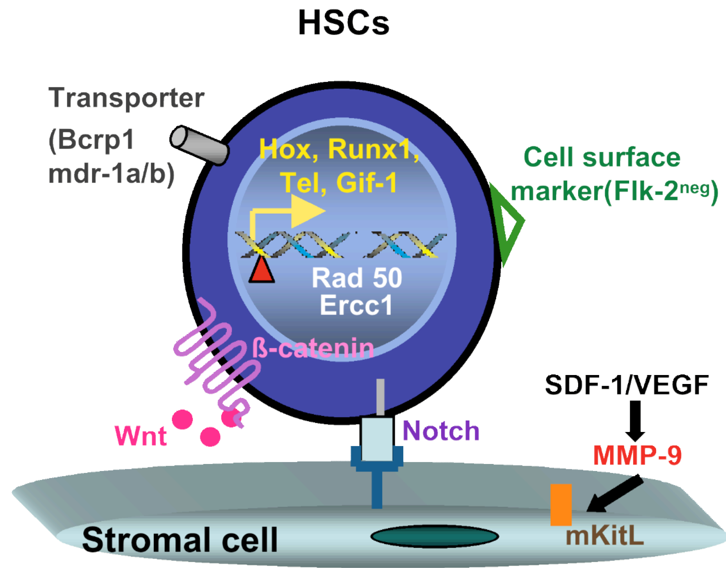

Figure 1.3. Intrinsic and extrinsic regulation of hematopoietic stem cells. Stem cell-autonomous modulators, such as transcription factors (Hoxb4, Runx1) and cell surface markers (Bcrp1, Flk-2) confer HSC-specific phenotypes and functions. External signals from stromal cells in bone marrow niche also exert roles on HSCs through secretion of cytokines (SDF-1), cell-cell interactions (Notch-delta, Wnt-frizzled), and cell-matrix interactions (MMP-9). Note: references are cited in the text.

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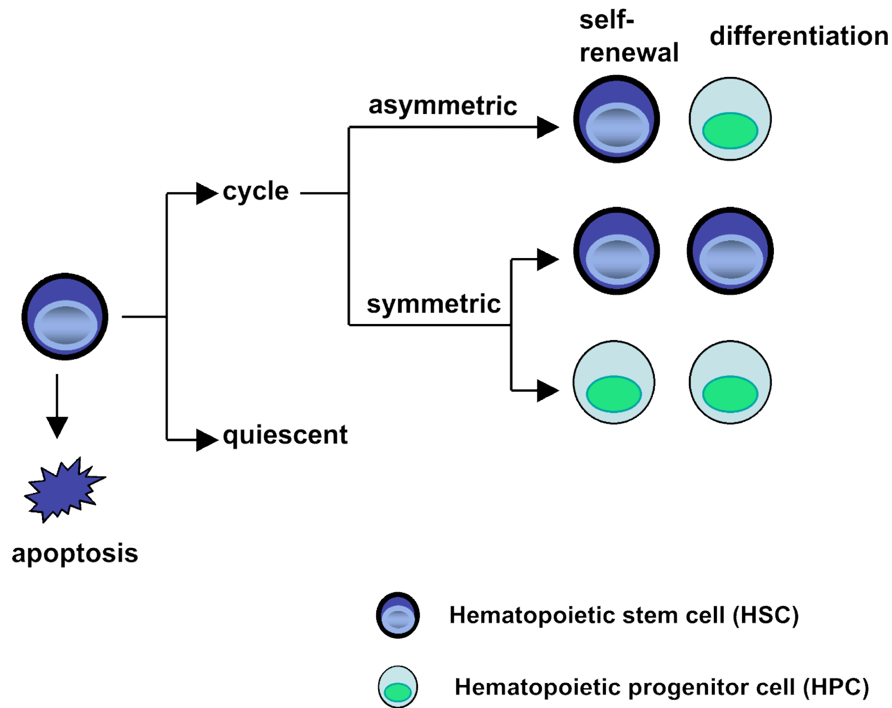

Figure 1.4. HSCs fate outcomes. Under the influence of intrinsic and extrinsic regulators, HSCs have three possible fate outcomes: apoptosis, quiescence and cycling. Apoptosis will cause the loss of HSCs. For a cycling HSC, there are three potential outcomes: (i) an asymmetrical division leading to the maintenance of the HSC population by generating one HSC (self-renewal) and one differentiated daughter cell, (ii) a symmetrical division leading to net expansion of HSCs by generating two identical HSCs and (iii) a symmetrical division leading to loss of HSCs by producing two committed progenitor cells.

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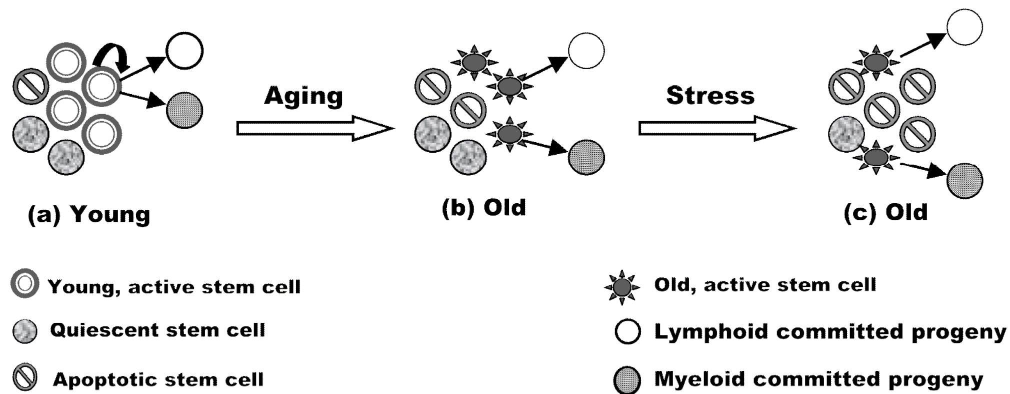

Figure 1.5 Diminished functional capacity of hematopoietic stem cells during aging. (a), a young stem cell population is characterized by few apoptotic cells, a mix of quiescent and active cells, the latter with robust self-renewal and differentiation capacity. Young stem cells show balanced differentiation into lymphoid and myeloid lineages. (b), an aged stem cell population has a higher rate of apoptosis due to acquired cellular damage. There are fewer quiescent cells in the stem cell reserve and the active stem cells have restricted lineage potential. (c), when an old stem cell population comes under hematopoietic stress, the rate of apoptosis increases as quiescent cells unsuccessfully make the activation step, further depleting the quiescent reserves. Active stem cells not only are hampered by lineage restrictions, but the number of differentiated progeny each produces is diminished, leading to a slow and blunted recovery.

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CHAPTER TWO

Mapping and confirmation of quantitative trait loci linked to strain-specific difference in hematopoietic stem cell number

Summary

Accumulating evidence has shown that there is a large natural variation among laboratory mouse strains in the number of bone marrow hematopoietic stem cells (HSCs). By using *in vitro* long-term cobblestone-area forming cell assay (CAFC), we have found that two inbred strains, C57/BL6 (B6) and DBA/2 (D2), demonstrated the strain-specific difference in this parameter, in which B6 mice have fewer marrow HSCs (CAFC day35) than do D2 mice. In order to understand the genetic basis for this variation, we used a forward genetic approach and first performed linkage analysis in BxD recombinant inbred strains and identified three quantitative trait loci (QTL) that linked to this difference. They were on chromosomes 3, 5 and 18 and correlated most highly with microsatellite markers D3Mit5, D5Mit352 and D18Mit53, respectively. The QTL mapping was further confirmed in congenic mouse strains in which a genomic segment surrounding the QTL was introgressed either from B6 strain mice into D2 genetic background, or vise versa. In the chromosome 3 "B6 onto D2" (symbolized as D.B Chr3) congenic strain and its reciprocal "D2 onto B6" strain (B.D Chr3), CAFC analyses demonstrated that D.B Chr3 mice had more than a 50%

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decrease in CAFC day35 number compared to their D2 background mice, whereas B.D Chr3 mice had a ~80% increase compared to B6 background strain ($p<0.05$). In addition, *in vivo* repopulation assay showed that 73% more long-term repopulating HSCs were present in B.D Chr3 mice. However, the numbers of peripheral blood cells (erythrocytes, leukocytes and platelets) and HPCs (CAFC day7 and day21) were not different between Chr.3 congenic and their background strains. Therefore, we conclude that (i) QTL on chromosome 3, 5 and 18 contribute to the strain-specific difference in HSC number between B6 (lower) and D2 (higher) mouse strains, (ii) Chr.3 QTL mapping is confirmed in congenic mouse strains in which introgression of the B6 allele onto a D2 background confers onto the D2 strain a decreased HSC number, i.e. the B6 strain phenotype, and vice versa, (iii) Chr. 3 QTL specifically regulates HSC compartment size and has no effects on differentiated hematopoietic cells.

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Introduction

The number of stem cells in a self-replenishing organ may significantly impact the maintenance of normal organ function. There is a large natural variation among laboratory mouse strains in the absolute number of hematopoietic stem and progenitor cells (HSCs and HPCs) in bone marrow (de Haan and Van Zant 1997; Liang and Van Zant 2003). Two such inbred strains, C57BL/6 (B6) and DBA/2 (D2) have been shown to have significant differences in some characteristics of hematopoietic cells. For example, young D2 mice have a 2- to 11-fold higher numbers of bone marrow HSCs than do B6 mice, depending on the assay for stem cell used. However, this trend is reversed in aged mice, i.e. ~2.5-fold more HSCs are found in old B6 mice than in D2 mice, indicating HSC compartment size contracts with age in D2 mice whereas it expands in B6 animals. The same age-related changes were also observed in the HPC population (de Haan and Van Zant 1999; Geiger et al. 2001). Other evidence showing the strain-specific differences in primitive heamtopoietic cell includes the higher fraction of cycling HPCs (de Haan et al. 2002) and lower proliferation response of HSCs to exogenous early-acting cytokines in D2 mice compared to B6 mice (Henckaerts et al. 2002). In addition, the proportion of mature blood cells, such as peripheral B and T lymphocytes (Boulton et al. 2003; Chen and Harrison 2002b), were also reported to be different in the two strains. Therefore, the broad spectrum of phenotypic

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differences in hematopietic cells between B6 and D2 strains, plus the availability of recombinant inbred stains derived by crossing them, have become the most powerful genetic models to investigate genetic basis for the natural variations of HSCs and HPCs.

As discussed previously, the first step in forward genetics approach is to map quantitative trait loci (QTL) that contribute to the strain-specific phenotypic differences in B6 and D2 by linkage analysis. BXD recombinant inbred mouse strains derived from crossing B6 and D2 mice may be used for this purpose (Van Zant and de Haan 1999; Williams et al. 1998; Williams et al. 2001). As shown in Figure 2.1, B6 and D2 mice are mated to produce F1 generation, which inherits one complete set of B6 chromosomes from one parent and one complete set of D2 chromosome from the other parent. F1 brother and sister mating produces F2 generation, whose genomes are chimeric with alternating stretches of B6 and D2 alleles due to meiotic recombination between aligned B6 and D2 chromatids. Mating pairs of F2 mice are randomly chosen and become the founders of individual BXD strains. The brother-sister breeding continues for 20 generations and creates BXD recombinant inbred (RI) strains that are homozygous at all loci. Because about 1000 polymorphic microsatellite markers have been identified throughout the B6 and D2 genome, the genotype at each locus (B6 or D2) can be identified, and the strain distribution pattern (SDP) in all BXD RI strains can be obtained for all markers. In order to map loci responsible for phenotypic variations, specific phenotypes are measured in each BXD strain individually. This results in a phenotype distribution pattern (PDP) for the entire BXD RI set. A

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sophisticated statistic program compares the PDP with the SDP and identifies marker SDPs with a high correlation to the PDP. The strength of the correlation is reflected by LOD (logarithm of the odds ratio) value. These selected markers, whose genomic location is known, then assign a chromosomal location to a locus that might be involved in the regulation of specific phenotype.

Moving to the generation of congenic animals is the critical step towards identifying and studying the function of phenotype-associated gene (Markel et al. 1997; Visscher 1999). The underlying idea is to transfer and isolate a specific interval surrounding the QTL of interest from the donor strain to a recipient or background strain (Figure 2.2). For example, in order to transfer a QTL on a certain chromosome from the genome of B6 onto the D2 strain, B6 and D2 are crossed to generate F1 hybrids, which are subsequently backcrossed to D2 again to produce a N2 generation. After N2 animals are genotyped at markers on both sides of the QTL, those that inherit one B6 allele are chosen and then crossed back to D2 parental strain again. With the process of backcrossing and genotyping being repeated for 8 generations, the homozygosity for D2 alleles is increased from 75% at N3 to 87.5% at N4 and eventually to 99.8% at N10. N10 progeny heterozygous for selected markers are then intercrossed to produce congenic mice that are homozygous for the B6 allele of QTL in the D2 genome background. By using the same method, we also can transfer D2 QTL allele to B6 background to generate what is called the reciprocal congenic strain. Therefore, congenic animals are genomic chimeras: they have donor strain genes only in the prescribed genomic segment of interest, all other genomic loci

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are homozygous for the recipient strain alleles. Thus, it is possible to determine the effect of individual, selected genes on a trait in the context of a fixed genetic background. Our lab has found that young D2 mice have higher HSC numbers than do B6 mice (de Haan et al. 2000; Geiger et al. 2001). In the current study, we aimed to investigate the QTL responsible for this strain-specific trait, generate congenic mice bearing QTL of interest, and confirm QTL mapping in congenic mouse models.

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Materials and Methods

Animals

Background strains: C57BL/6J (B6) ($Ptprc^b$ [Ly-5.2]), DBA/2J (D2) mice were purchased from the Jackson Laboratories (Bar Harbor, ME).

Congenic stains: Chromosome 3 congenic mice were generated by Geiger *et al.* as described previously (Geiger et al. 2001). Genotypes of congenic mice were checked every 6 months through genetic marker–based polymerase chain reaction. All primers for simple sequence repeat (SSR) element markers were bought from Research Genetics, Huntsville, AL. Two strains of chromosome 3 congenic mice were generated and maintained: B.D Chr3 (14cM~33cM) congenics with D2 QTL being introgressed onto B6 background, and their reciprocal congenic strains D.B Chr3 (19cM~60cM) with B6 QTL onto D2 background.

Transplantation recipient mice: B6.SJL ($Ptprc^b$ [Ly-5.1]) mice from Charles River laboratories (Frederick, MD) were used as recipients in transplantation experiments.

All mice were female and are 6 to 10 weeks old and they were kept in the animal facilities of the University of Kentucky under pathogen-free conditions according to NIH-mandated guideline for animal welfare. They were fed with water and food ad libitum.

Preparation of bone marrow nucleated cells suspension

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Mice were killed by cervical dislocation after isofluorane anesthesia. Femora were removed and flushed with Iscove modified Dulbecco media (IMDM) containing 2% fetal calf serum (FCS), 80 U/mL penicillin, and 80 mg/mL streptomycin. Cell counts were taken on a Coulter MD8 (Beckman Coulter, Fullerton, CA). The cells were subsequently used for the cobblestone area-forming cell (CAFC) assay without any further processing.

Cobblestone area-forming cell (CAFC) Assay

CAFC assay were carried out as described previously (Geiger et al. 2001). In brief, FBMD-1 stromal cells were plated into 96-well tissue culture-treated plates (Costar, Cambridge, MA) and they subsequently formed a confluent monolayer after 7 to 10 days. Bone marrow cells were flushed and pooled into Iscove Modified Dulbecco media (IMDM) with 2% fetal calf serum (FCS) (Life Technologies). Cells were then seeded into FBMD-1 coated culture plates in IMDM, 20% horse serum, 80 U/mL penicillin, 80 mg/mL streptomycin (all from Life Technologies), 10^{-4} ß-mecaptolethanol, 10^{-5} M hydrocortisone (both Sigma, St. Louis, MO) at doses of 81,000, 27,000, 9,000, 3,000, 1,000, or 333 cells per well. Typically 20 replicate wells per dilution were evaluated. Individual wells were screened at day 7,14,21,28 and 35 for the presence of a cobblestone area, defined as a colony of at least 5 small, non-refractile cells growing underneath the stroma (as shown in Figure 2.3). The longer the latency before cobblestones appear, the more primitive the nature of cells forming that cobblestone. Therefore, the most primitive hematopoietic stem cells (HSCs) show

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cobblestones at day 35, whereas colonies that appear earlier are derived from more committed progenitor cells (HPCs). Frequencies of CAFCs were calculated by using maximum likelihood analysis. The frequency of CAFCs equals 1 divided by the number of cells yielding 37% negative wells according to the equation of $ln p_0 = -m$.

Linkage analysis

The frequencies of CAFC day35 in BXD recombinant inbred mouse strains were measured and published previously (de Haan and Van Zant 1997) and were used to perform genome-wide searches for linked loci through “Interval Mapping” and using the “Marker Regression” tool on the WebQTL website (http://www.webqtl.org/cgi-bin/WebTL.py) (Chesler et al. 2004; Wang et al. 2003). Interval Mapping tool computes linkage maps for the entire genome or single chromosomes and uses permutation and bootstrap tests to assess strength and consistency of linkage for the CAFC day 35 trait. This results in computation of likelihood ratio statistics (LRS) values for genome-wide levels for at least suggestive linkage. The Marker Regression tool plots permutation results, lists those markers linked to trait variation, and provides access to composite mapping functions

***In-vivo* long-term limiting dilution analysis (competitive repopulation unit assay, CRU assay)**

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Long-term limiting-dilution analysis in competitively repopulated hosts is an *in vivo* method to functionally identify HSCs and measure their frequencies (Szilvassy et al. 1990). The basic idea for the experimental setup is to transplant graded numbers of “test” cells into myelo-ablated recipients together with a fixed number of “competitor” cells. In order to distinguish different cell sources, a hematopoietic cell maker, Ly-5 (or CD45), is commonly used in such kind of experimental design, in which B6 mice naturally express the Ly-5.2 allelic variant, whereas competitor cells and recipient mice express the Ly-5.1 allele. The Ly-5.1-bearing mice are B6 background animals congenic for the Ly-5.1 allele introgressed from the SJL strain. Since these allelic variants don’t exist between D2 and D2-based congenic mice, this assay can’t be performed on these stains. As shown in Figure 2.3, graded numbers of B.D Chr3 congenic- and B6-derived Ly-5.2 “test” cells (6,000; 20,000; 60,000) were admixed with a radioprotective dose ($2x10^5$) of competitor cells (Ly-5.1) and injected intravenously into lethally irradiated (9 Gy) Ly-5.1 recipient mice. Recipients were bled from the retro-orbital sinus at 5, 10 and 20 weeks after transplantation. Erythrocytes were depleted from each peripheral blood (PB) sample by hypotonic lysis using NH_4Cl, and the remaining leukocytes then stained in triplicate with a donor (B6)-specific anti-Ly-5.2 monoclonal antibody (mAb) conjugated with fluorescein isothiocyanate (FITC) (clone ALI4A2) and phycoerythrin (PE)-conjugated mAbs (Becton-Dickinson-PharMingen, San Diego, CA) specific for either B (anti-CD45R/B220; clone RA3-6B2) or T lymphocytes (anti-Thy-1.2; clone 30H12), or granulocytes (anti-Ly6G/Gr-1; clone RB6-8C5) and macrophages (anti-CD11b/Mac-1; clone

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M1/70). Samples were analyzed using a FACScan instrument (Becton-Dickinson Immunocytometry Systems, San Jose, CA). The frequencies of CRU were calculated from the proportions of negative recipients (in which <5% of the circulating B, T and myeloid cells were regenerated by Ly-5.2$^+$ stem cells) in each cell dose group using L-Calc software (StemCell Technologies Inc., Vancouver, BC).

Peripheral blood cell counts

Anesthetized mice were bled from the retro-orbital venous plexus. Circulating leukocyte, erythrocyte and platelet counts were measured by analysis of 40ul blood using a System 9118$^+$ Hematology Series Cell Counter (Biochem Immunosystems, Allentown, PA).

Statistical analysis

All experiments were preformed at least in triplicate with a minimum sample size of 3 mice per group. Data were analyzed by either two-tailed *t*-test assuming unequal variances or one-way ANOVA.

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Results

Quantitative trait loci responsible for strain-specific difference in HSC number were mapped on murine chromosome 3, 5 and 18

By using an *in vitro* cobblestone-area forming cell assay (CAFC), we have found that young D2 mice have ~2-fold higher number of marrow hematopoietic stem cells (HSCs) than do B6 mice (de Haan et al. 2000; Geiger et al. 2001). We hypothesized that single or multiple genomic regions might contribute this strain-specific quantitative trait. Genome-wide linkage analysis was therefore performed by comparing phenotype (HSC number per femur) distribution pattern (PDP) with strain distribution pattern (SDP) in BXD recombinant inbred mice to search for linked loci (Wang et al. 2003). Three quantitative trait loci (QTL) were mapped on chromosome 3, 5 and 18 with microsatellite markers D3Mit5 (25cM), D5Mit352 (20cM) and D18Mit53 (27cM) (Figure 2.5), respectively, having the highest linkage. There are several points to be made from this linkage analysis. First are the linkage peaks pointing to these 3 chromosomes, whose amplitudes are above the statistical suggestive threshold (9.2), but below the significant threshold (16.3). Second, the additive effects, indicated by the arrow in the figure, are all positive for these 3 makers (D3Mit5, D5Mit352 and D18Mit53), suggesting that the D2 allele increases, whereas the B6 allele decreases, the trait value (HSC number). Third, more than one locus affects femoral marrow HSC number, implying that multiple regulatory pathways either synergistically or independently are involved in the control of HSC pool size.

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Congenic strains confirm QTL mapping

In order to experimentally validate the mapping, we have generated congenic mouse strains in which a genomic interval containing the QTL from one strain is isolated and transferred onto the genomic background of the other strain, and vice versa. Therefore, the only genotypical difference between congenic and background animals is the introgressed segment, a configuration which is helpful to study the independent influences of each QTL. Generation of congenic strains has been accomplished for each of 3 QTL responsible for D2/B6 variation in HSC numbers. Studies concerning the chromosome 3 (Chr.3) QTL in congenic mice will be primarily discussed in the following chapters.

In this part of the study, we hypothesized that congenic B.D Chr3 mice, in which an interval on Chr.3 in B6 background has been replaced by the D2 QTL segment, would confer the D2 phenotype for HSC number (higher). Conversely, congenic D.B Chr3 mice, in which the B6 QTL was introgressed into D2 background, would show lower HSC numbers. Therefore, we measured CAFC day35 frequency in both congenics and their respective background mice. As predicted from the mapping data, congenic B.D Chr3 mice showed statistically significant ($P<0.005$) increases in CAFC day35 numbers compared to B6 background; in fact, more than doubling them. Conversely, reciprocal D.B Chr3 congenic mice had more than a 50% decrease in this parameter ($P<0.05$) (Figure 2.6). Similar results were also observed in reciprocal congenic strains for the Chr.5 QTL, demonstrating that each of the QTL have profound effects on stem cell number. Moreover, mice congenic for both QTL (Chr.3/Chr.5) showed a

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quantitatively similar phenotype as the singly congenic mice and thus the effects of the QTL are not additive or synergistic.

The differences in CAFC day35 frequency (HSC numbers) shown between the congenics and their background should also be reflected by an in vivo analysis. Long-term limiting-dilution analysis in competitively repopulated hosts is the gold standard method to functionally identify and quantify HSCs *in vivo* (Szilvassy et al. 1990). Briefly, graded numbers bone marrow cells from B.D Chr3 or B6 mice were co-transplanted into myeloablated mice together with a radioprotective dose of competitor BM cells containing an allelic variant of the hematopoietic cell-specific marker, Ly-5 (also known as CD45 and Ptprc). The frequency of HSCs in the test population, measured as competitive repopulating units or CRUs, was then determined by applying maximum likelihood analysis and Poisson statistics. As shown in Table 2.2, the long-term repopulating stem cells were present at a frequency in B6 mice of 1 in 17,424 marrow cells, whereas this frequency increased to 1 in 10,615 marrow cells in the B.D Chr3 congenics ($P<0.05$). When these frequencies were calculated into the absolute number of CRU per femur, B.D Chr3 congenic mice had almost ~80% more long-term repopulating stem cells than did B6 mice, a result consistent with those from *in vitro* CAFC assays.

Taken together, both *in vitro* and *in vivo* results validate the linkage analysis, i.e., Chr.3 and Chr.5 QTL are responsible for the strain-specific difference in HSC number between B6 and D2 strains: D2 alleles increase, whereas, B6 alleles decrease HSC numbers.

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Stage-specific regulation of HSCs by Chr.3 QTL

HSCs are the major source of downstream progenitors and all lineages of mature blood cells. If D2 or B.D Chr3 mice have more HSCs in the bone marrow, do they give rise to more hematopietic progenitor cells (HPCs), and peripheral blood cells along their differentiation pathways? Because CAFCs from day 7 to 21 represent different stages of HPCs (Ploemacher et al. 1991; Ploemacher et al. 1989), we therefore compared CAFC frequencies at day 7 and day 21 among congenic and background strains. Together with cell counts of peripheral leukocytes, erythrocytes and platelets, we tested the hypothesis whether Chr.3 QTL also affect the numbers of committed and differentiated cells. The results summarized in Table 2.3 showed that there were no statistically significant differences in either CAFC day7 or day21 frequencies per femur or peripheral mature blood cell counts among Chr.3 congenic and their background mice. These data suggest that HSC population size is regulated in a stage-specific way, i.e., the gene underlying chromosome 3 QTL specifically control HSC compartment size, and they have limited or no effects on downstream progenies along the differentiated pathways. This stage-specific regulation model and related differentiation gene expression profiles between HSCs and HPCs has already been suggested by other studies (Geiger et al. 2001; Lawrence et al. 1995; Park et al. 2002; Zhong et al. 2005).

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Discussion

Although increasing numbers of genes have been implicated in the regulation of HSCs, the signaling pathways and underlying mechanisms that govern the self-renewal and differentiation of HSCs, and thus determine the number of bone marrow HSCs, is still poorly understood (Lessard et al. 2004). In a forward genetic approach, using the BXD recombinant inbred set and quantitative trait loci (QTL) mapping, we identified multiple loci on chromosome 3, 5 and 18, that were linked to the variations in HSC number of C57BL/6 (B6) and DBA/2 (D2) mouse strains. We also confirmed Chr.3 QTL mapping in congenic mouse models.

That the size of the stem cell pool shows natural variation between B6 and D2 mice has been previously shown. Henckaerts *et al.* demonstrated that young B6 mice had more $Lin^{-}Sca\text{-}1^{++}c\text{-}kit^{+}$ cells than did D2 mice and suggested that the stem cell pool in B6 was larger than in D2 mice (Henckaerts et al. 2002). These results are contradictory to what we and others have found using a variety of functional assays. Experience and our previous findings have shown that although $Lin^{-}Sca\text{-}1^{++}c\text{-}kit^{+}$ cell population is highly enriched in stem cells, it is not homogenous (de Haan et al. 2000). Therefore, identification of stem cells solely on the basis of phenotypic markers is risky, particularly when different strains are involved. In our study, we used a functional assay, the CAFC assay, to quantify stem and progenitor cells in B6, D2 and chromosome 3 congenic strains and found that D2 alleles increased, whereas B6 allele decreased HSC numbers.

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Most importantly, similar results were obtained when we performed more rigorous in vivo competitive repopulating unit (CRU) assay to measure the number of long-term repopulating stem cells. Therefore, our findings from both in vitro CAFC and in vivo CRU assays strongly support the accuracy of our measurement of stem cell numbers.

The genetic basis for natural variations of HSC number has been previously reported by Muller-Sieburg and colleagues (Muller-Sieburg and Riblet 1996). They demonstrated similar findings as ours concerning the HSC number difference between B6 (lower) and D2 (higher) strains. By using the same BXD recombinant inbred mice, however, they mapped two completely different loci accounting for the inter-strain variation on chromosomes 1 and 11. This mapping discrepancy is most likely due to the following reasons. First, the strategies to define HSCs are different in the two studies. Although they used a similar long-term in vitro culture system with the same stromal cells (FBMD-1) as ours, they considered cells producing at least 500 non-adherent differentiated cells after 4 weeks of culture as HSCs (called long-term culture initiating cells, LTC-IC). Therefore, the cells they measured are probably less primitive than those we identified. This explanation is strongly supported by our previous findings that loci responsible for the early progenitor cell (CAFC day21) number difference in B6 and D2 mice mapped to chromosome 1 (Geiger et al. 2001). Secondly, the phenotype used for linkage analysis is different. In their study, they quantified the frequency of stem cells contained in every 10^5 bone marrow cells. Since we already found that B6 mice had more bone marrow cells in the long bone of the

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back legs than do D2 mice (data not shown), in order to exclude the effects of bone marrow cellularity and provide more accurate and real measurement of stem cell pool size, we used stem cell frequency *per femur* as our phenotype to perform QTL mapping.

In the present study, several loci on chromosome 3, 5 and 18 were genetically linked to the natural variations in HSC number. Moreover, QTL mapping were further confirmed in each of our QTL congenic mouse models (Figure 2.6 and unpublished data for Chr18 congneics). Additional evidence for the existence of a locus on chromosome 18 regulating HSC number comes from recently published studies of 5q-deleted myelodysplastic syndromes (Nilsson et al. 2000). The human 5q chromosomal region is syntenic to the linked locus on chromosome 18. It was reported that HSCs are dysregulated in this human disorders, a finding that links this region to the stem cell regulation. Therefore, multiple, rather than a single QTL, regulate HSC numbers. Whether genes in and around these QTL regions act independently or synergistically needs to be further investigated. Our CAFC day35 analyses in Chr.3, Chr.5 and Chr.3/Chr.5 double congenic (Figure 2.6) suggest that the effects of Chr.3 and Chr.5 QTL are not additive and they may act through a common mechanistic pathway. Taken together, a forward genetic approach leads to the discovery of multiple genes involved in the same phenotype, which helps to reveal the interactions of these genes and the roles of each gene in the regulatory pathway. This can be viewed as an advantage of forward genetics over reverse genetic approach, which mainly focuses on the functional effect of an individual gene on stem cells.

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Our QTL mapping, CAFC, CRU analyses and peripheral blood cell counts in congenic mice further demonstrate that Chr.3 QTL specifically regulates stem cell pool size, without any observable effects on progenitor and differentiated cell populations. Stage-specific regulation of stem/progenitor cells is complex in that several distinct loci have been shown to be involved in this process. Some QTL specifically determine hematopoietic progenitor cell (HPCs) number, such as loci on chromosome 1 and 7. Some of them affect both progenitor and stem cells. Other QTL on chromosome 2 and 18 have been reported to link to the dynamic changes of stem and progenitor cell number with age (Geiger et al. 2001). In addition, loci on chromosome 1, 8 and 19 specifically determine the proportions of peripheral B and T lymphocytes in B6 and D2 strains (Chen and Harrison 2002a). In aggregate, different genomic loci are responsible for the stage- and strain-specific regulation of hematopoietic cells. Congenic animal models lead to the further investigation of the roles of individual loci and to the discovery of underlying gene (de Haan et al. 2002). Furthermore, recent studies about differential gene expression profile between stem and progenitor cells based on microarray analyses also help to identify the genes in our QTL that specifically regulate stem cell population (Park et al. 2002; Zhong et al. 2005).

Even though our analysis revealed significant differences in stem cell levels, all of the strains tested apparently had normal proportions of mature blood cells. It is clear that the relatively lower number of HSCs in B6 and congenic D.B Chr3 mice is entirely sufficient to provide blood cells for the lifespan of the animal. Therefore, in D2 and B.D Chr3 mice, the excess stem cells might remain

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quiescent and thus would not be evident in the periphery. However, under a hematopoietic stress condition, such as bone marrow transplantation, the subpopulation of stem cells might be reactivated and contribute to the hematopoietic reconstitution. Evidence supporting this explanation comes from studies of chimeric mice generated by embryo aggregation of B6 and D2 strains (Van Zant et al. 1983). The hematopoietic contribution of D2 origin gradually decreased with time and completely stopped after approximately two years. However, transplantation of the B6/D2 chimeric bone marrow cells into myelo-ablated recipient mice showed that a higher proportion of D2-derived blood cells were present in the periphery again, indicating that D2-derived HSCs became quiescent, rather than extinct. These quiescent D2 HSCs could still be reactivated and reconstitute the hematopoietic system. Another possible reason for the existence of "extra" stem cells in D2 and B.D Chr3 mice is that these cells might be required for a defective hematopoietic compartment. We have found that a higher proportion of progenitor cells in D2 and B.D Chr3 mice were killed by hydroxyurea (De Haan and Van Zant 1999 and unpublished data), a chemical causing DNA damage and consequently interfering with DNA synthesis of cycling cells. The results suggest that high turnover rate and/or sensitivity to DNA damage of progenitor cells place increased demand of stem cells in these two strains and thus require more stem cells to replenish the blood cells and maintain hematopoietic system.

Stem cell transplantation has become increasingly important in the clinic for the treatment of a variety of diseases in humans. One obstacle to this

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procedure is the difficulty in procurement of adequate numbers of stem cells (Kondo et al. 2003). In the present study, we demonstrate that hematopoietic stem cell numbers are under strict genetic control in mouse and that several genomic loci are involved. Because of the genomic synteny between human and mouse (McPeek 2000; Miller et al. 2004), the results may be important to identify genes regulating stem cell number in humans. If so, these genes may well provide a desperately needed strategy for stem cell expansion and transplantation.

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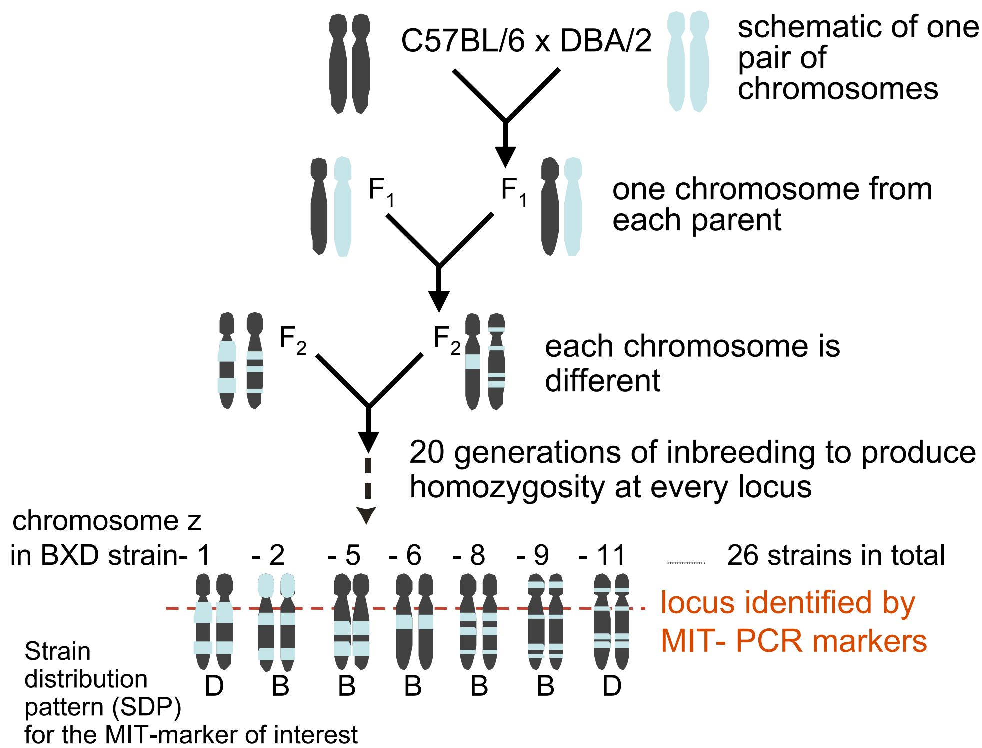

Figure 2.1 Schematic illustration of derivation of BXD recombinant inbred mouse strains. BXD recombinant inbred (RI) strains are derived from interbreeding of progeny of C57BL/6 and DBA/2 mice for 20 generations. Every locus throughout the genome, identified by microsatellite (MIT) marker, is homozygous for either the C57BL/6 (B) allele or the DBA/2 (D) allele. The presence of “B” or “D” at each locus in all BXD RI strains forms strain distribution pattern (SDP) for this marker or locus.

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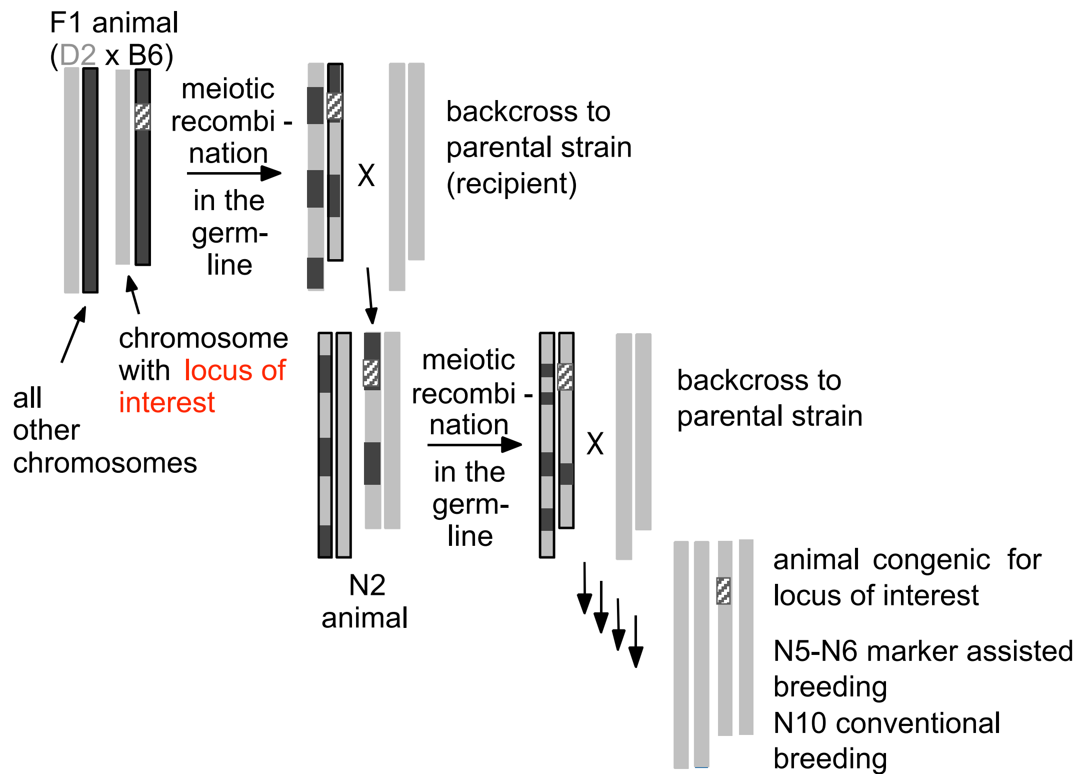

Figure 2.2 Scheme depicting generation of congenic animals. F1 progeny of C57BL/6 (B6, gray chromosome) and DBA/2 (D2, black chromosome) are selected for the presence of the locus of interest (dashed box) and the mice are backcrossed to either of the parental strains (D2 is the recipient strain and B6 is the donor strain in this figure). The selection and backcross are performed for 10 generations. In the N10 generation, contaminating donor strain alleles, except for the locus of interest, have been reduced to a statistically negligible level; that is $(1/2)^{10} \leq 0.0005$.

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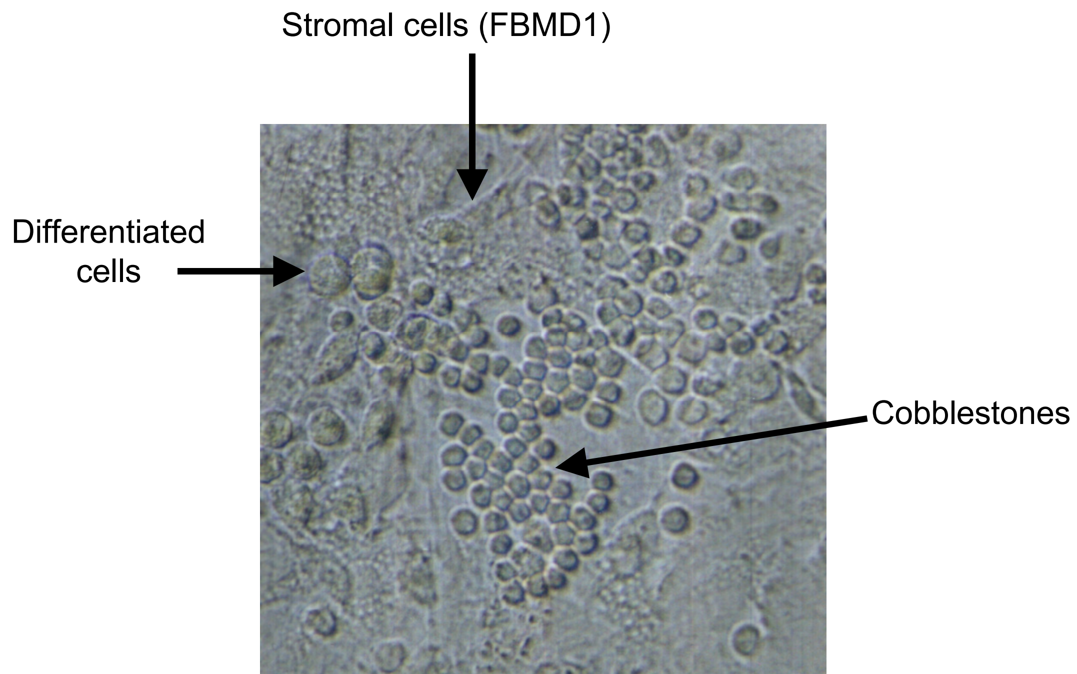

Figure 2.3 Illustration of cobblestone area under microscope. A cobblestone area derived from primitive hematopoietic cells is defined as a colony of at least 5 small, non-refractile cells growing underneath the stroma (FBMD1 cells). The differentiated cells are above the stromal layer and are usually bigger and birefringent.

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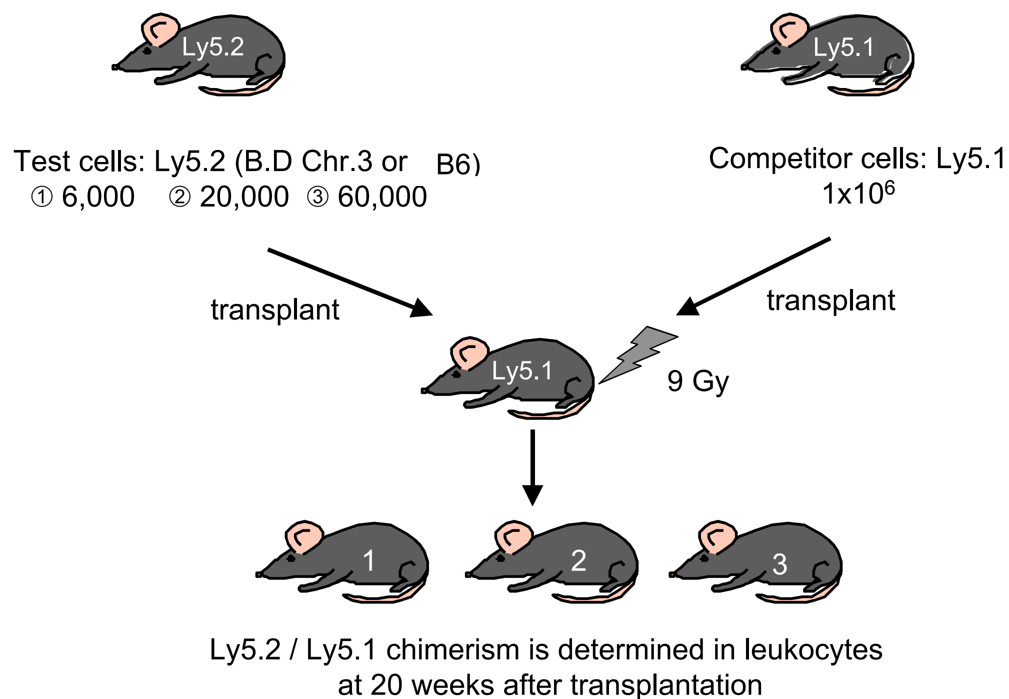

Figure 2.4 Experimental design for Competitive Repopulation Unit (CRU) assay. Graded number of B.D Chr3 congenic or C57BL/6 (B6) Ly-5.2 bone marrow cells are transplanted into lethally-irradiated recipient Ly-5.1 mice, together with $1x10^6$ Ly-5.1 competitor cells. Test (Ly-5.2)-derived leukocytes in peripheral blood are measured at 20 weeks after transplantation. CRU frequency is calculated from the proportion of negative mice in each cell dose group as described in the Methods.

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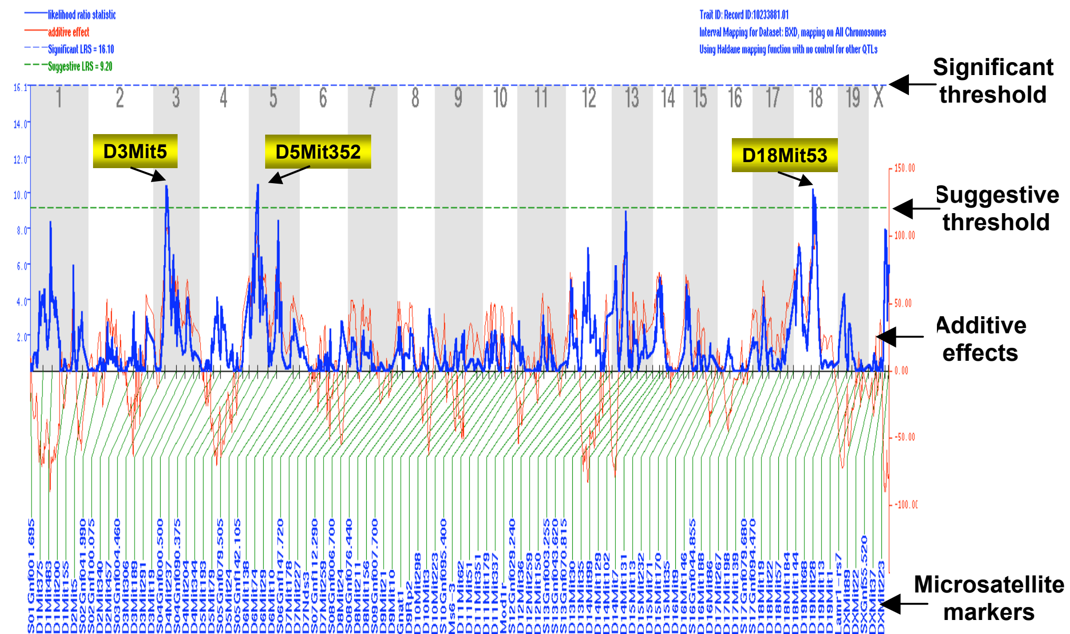

Figure 2.5 Genome-wide quantitative trait loci (QTL) mapping. A murine genome-wide scan of linkage of CAFC day35 number to microsatellite markers, some of which are listed across the bottom of figure, but illegibly at this scale of reproduction. Each of the 19 autosomes and the X chromosome is labeled across the top of the figure. Three peaks above the threshold of suggestive linkage are labeled on chromosomes 3, 5 and 18, indicating that these 3 loci are linked to HSC number regulation. The additive effects demonstrate the effects of B6 or D2 allele on the phenotype: positive additive value represents D2 allele increase the phenotype whereas negative value indicates B6 allele increase the phenotype.

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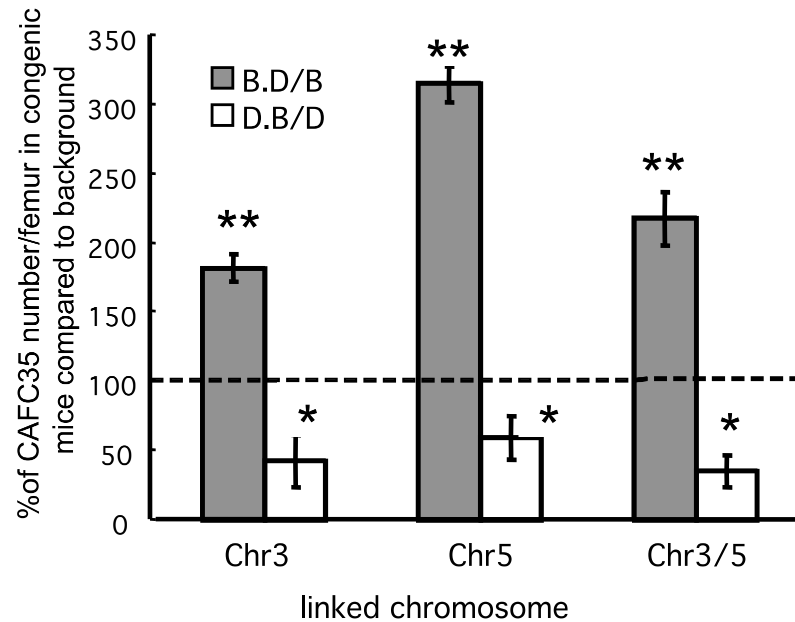

Figure 2.6 CAFC day35 analyses in chromosome 3, 5 congenic and background mice. Relative numbers of HSCs (CAFC day35) per femur are measured in 6 congenic strains compared with their background strains (either B6 or D2). Congenic mice include single reciprocal congenics (B.D Chr3 (14cM~33cM), B.D Chr5 (8cM~29cM), D.B Chr3 (19cM~60cM), D.B Chr5 (5cM~38cM)) and double congenics (B.D Chr3 (14cM~33cM)/B.D Chr5 (8cM~29cM), and D.B Chr3 (19cM~60cM), D.B Chr5 (5cM~38cM)). The values derived from at least 9 samples are shown as mean ± 1SEM. The dashed line (100%) indicates the same value between congenic and background mice. ** represents P <0.005, and * represents P<0.05.

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Table 2.1 Determination of long-term competitive repopulating units (CRUs) frequency in B6 and B.D Chr3 mice.

No. cells transplanted per recipient mouse	Number of negative mice[#]	
	B6	B.D Chr3
6,000	13/21[&]	11/21
20,000	7/22	4/22
60,000	1/12	0/12
1/CRU frequency[‡] (±SEM)	17424 (14221~21349)	10615 * (8661~13009)
No. CRU per femur[¶] (±SEM)	1326 (1082~1624)	2289 * (1806~2713)

Negative mice are defined as animals in which < 5% of the peripheral blood B and/or T lymphocytes and/or myeloid cells were derived from donor (Ly5.2+) stem cells.

& Values are shown as the number of negative mice out of total number of recipient mice at the 20-week-timepoint. The data were pooled from 3 independent experiments in which serial dilutions of Ly5.2+ donor cells were transplanted into lethally irradiated Ly5.1+ recipient mice along with $2X10^5$ Ly5.1+ competitors, and peripheral blood of recipients was analyzed for lymphomyeloid engraftment 20 weeks after transplantation.

‡ CRU frequency is calculated by applying Poisson statistics to the proportion of negative recipients at different dilutions using L-Calc software, and represented

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by the number of bone marrow cells containing 1 CRU. Numbers in parentheses give the range of CRU frequency defined by ± 1 SEM.

¶ Values shown are calculated by multiplying CRU frequency with femoral cellularity. The cellularity per B6 mouse femur averaged 23.1 (± 1.1) $X10^6$, and that of B.D Chr3 was 23.5 (± 1.7) $X10^6$. Numbers in parentheses give the range of CUR frequency defined by ± 1 SEM.

* Statistical analysis was performed using L-Calc software. $P<0.05$ is considered significant difference.

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Table 2.2 Comparison of hematopoetic progenitor cells (HPCs) and peripheral blood cell numbers among congenic and background mouse strains.

Mouse strains	Compared to background strains		Peripheral blood cell accounts		
	CAFC day7 per femur (%)	CAFC day21 per femur (%)	Leukocytes ($x10^3$/ul± 1 SEM)	Erythrocytes ($x10^6$/ul± 1 SEM)	Platelets ($x10^5$/ul± 1 SEM)
B6			5.1±0.3	9.9±0.2	11.1±0.3
B.D Chr3	89.9±7.4	89.0±7.3	6.2±0.7	9.4±0.3	10.5±0.5
D2			7.8±0.3	10.3±0.1	11.1±0.2
D.B Chr3	95.0±11.5	87.3±6.8	7.6±0.3	10.3±0.2	11.1±0.2

HPC numbers are represented by CAFC day7 and day 21 frequencies. A differentiated cell count of peripheral blood samples from congenic and background mice (n≥9) was performed for leukocytes, erythrocytes and platelets. Data shown are the average values ± 1 SEM. The normal ranges of the leukocytes, erythrocytes and platelets are 5~8$x10^3$/μl, 9~11 $x10^6$/μl and 11~14 $x10^5$/μl respectively. No significant differences were shown in these parameters between Chr. 3 congenic and their respective background mouse strains by a student t-test using two samples comparison analysis.

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CHAPTER THREE

Changes in cycling, apoptotic and self-renewal capabilities of HSCs in congenic mouse models

Summary

The strain-specific difference in hematopoietic stem cell (HSC) number between C57BL/6 and DBA/2 has been shown to link to several genetic quantitative trait loci (QTL), which have been further confirmed in congenic mouse models. However, the underlying biological processes that are involved in the regulation of stem cell number are not yet clear. Therefore, in this study we aimed to investigate the cell cycling, apoptotic and self-renewal status of HSCs in chromosome 3 congenic mouse strains by using immunostaining, flow cytometry and serial bone marrow cell transplantation strategies. HSCs in the S/G2/M phases of the cell cycle detected by DAPI staining comprised 5-7% of the population and didn't show any differences among Chr3 congenic and control mice. However, measurement of cell cycle kinetics by in vivo BrdU incorporation showed that B.D Chr3 congenic HSCs replicated at a faster pace than did B6 cells. Our studies also demonstrated that under steady-state conditions B6 HSCs underwent apoptosis at a 3-fold higher frequency (2.67%) than D2 stem cells (0.86%). The introgression of D2 alleles around chromosome 3 QTL onto a B6 genetic background (B.D Chr3) resulted in a D2-like apoptotic phenotype,

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whereas reciprocal D.B Chr3 HSCs had a further reduced apoptotic rate (0.29%). In addition, competitive repopulation advantages of B.D Chr3-derived HSCs over B6 cells in recipient mice were significantly higher and were retained in successive transplants, indicating that the D2 chromosome 3 QTL conferred higher self-renewal capability to stem cells. Taken together, these findings indicate that the chromosome 3 QTL also plays roles in regulating the cell cycle, apoptosis and self-renewal of HSCs, which may in turn account for the natural variation of stem cell number between B6 and D2 mouse strains.

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Introduction

Hematopoietic stem cells (HSCs) are rare cells, comprising only a very small proportion of bone marrow cells. Different methods of enumeration give somewhat different estimates of the exact numbers (Coulombel 2004). Moreover, there are clear strain variations with respect to stem cell frequency (Liang and Van Zant 2003). Using in vivo and in vitro functional assays, a typical HSC frequency is 1 per 10,000-15,000 marrow cells (Conneally et al. 1997; Szilvassy et al. 1990). Similarly, immunostaining-defined HSCs comprise 0.01% to 0.02% of the whole marrow population (Christensen and Weissman 2001; Morrison et al. 2002; Morrison and Weissman 1994; Zhou et al. 2002)). Assuming $3x10^8$ bone marrow cells in an adult mouse, about $3\text{-}6x10^4$ HSCs should be present in all marrow spaces.

How do such a small number of HSCs maintain themselves as well as produce all lineages of blood cells in the life history of an animal? Initial retroviral marking studies pointed toward a largely quiescent stem cell pool with only one or a few HSC clones actively cycling and giving rise to mature blood cells at any time. Once one HSC clone was exhausted a new clone appeared to be activated, thus following a clonal succession pattern (Becker et al. 1963; Lemischka et al. 1986). However, this view has been challenged by recent 5-bromodeoxyuridine (BrdU) labeling experiments in defined stem cell populations. Bradford et al. analyzed the proliferation kinetics of highly enriched HSCs that were defined by Hoechst (Ho) and Rhodamine 123 (Rho) exclusion (Bradford et al. 1997). They

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demonstrated that around 90% of Rh/Ho^{low} primitive hematopoietic stem cells had incorporated BrdU and thus underwent at least one round of DNA replication over a period of 12 weeks, with an average turnover rate time of 30 days. Similar studies by Cheshier et al. extended the previous observations and found more than 99% of long-term HSC-enriched population, c-kit^{bright}, Thy1.1^{lo}, Sca-1^{+}, Lineage^{-} (KTSL-), had incorporated BrdU by 6 months, and approximately 8% of them entered the cell cycle each day in an asynchronous manner (Cheshier et al. 1999). Therefore, around 2400~4800 murine HSCs (3~6x10^4 x 8%) begin dividing and generate hematopoietic cells each day. All studies are consistent with the idea that a majority of HSCs are not dormant but divide slowly and regularly to maintain themselves as well as produce differentiated cells.

Specific components of the cell cycle machinery that determine whether cells continue proliferating or cease dividing have been reported to regulate stem cell pool size. For example, in knockout model for cyclin-dependent inhibitors (CKIs), the absence of *p21* increased the proliferation of HSCs and led to expanded stem cell numbers. However the self-renewal of these stem cells was impaired (Cheng et al. 2000a; Cheng et al. 2000b). Interestingly, defective *p18* had similar effects on the proliferation and number of stem cells, but enhanced their renewal capability (Cheng et al. 2000a; Yuan et al. 2004). Another important cell cycle regulator, *cyclin D*, has also been studied for effects on hematopoietic systems. *Cyclin D1/D2/D3*-deficient mice demonstrated a dramatic decrease in numbers and functions of HSCs and HPCs in fetal liver. Moreover, the ability of these cells to proliferate was profoundly impaired (Kozar et al. 2004). Other

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regulators, which are not included in cell cycle machinery, may also affect stem cell numbers by indirect mechanisms. A recent report indicated that Bmi-1, an upstream inhibitor of p16 and p19^{ARF}, was critical for HSC self-renewal and population size (Iwama et al. 2004). Taken together, data indicate that HSCs are always cycling and that cell cycle control is one of the critical regulators of stem cell number and function. However, as discussed previously, more HSCs are generated each day than are apparently necessary to replenish mature blood cells lost through “wear and tear”. In order to keep the normal level of stem cell number, other mechanisms, such as apoptosis, self-renewal and/or the rate of differentiation, must be involved to physiologically balance with cell cycle control (Sommer and Rao 2002).

Several lines of evidence have indicated that apoptosis acts as an important regulator of stem cells. First of all, expression of some apoptosis-related genes were detected in human and/or murine HSCs (Domen 2001). Secondly, targeted disruption of some of these genes in null and dominant negative mutant mice interfered with normal apoptotic processes in HSCs. For example, overexpression of Bcl-2, a negative regulator of apoptosis, increased not only the numbers and competitive repopulation capabilities of HSCs, but also the resistance of HSCs to apoptosis induced by ionizing radiation (Domen and Weissman 2003). Inactivation of another anti-apoptotic gene, Bcl-X_L, displayed massive cell death in immature hematopoietic cells at embryonic day 13 (Motoyama et al. JUN 7 1999). Furthermore, of the caspase null mutants studies,

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clear abnormalities during fetal hematopoiesis at the progenitor level have also been reported for the caspase 8 null mutant (Varfolomeev et al. 1998).

As described above, there are three potential outcomes for a dividing HSC: (1) a symmetric division produces two HSCs, resulting in net expansion of stem cell population, or (2) produces two differentiated cells, leading to the loss of HSCs; (3) an asymmetric division produces a stem cell and a differentiated cell, leading to maintenance of HSCs (Faubert et al. 2004; Liang and Van Zant 2003). Therefore, the choices between symmetric and asymmetric division, resulting in self-renewal versus differentiation, are important in the regulation the number of HSCs. Although the molecular mechanisms determining symmetric or asymmetric division in mammalian HSCs are not yet clear, a growing list of genes have been found recently to be involved in the regulation of stem cell renewal and their numbers. Overexpression of some positive regulators in HSCs, such as *HoxB4, Bmi-1, ß-catenin*, resulted in net expansion of HSC population as well as an enhancement of their repopulating/self-renewal capabilities (Antonchuk et al. 2001; Antonchuk et al. 2002; Beslu et al. 2004; Karanu et al. 2000; Krosl et al. 2003; Reya et al. 2003; Sauvageau et al. 1995; Stier et al. 2002; Thorsteinsdottir et al. 1999; Vercauteren and Sutherland 2004). Similarly, knockout or inactivation of such genes impaired both numbers and functions of HSCs (Brun et al. 2004; Duncan et al. 2005). Recent studies concerning the roles of the *Gfi1* transcription factor in murine HSCs demonstrated that deletion of this gene led to both a dramatic decrease in stem cell frequency and in repopulating ability in serial transplantation assays. These defects were

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counterbalanced by an increase in proportion of cycling HSCs, indicating that *Gfi1* restrains proliferation of HSCs and thereby preserves their functional integrity in terms of self-renewal and engraftment abilities (Hock et al. 2004a; Zeng et al. 2004).

Altogether, most studies investigating the functions of genes in HSCs are performed by a reverse genetic approach; that is, through enforced overexpression or knockout of a specific gene in transgenic mouse models. In contrast, in forward genetics, genetic manipulations in congenic mice are through natural breeding processes and gene expression is usually not outside of physiological range (Wakeland et al. 1997). Therefore, congenic mouse models are very useful for investigations of stem cell-related genes and their functions under physiological conditions. In spite of this, very little work has been done to characterize HSC population using these models. In our chromosome 3 congenic mice, we have shown that a QTL on this chromosome specifically regulates HSC numbers. Although most of the genes discussed previously that regulate cycling, apoptosis and renewal of HSCs are not located within this QTL congenic interval, it is highly probable that these processes might be directly or indirectly altered such that they affect stem cell pool size. Therefore, this study was aimed at the analysis of these stem cell number-related phenotypes and to determine whether the chromosome 3 QTL also has an effect on these parameters.

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Materials and Methods

Animals

Background strains: C57BL/6J (B6) (Ptprc^{b} [Ly-5.2]), DBA/2J (D2) mice were purchased from the Jackson Laboratories (Bar Harbor, ME).

Congenic stains: Chromosome 3 congenic mice were generated by Geiger *et al.* as described previously (Wakeland et al. 1997). Genotypes of congenic mice were checked every 6 months through genetic marker–based polymerase chain reaction. All primers for simple sequence repeat (SSR) element markers were bought from Research Genetics, Huntsville, AL. Two strains of chromosome 3 congenic mice were generated and maintained: B.D Chr3 (14cM~33cM) congenics with D2 QTL being introgressed onto B6 background, and their reciprocal congenic strains D.B Chr3 (19cM~60cM) with B6 QTL onto D2 background.

Immunofluorescent staining of hematopoietic stem cells (HSCs)

Bone marrow cells were flushed and pooled into Hank's balanced salt solution (HBSS, Gibco, Grand Island, NY) containing 2% fetal calf serum (FCS) (Life Technologies). They were then blocked with anti-CD16/32 (clone 2.4G2, Fc Block), and stained with biotin-conjugated rat monoclonal antibodies specific to lineage antigens on the surface of murine mature blood cells, including CD5 (clone 53-7.3), CD8a (clone 53-6.7), CD45R/B220 (clone RA3-6B2), CD11b/Mac-1 (clone M1/70), Ly-6G/Gr-1 (clone RB6-8C5), and TER119/Ly-76

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(clone TER-119). In addition, cells were also incubated with fluorescent-labeled stem cell-specific markers, phycoerythrin (PE)-conjugated anti-Ly-6A/E (Sca-1; clone E13-161.7) and allophycocyanin (APC)-conjugated anti-CD117 (c-kit; clone2B8) monoclonal antibodies. Following washing, they were resuspended in medium containing streptavidin-APC-Cy7, which specifically bound to biotin-conjugated lineage antibodies. After 30 minutes incubation, the cells were finally resuspended into 5ug/mL propidium (PI) solution to distinguish viable and dead cells. All monoclonal antibodies were purchased from Pharmingen, San Diego, CA. Flow cytometric analysis was performed on a dual-laser FACSVantage (Becton Dickinson Immunocytometry Systems, San Jose, CA) to select PI negative, lineage negative, Sca-1 and c-kit positive cells (Pi-, Lin-, Sca-1+, c-kit+ (LSK) cells), which highly enriched viable hematopoietic stem cell (HSC) population (Figure 3.1).

Cell cycle analysis of HSCs

Flow cytometry was used to quantify the cell cycle status in HSC compartment. Bone marrow cells were isolated and immunofluorescetly stained as described previously except that different fluorochromes were conjugated to lineage (CD5, CD8a, CD45R/B220, CD11b/Mac-1, Ly-6G/Gr-1 and TER119/Ly-76) and stem cell-specific (Sca-1 and c-kit) monoclonal antibodies. Phycoerythrin (PE)-conjugated antibodies were used here to stain all lineage-positive mature blood cells, and phycoerytherin Texas Red (PETR)-conjugated anti-Sca-1 and fluorescein isothiocyanate (FITC)-conjugated anti-c-kit antibodies detected the

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existence of stem cells. Following the immunofluorescent staining, cells were fixed using 1% formaldehyde (Polysciences, Inc. Warrington, PA) and permeabilized by 0.2% Triton X-100 (Bio-Rad Laboratories, Richmond, CA). 10µl/ml 4', 6-diamidino-2-phenylindole, dilactate (DAPI, Sigma, St. Louis, MO) was used to determine the DNA content and the percentage of cycling stem cells. DNA content, and thus stoichiosmetric DAPI signal, in cells in G_2/M phase of the cell cycle is 2 times higher than that of cells in G_0/G_1 phase, with S phase cells possessing an intermediate amount. Flow cytometric analysis was performed on a dual-laser FACSVantage (Becton Dickinson Immunocytometry Systems, San Jose, CA).

In-vivo bromodeoxyuridine (BrdU) incorporation

Cell cycle analysis using DAPI staining only revealed the percentage of HSCs in S/G2/M phase at a specific timepoint in vitro, but couldn't determine cell cycle kinetics in vivo. The long-term administration of BrdU was used to investigate in a dynamic way, the cycling history and turnover rate of HSC population.

BrdU stock solution was made up at a concentration of 10mg/mL in PBS (Sigma, St. Louis, MO), and frozen at -80ºC. Age-matched chromosome 3 congenic and background mice were given BrdU-containing drinking water at a concentration of 0.5mg/mL for 1, 2, 3, 4, 5 days (4 mice in each group at each timepoint). Containers of BrdU water were wrapped in aluminum foil to prevent light exposure and water bottle were changed every 5 days. For the zero time

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point, mice were injected intraperitoneally with 100ul (0.5mg) BrdU and sacrificed 90 minutes later.

Harvesting of bone marrow cells and immunofluorescent staining of lineage and stem cell markers were as described previously. The cell cycle analysis was performed using the BrdU Flow Kit (Pharmingen, San Diego, CA). Briefly, labeled cells were fixed and permeabilized with Cytofix/Cytoperm Buffer for 40 minutes. After washing with Perm/Wash Buffer, cells were treated with DNase at 37ºC for 1 hour to expose BrdU epitopes. Cells were subsequently resuspended in FITC-anti-BrdU solution for 30 minutes at room temperature. Flow cytometric analysis was carried out to determine the proportion of BrdU positive Lin-Sca-1+c-kit+ (LSK) cells.

Apoptotic analysis of HSCs

Bone marrow cells were prepared and immunofluorescently stained as described previously. In brief, LSK cells were selected by HSC-specific antibodies that were tagged with APC-Cy7, phycoerythrin (PE) and allophycocyanin (APC) respectively. FITC-conjugated Annexin V and 7-AAD were utilized to identify early apoptotic cells. Thymocytes were used as the control to distinguish Annexin V positive and negative cells (all from Pharmingen, San Diego, CA). 3 independent experiments were performed in each strain, and at least 2 mice were analyzed in each experiment. All experiments were carried out on ice and completed within 1.5 hours. Flow cytometric analysis was

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performed on a dual-laser FACSVantage (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Competitive repopulation and serial transplantation assay

Recipient mice, B6.SJL ($Ptprc^b$ [Ly-5.1]), were given drinking water ad libitum containing 0.16mg/ml sulfamethoxazole and 0.004mg/ml trimethoprim for at least 5 days before irradiation. They were then exposed to 9 Gy total-body γ irradiation from a $^{137}C_s$ source administered as a single dose at least 6 hours before transplantation. Unfractionated bone marrow cell suspensions were prepared as described before. $1x10^6$ B.D Chr3 congenic- and B6-derived $Ptprc^b$ [Ly-5.2] donor cells were injected retro-orbitally into recipients, together with equal number of competitor Ly5.1 cells. There were 8 recipient mice in each group and 3 independent experiments were performed. At 16 weeks after transplantation, recipient mice were bled from the retro-orbital venous plexus. Peripheral blood was analyzed to determine the hematopoietic reconstitution capability of donor cells. Erythrocytes were eliminated by hypotonic lysis. The remaining leukocytes were stained with FITC-anti-Ly5.2 (CD45.2, clone 104) and PE -anti-Ly5.1 (CD45.1, clone A20) to detect the presence of Ly5.1- or Ly5.2-positive cells. In addition, the percentage of donor-derived myeloid and lymphoid cells was determined by labeling cells with FITC-anti-Ly5.2 and PE-conjugated lineage antibodies, which are specific to macrophages (anti-CD11b/ Mac-1, clone M1/70), granulocytes (anti-Ly6G/Gr-1, clone RB6-8C5), B lymphocytes (anti-CD45R/B220, clone RA3-6B2) and T lymphocytes (anti-Thy-1.2; clone 30H12)

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respectively (all from Pharmingen, San Diego, CA). Samples were then analyzed by a FACScan instrument (Becton-Dickinson immunocytometry System, San Jose, CA, USA). At the last timepoint (16th week), recipients' bone marrow cells were also analyzed to determine the Ly5.1/ Ly5.2 chimerism in whole bone marrow and in the Lin-, Sca-1+ and c-kit+ (LSK) cell population. FITC-anti-Ly5.2 labeling was used to identify the chimerism, and immunofluorescent staining of LSK cells was performed as described before.

In serial transplantation experiments, as shown in Figure 3.2, primary recipient mice were sacrificed at 16 weeks after transplantation. Bone marrow cells were pooled from 16 mice, and $1x10^7$ cells were retroorbitally injected into 16 (8 for each group x 2 groups) lethally irradiated secondary Ly5.1 recipients (9Gy). Peripheral blood and bone marrow were assayed for Ly5.1/Ly5.2 chimerism 16 weeks post-transplant. The same procedure was reiterated for the tertiary and quaternary recipients.

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Results

Cell cycling status of HSCs

A small proportion of HSCs in the bone marrow is always cycling to maintain a steady-state level of stem cells and mature blood cells (Bradford et al. 1997; Cheshier et al. 1999; Pietrzyk et al. 1985). The cycling status of HSCs is one of the important determinants to control their population size and the number of differentiated progeny they produce (Steinman 2002). An increased proportion of actively dividing stem cells and/ or a shortening of their cycling time will lead to HSC expansion. Since D2 and B.D Chr3 congenic mice have more bone marrow HSCs, I therefore hypothesized that a higher proportion of cycling HSCs may exist in these strains. Flow cytometric analysis was performed to measure the cell cycle status of the stem cell-enriched Lin-Sca-1+c-kit+ (LSK) population. A DNA dye, DAPI, which binds strongly and stoichiometrically to AT-rich regions in the minor groove of DNA, was used to determine the DNA content of the cells at different cell cycle stages. As shown in figure 3.3 A and B, cells in G_2/M phase have a 2 times higher DNA content than those in G_0/G_1, with S phase cells possessing an intermediate amount. Differential DNA content enables the measurement of percentage of cycling stem cells by simply comparing the profile of DAPI signals in a cell population. The results summarized in table 3.1 demonstrate that in all studied strains, around 5-7% of LSK cells were in S/G2/M phase of the cell cycle, a number similar to previous reports by other groups, indicating that a small number of HSCs are always dividing in order to maintain

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the steady-state hematopoiesis. However, no significant differences were observed in the fraction of cycling LSK cells among chromosome 3 congenic and their respective background mouse strains, suggesting that the effects of chromosome 3 QTL on HSC number may not be through regulation of HSC cycling.

In vivo cell cycle kinetics of HSCs

Cell cycle analysis by using DAPI staining could only tell us the percentage of HSCs in S/G2/M phase at a specific timepoint in vitro, but couldn't determine cell cycle kinetics of HSCs in vivo. The BrdU incorporation experiment is an alternative way to achieve this goal. BrdU is a thymidine analog that can be incorporated into newly synthesized DNA of replicating cells (Pietrzyk et al. 1985; Willis et al. 1993). When BrdU was added to the drinking water of the chromosome 3 congenic mice and their controls for 5 days, the continuous BrdU incorporation into LSK bone marrow populations allowed me to measure accumulations of replicating cells, and thus stem cell turnover rates over the long-term (Forster and Rajewsky 1990; Hagan and MacVittie 1981; Kriss and Revesz 1962; Tice et al. 1976). Two populations of LSK cells were analyzed according to the brightness of Sca-1 staining: the entire LSK population which contains all long-term HSCs as well as some short-term HSCs and multipotent progenitor cells not as brightly stained with Sca-1 (LSK), and a population of only the brightest Sca-1 staining LSK cells (LSK++) which are most highly purified long-term HSC subset (Okada et al. 1991; van de Rijn et al. 1989).

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Figure 3.4 shows different BrdU labeling patterns of the two population LSK cells in different mouse strains. In both B.D Chr3 congenic and B6 LSK cells (upper curve), the rate of BrdU incorporation increased dramatically in the first 2 days, and by day 2 ~70% of LSK population was labeled. Subsequently, the BrdU uptake slowly increased, and only around 20% more LSK cells were labeled in the following 3 days. 90% of LSK cells were BrdU positive at 5 days. Even though the overall pattern of labeling was similar in all mouse strains, the more striking difference was observed in LSK populations between the congenic and background strains. An average of 15% of B.D Chr3 congenic LSK cells were labeled 1 hour after a single pulse of BrdU, and 46% were BrdU positive by day 1. In contrast, labeling of the analogous population of B6 cells proceeded at a slower pace, showing a 3-fold lower labeling in 1 hour (~5%), and 50% less labeling at day1. In the more primitive LSK++ cell population (lower curve), the overall BrdU labeling rate was significantly slower compared to LSK population, which is consistent with the turnover dynamics of the hematopoietic hierarchy (Cheshier et al. 1999; Pietrzyk et al. 1985). During the pulse-labeling period (T_0), 1.6±1.2% of LSK++ cells in B.D Chr3 mice and 3.1±1.5% in B6 cells were undergoing DNA synthesis and incorporating BrdU, but the difference was not statistically significant. B.D Chr3 LSK++ cells showed a 2-fold higher labeling than analogous B6 cells (10.8±4.9% vs 5.4±1.1%) by day1 but also without statistical significance. During the 5 days period of BrdU exposure, nearly one third of LSK++ cells were labeled in 3 days and more than half were labeled by day 5. All BrdU labeling data derived from B6 mouse strain are very similar to the

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published results obtained by other labs (Bradford et al. 1997; Cheshier et al. 1999). Although extended periods of time need to be further investigated, these results underscore that (1) HSCs divide regularly during steady-state hematopoiesis. (2) Within heterogeneous HSC populations, different subsets of cells possess different proliferative rates. In general, the more primitive cells are, the slower they proliferate. And (3) B.D Chr3 congenic LSK stem cells replicate faster than do B6 cells, a finding that is mainly attributed to the presence of less primitive cells in this population.

Apoptosis status of chromosome 3 congenic HSCs

Studies have previously shown that apoptosis is an important pathway in regulating HSC numbers. Apoptosis can be identified by a series of morphologic changes, including cell shrinkage, membrane blebbing, chromatin condensation, caspase activation, and enzymatic internucleosomal DNA destruction (Brown and Attardi 2005). Early in this process, cells lose their phospholipid membrane asymmetry and expose phosphatidylserine (PS) to the outer leaflet of the cell membrane (Martin et al. 1995). This change can be monitored by Annexin V, a Ca^{2+}-dependent, phospholipid-binding protein with high affinity for PS (Vermes et al. 1995). The DNA stain 7-AAD is used to identify viable cells able to actively exclude the dye (Schmid et al. 1994). Therefore, early apoptotic cells are Annexin V positive and 7-AAD negative, whereas necrotic cells are double positive for both Annexin V and 7-AAD. By using a flow cytometry protocol that simultaneously identifies LSK cells, apoptosis can thus be measured in this

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primitive population (Anthony et al. 1998). Figure 3.5 depicts the Annexin V and 7-AAD staining profile of B6 (panel A) and B.D Chr3 (panel B) LSK cells. Panels C to E are a control series using freshly isolated thymocytes, which contains significant numbers of apoptotic and necrotic cells, to determine quadrant positions. In all profiles, the apoptotic cells are in the lower right quadrant and necrotic cells are in the upper right quadrant. Table 3.2 summarizes the apoptotic analysis of stem cells in chromosome 3 congenic and control strains. Under steady-state conditions B6 and D2 apoptotic LSK cells comprised 2.67±0.42% and 0.86±0.2%, respectively, of the population ($P<0.001$). The replacement of D2 chromosome 3 QTL alleles in a B6 genetic background (B.D Chr3) reduced the apoptotic LSK fraction to 0.55±0.1% ($P<0.001$), resulting in a D2-like phenotype. However, introgression B6 alleles around QTL onto the D2 background (D.B Chr3), which might be expected to increase apoptosis, further reduced the already low D2 apoptotic rate to 0.29±0.03 ($P<0.001$). These data suggest that strain-specific apoptotic rates exist in hematopoietic stem and progenitor populations and that they can be altered by genetic manipulation. The dramatic range (9-fold) of this important parameter among these strains underscore the critical role it may play in the determination of stem cell population size.

Measurements of self-renewal capabilities of congenic HSCs

In order to examine the self-renewal capability of HSCs, serial transplantation experiments were performed to partially mimic the replicative demands on HSCs during a lifetime of hematopoiesis (Becker et al. 1963;

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Hellman et al. 1978; Krause et al. 2001; Siminovitch et al. 1964). As illustrated in Figure 3.2, B6 control and B.D Chr3 congenic bone marrow cells (Ly-5.2 donor cells) were injected into 2 separate groups of irradiated Ly-5.1 recipients along with Ly-5.1 competitor cells. Sixteen weeks post-transplant, Ly5.1/Ly5.2 chimeric bone marrow cells from primary recipients were transplanted into secondary recipients without the addition of competitor cells, and such serial transplantation was continued until the 4th generation of recipients. At each transplant, the peripheral blood and bone marrow of recipient mice were analyzed and quantified for initially injected B6 or B.D Chr3 (Ly-5.2) cells, which are the sole source of stem cells to reconstitute hematopoietic system in serial myelo-ablated recipients. The competitive repopulation advantage of HSCs of one strain relative to the other is reflected by the percentage of donor-derived cells. The more donor-derived hematopoietic cells, the higher repopulation or self-renewal capability HSCs have (Harrison et al. 1993; Harrison and Zhong 1992; Szilvassy 2003)). As shown in Figure 3.6, higher numbers of B.D Chr3 congenic-derived cells were found in peripheral blood leukocytes (panel A), unfractionated bone marrow (panel B), and LSK stem cells (panel C) than those derived from transplanted B6 cells. All of the differences are highly significant ($P<0.001$) and sustained with each successive transplant. Therefore, the B.D Chr3 stem cells clearly have a numerical competitive repopulation advantage relative to B6. Moreover, only 8 of initial 16 (50%) survived in the group of quaternary recipients that were transplanted with B6 cells, whereas all 16 mice survived in B.D Chr3 group. All data suggest that B.D Chr3 congenic HSCs replicate with a

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competitive advantage that permits serial transplantation well beyond that of B6-derived stem cells, and importantly, that the D2 chromosome 3 QTL confers this higher self-renewal capability to HSCs.

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Discussion

Stem cell population size could be affected by as least 3 fundamental factors: the rate of proliferation, apoptosis, and the capability for self-renewal (Morrison et al. 1997). Studies were therefore performed to characterize these parameters in chromosome 3 congenic strains in order to understand which factor involves in chromosome 3 QTL-mediated HSC number regulation.

Two strategies were used to measure the proportions of HSCs entering the cell cycle: DAPI staining of LSK cells and BrdU labeling of LSK and LSK++ cells. The results from the first method showed that, consistent with previously published data (Cheshier et al. 1999; Morrison and Weissman 1994), ~5% of LSK cells were in S/G2/M phase of the cell cycle, and no significant differences were observed among all 4 strains (Table3.1). In contrast, BrdU incorporation experiments demonstrated that ~15% of B.D Chr3 congenic LSK cells were labeled in 1 hour after BrdU administration, a value 3-fold higher than that of B6 LSK cells (Figure 3.4). This discrepancy between DAPI results and the BrdU labeling might be due to the following considerations: (1) The experimental techniques are different between two methods: DAPI staining is done on fixed cells in vitro whereas the BrdU labeling is in vivo; (2) the DAPI method measures the fraction of cells in S/G2/M phase only at the precise time that the bone marrow is harvested, whereas BrdU cumulatively labels the cells that undergo at least one division during the period of exposure to BrdU (Dolbeare et al. 1983); and (3) the LSK population identified by both methods are not precisely the same

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because different combination of antibodies and fluorochromes had to be used as described in Methods.

The time-course results from 5 days of BrdU administration are comparable with those of Cheshier et. al (Cheshier et al. 1999). The overall experimental strategy in their study was similar to ours, except that they used one additional marker, Thy1.1, to identify B6 HSCs. LSKThy1.1^{negative} and LSKThy1.1$^{negative/low}$ cell populations, whose contents are comparable to our LSK++ (highly purified long-term HSCs) and LSK (heterogeneous HSCs) cells respectively, were studied for their BrdU incorporation rates. Within the overlapping periods of labeling, both studies showed similar patterns and extents of BrdU labeling in long-term and heterogeneous HSC populations. When the labeling period was extended in Cheshier's study, the time for virtually all LSKThy1.1$^{negative/low}$ and LSKThy1.1^{negative} cells to incorporate BrdU were 30 and 180 days respectively, times very similar to what we observed in B6 background HSCs (data not shown). The fact that the more primitive HSC subset possesses a longer turnover time is further supported by previous studies of Pietrzyk et.al (Pietrzyk et al. 1985). Although all HSCs theoretically enter the cell cycle by a certain timepoint, the slow replicating feature of more primitive HSCs supports the idea that the HSC population is hierarchically organized on the basis of their cycling or quiescence state (Spangrude and Johnson 1990). In another point of view, the specific stage in a cell cycle might in turn determine whether primitive cells function as a stem cell or a progenitor cell (Habibian et al. 1998; Lambert et al. 2003; Quesenberry et al. 2002). Nevertheless, the low cycling rate is a typical

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feature of long-term HSCs and is necessary for the preservation of stem cell function as well as potentiality in the hematopoietic system.

The effects of the chromosome 3 QTL on HSC proliferation are only present in LSK cells in the 24-hour BrdU pulse-labeling. The fact that D2 alleles enhanced the cycling rate and this effect was restricted to a subset of HSCs suggest that the chromosome 3 QTL interval must contain specific gene that regulate stem cell proliferation. An increasing numbers of regulatory molecules have been implicated in the control of cell cycle progression in different HSC compartment. One of them is *cyclin D2*, which was found to be highly expressed in most of LSKThy1.1^{negative} cells in Cheshier's study (Cheshier et al. 1999). Furthermore, absence of *cyclin D1/D2/D3* profoundly impaired the proliferation of both HSCs and multi-lineage hematopoietic progenitor cells, suggesting that *cyclin D* might act as one of the controllers (Kozar et al. 2004). Other cell cycle-related genes, such as p21, p18 and p27, were also reported to be involved in regulating different types of hematopoietic cells (Cheng 2004; Steinman 2002). For example, p21 and p18 specifically control HSC proliferation, whereas p27 only affects hematopoietic progenitor cells. Further study of the chromosome 3 QTL interval in the congenic mouse model may provide a platform leading to the discovery of novel cycle-active gene and/or functions of already known genes.

The apoptotic analyses shown in Table 3.2 are novel. It is the first report that natural variations in steady-state apoptosis exist in the HSC compartment, and apoptosis is a more important natural regulator of stem cell population size than expected. The proportion of apoptotic HSCs in B6 mice was 3-fold higher

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than that of D2 mice, and the difference was confirmed in 3 replicate and independent experiments. This observation is consistent with the idea that a high rate of apoptosis leads to a decrease in cell numbers in a given population and indicate that apoptosis might be one of mechanisms causing a lower HSC frequency in young B6 mice (Domen 2001). Another interesting finding in this analysis is that D2 alleles in the chromosome 3 congenic interval completely conferred the D2 phenotype to B6 stem cells, resulting in a similar fold decrease in apoptotic rate in B.D Chr3 HSCs. In contrast, introgression of B6 alleles onto a D2 background further decreased the already low apoptosis in D2 mice, which was opposite to what was expected. This discrepancy might be due to the fact that the B6 chromosome 3 congenic interval (19cM~60cM) introgressed onto D2 background is not identical to the D2 interval (14cM~33cM) on the B6 genome, although both contain the QTL at 25cM. It is highly possible that another locus or loci located in this congenic interval and/or in the genetic background might also have profound effects on apoptosis of HSCs in vivo perhaps in conjunction with the QT gene (Bystrykh et al. 2005). Nevertheless, there is clearly a locus or loci in the chromosome 3 congenic intervals that regulate apoptosis of HSCs and this locus/loci might be responsible for natural variations in this parameter. Furthermore, the interactions between this locus/loci and the genetic background also exert their effects in vivo. However, whether the variations in both apoptosis and stem cell number are attributed to the same locus on chromosome 3, and whether there is a cause-effect relationship between these two parameters is still not clear yet. To answer these questions, further studies, such as fine mapping,

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positional cloning and/or microarray analysis, may be necessary to discover underlying mechanisms (Biola et al. 2003; Mackay 2001).

In order to study the effects of chromosome 3 QTL on stem cell renewal, serial transplantation experiments, the “gold-standard” method to measure this parameter, were performed in B.D Chr3 congneic and their B6 background mice. Although the same numbers of bone marrow cells from both strains were injected into primary recipient mice, the peripheral blood leukocytes and bone marrow nucleated cells derived from congenic mice were significantly higher than those of B6 cells at 16 weeks post-transplant, a time at which long-term HSCs are considered to be the only source of hematopoietic reconstitution (Harrison et al. 1993). Two mechanisms might be responsible for these competitive repopulating advantages: high proliferation rate of HSCs and/or high stem cell numbers (Szilvassy 2003). Since we have previously shown that a higher proliferation rate exists in B.D Chr3 congenic HSCs, we next determined whether the stem cell number difference also contributes to the differential engraftment levels. The results from analyses of chimeric bone marrow LSK stem cells showed that around 75% more LSK cells were derived from B.D Chr3 congenic HSCs compared to B6 stem cells, suggesting that a competitive advantage of congenic cells also results from the higher HSC frequency.

Several interesting results were observed in the serial transplant experiments. First of all, initially injected B.D Chr3 and B6 Ly-5.2 cells could be detected in both peripheral blood and bone marrow of all secondary, tertiary and quaternary recipient mice over a period of 64 weeks, indicating that these donor-

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derived cells contain an adequate number of HSCs to be able to self-replicate and repeatedly reconstitute the hematopoiesis over the long-term. Secondly, the competitive advantage of B.D Chr3 congenic cells over B6 cells existed in both differentiated and in stem cells of all serial passages, suggesting the B.D Chr3 LSK stem cells have a higher self-renewal ability than do B6 HSCs. This conclusion is further supported by the survival rate of quaternary recipients, in which only half of B6 recipients survived compared to B.D Chr3 recipients. It is well known that the demands on HSCs for regeneration of both stem cells (self-renewal) and mature cells (differentiation) are increased under conditions of hematopoietic reconstitution after transplantation. This replicative stress is amplified with serial transplants and results in stem cell exhaustion (Hellman et al. 1978; Krause et al. 2001). Stem cells with higher renewal abilities usually endure more serial transplants. Consistent with these theories, our results indicated D2 alleles around the chromosome 3 QTL increase HSC renewal at least 2-fold, which might consequently lead to an approximate 2-fold increase in HSC numbers. In other words, stem cell renewal might be one of the mechanisms by which chromosome 3 QTL regulated HSC numbers. Since Ly5 congenic mice don't exist on a D2 genetic background, such competitive repopulation and serial transplantation assays can't be performed with D2 and D.B Chr3 congenic cells. Whether B6 alleles would decrease the stem cell renewal is still a question that needs to be addressed. Finally, analysis of Ly-5.1/Ly-5.2 chimerism in bone marrow LSK stem cells has showed that both the B.D Chr3 congenic and B6 stem cells have a very strong competitive advantage

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over Ly5.1 competitor cells, and that by the 4th serial transplant almost all bone marrow LSK stem cells were derived from B.D Chr3 and B6 LSK cells (90%). The complete dominance of B.D Chr3 and B6 LSK cells over Ly5.1 competitor cells might come from the effects of Ly5 alleles and/or a passenger locus in the Ly5 congenic interval. The evidence supporting the first possibility comes from studies by van Os (van Os et al. 2001). They found that the engraftment level of Ly5.1 cells in Ly5.2 hosts was lower than that of Gpi-1^a cells in Gpi-1^b hosts under the conditions of low-dose irradiation (≤ 4Gy) and/or transplantation of small numbers of cells ($2x10^6$). Although both Ly5 and Gpi-1 act as cellular markers to distinguish donor from host cells, Ly5 has an extracellular protein domain that may induce an immune response whereas Gpi-1 is an intracellular protein (glucose phosphate isomerase) without immune activity. Further studies demonstrated that the disparity between Ly5.1 and Ly5.2 antigen could slightly induce a T cell-mediated immunological response directed against Ly5.1-derived cells, probably HSCs, and consequently impaired the engraftment capability of Ly5.1 cells. Therefore, the gradual loss of Ly5.1 cells, especially bone marrow LSK stem cells, during the serial transplants shown in our studies might be due to T cell-mediated immunity under certain conditions, such as a low number ($1x10^6$) of Ly5.1 cells were injected. This explanation is also supported by the findings from other researches (Chen et al. 2004; Xu et al. 2004).

In aggregate, all results demonstrate that B.D Chr3 HSCs have a high competitive self-renewal potential coupled with increased cycling and decreased cell death during steady-state hematopoiesis. Although some of these analyses

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were not performed in the reciprocal congenic HSCs (D.B Chr3), these data at least suggest that the regulation of HSC number by chromosome 3 QTL is complicated, probably involving several biological processes and multiple molecular mechanisms. The results in this chapter have provided a direction to screen the genes in the congenic interval based on these phenotypical analyses. Moreover, it also helps to understand the coordination of cell cycle regulation with cell differentiation and survival in HSCs.

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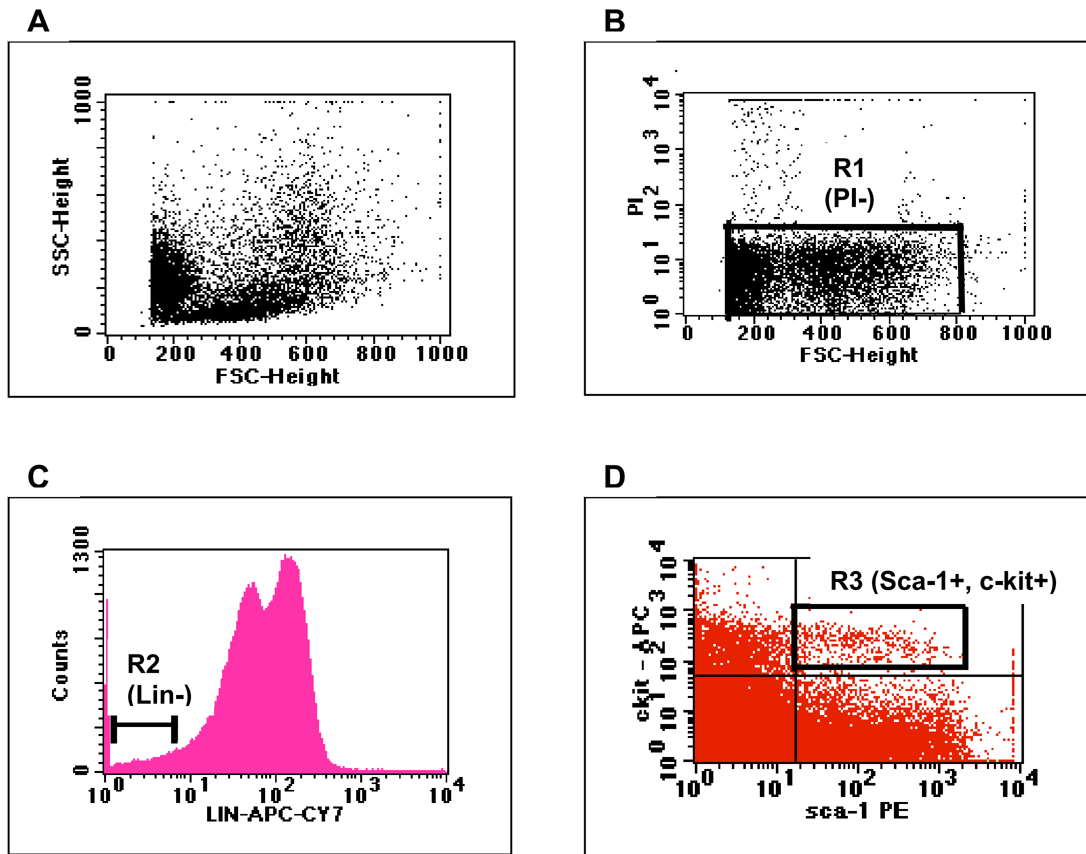

Figure 3.1 Identification of Lin-, Sca-1+, c-Kit+ (LSK) HSCs by flow cytometry. Bone marrow cells (A) were stained with antibodies against lineage and stem cell markers as described in Methods. The viability of HSCs was determined by PI staining (B), in which PI negative cells are viable (R1 region, PI- cells). The negative selection (C) of viable cells eliminated all lineages of differentiated cells and retained immature cells (R2 region, Lin- cells), whereas the positive selection (D) defined Sca-1+, c-kit+ stem cells (R3 region). Therefore, HSCs identified by flow cytometric analysis are PI-, Lin-, Sca-1+ and c-kit+ (LSK) cells.

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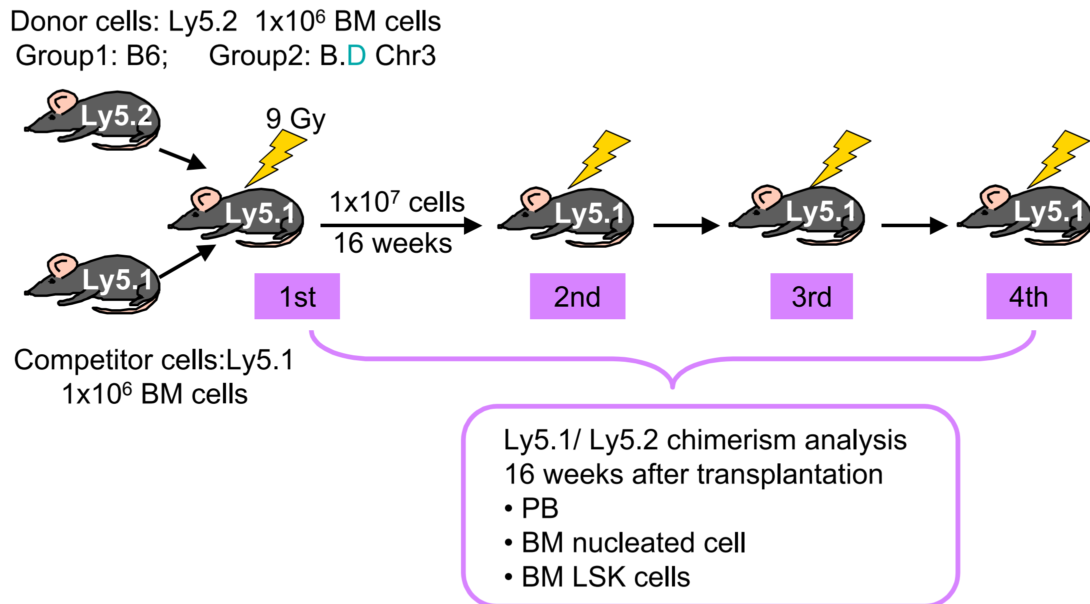

Figure 3.2 Schematic illustration of serial transplantation of bone marrow cells to measure self-renewal of HSCs. Bone marrow (BM) cells harvested from C57BL/6 (B6) and B.D Chr3 congenic (Ly5.2) mice were transplanted into two separate groups of lethally-irradiated Ly5.1 mice (primary recipients), along with equal numbers ($1x10^6$) of Ly5.1 competitor cells. 16 weeks after transplantation, $1x10^7$ BM cells pooled from 1° recipients were injected into 2° recipients. The procedures were reiterated in tertiary and quaternary transplantations. In each generation of recipients, peripheral blood (PB), BM and BM Lin-, Sca-1+, c-kit+ (LSK) stem cells were analyzed for Ly5.1/Ly5.2 chimerisms 16 weeks post-transplant. Two separate and independent experiments were performed for each strain and in each experiment, 8 recipient mice were included for each transplantation.

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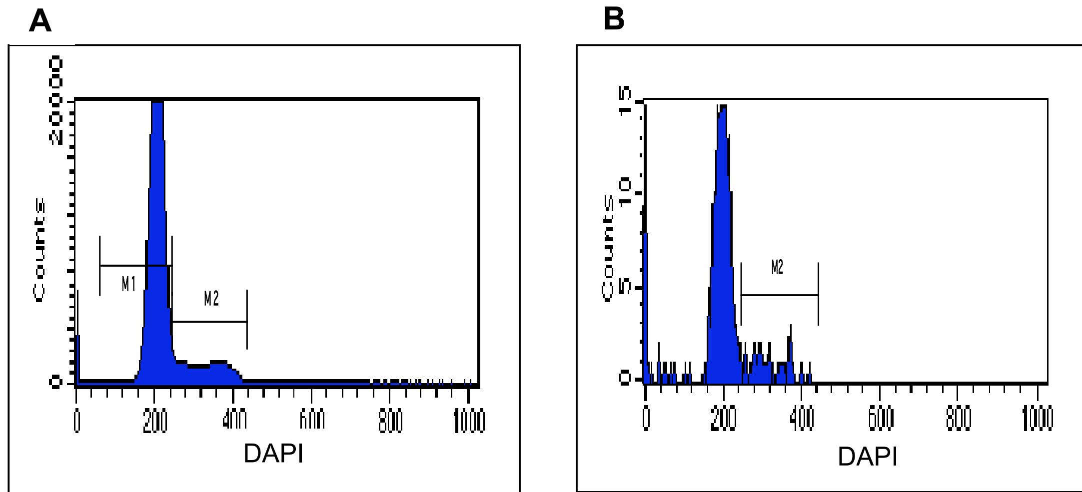

Figure 3.3 Cell cycle analysis of unseparated bone marrow cells and LSK stem cells by DAPI staining. Bone marrow cells and bone marrow LSK cells were identified as shown in figure 3.1. DNA content, and the percentage of cycling cells (S/G2/M phase of the cell cycle) was determined from the DAPI histogram of gated bone marrow (A) and LSK cells (B). Cells with greater than a diploid DNA content (M2 region) represent cycling cells, whereas other diploid cells (M1 region) are in G0/G1 phase.

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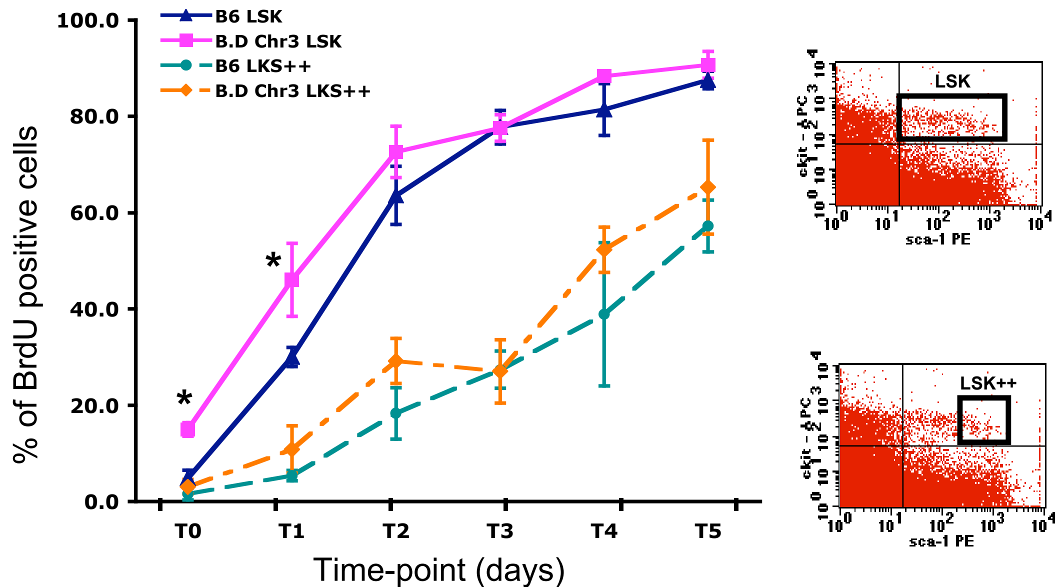

Figure 3.4 Cell cycle kinetics of HSCs measured by BrdU incorporation. BrdU was added to the drinking water of the B.D Chr3 congenic and C57BL/6 (B6) background mice for 5 days. The BrdU incorporation rate was measured daily in cohorts (4 mice at each timepoint) of mice and the time zero point was obtained 1 hour following an intraperitoneal injection of BrdU. Two populations of LSK cells were assayed for BrdU uptake: the entire LSK population and the LSK++ cells that were most strongly stained with Sca-1 antibody. The percentages of BrdU positive cells at each timepoint are the average of 4 mice and error bars represent ±1 standard deviation. *$p<0.05$ relative to background.

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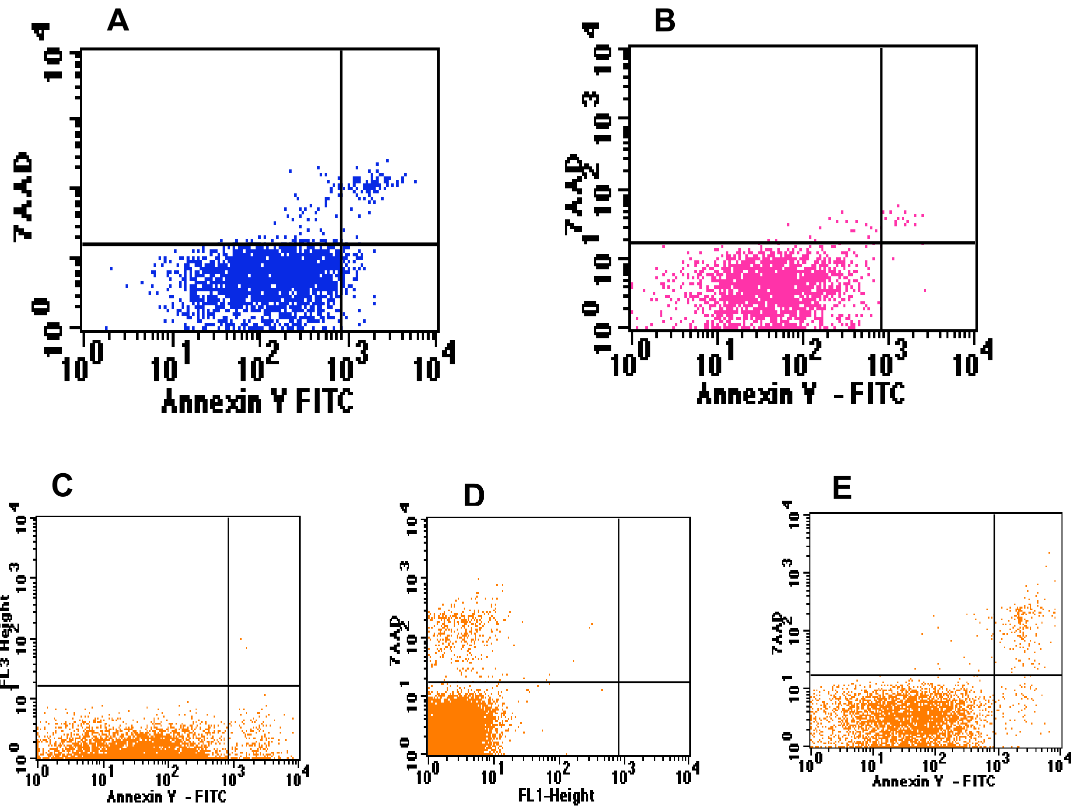

Figure 3.5 Measurement of apoptotic LSK cells by Annexin V and 7-AAD. LSK cells were identified as shown in figure 3.1. The Annexin V and 7 AAD staining profile of LSK cells from B6 background mice (A) and B.D Chr3 congenic mice (B) are shown. Apoptotic cells, which are annexin V positive and 7-AAD negative, are in the lower right quadrants, whereas necrotic cells fall in upper right quadrants (both Annexin V and 7-AAD are positive). The lower row of panels (C, D, E) is a control series run with each experiment using mouse thymocytes, which contains significant numbers of apoptotic and necrotic cells. Panels C and D show single staining of thymocytes for Annexin V and 7-AAD, respectively, whereas Panel E shows the double staining.

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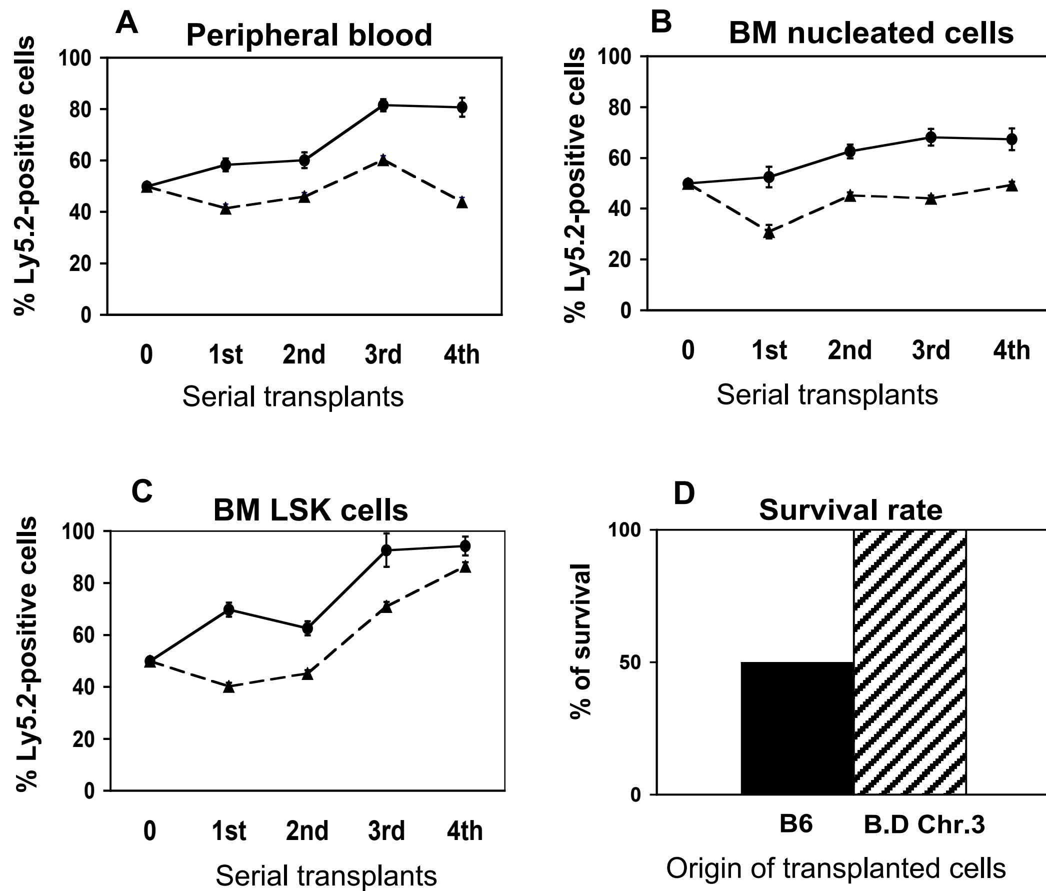

Figure 3.6 Serial transplants of B6 and B.D Chr3 congenic bone marrow cells to measure HSC self-renewal capability. Serial transplantation procedures were performed as described in figure 3.2. 16 weeks following each of the serial transplants, the percentages of Ly5.2-derived cells in recipients' peripheral blood (A), bone marrow (B) and bone marrow LSK (C) populations were determined and compared between B6 (solid line) and B.D Chr3 congenic groups (dashed line). The survival rate was also measured in quaternary recipients in both groups. The values at each timepoint are the averages of 16 recipient mice from two independent experiments. The percentages of B.D Chr3-

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derived cells are higher than those of B6-derived cells with a statistical significance ($p<0.05$) in every comparison.

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Table 3.1 Cell cycle analysis of LSK HSCs by DAPI staining in chromosome 3 congenic and their background mouse strains.

Mouse strains	Percentage of LSK cells in S/G2/M (% ± 1 SEM)
B6	5.20 ± 0.98 %
B.D Chr3	7.29 ± 1.88 %
D2	5.59 ± 0.79 %
D.B Chr3	5.62 ± 0.75 %

Bone marrow LSK stem cells were identified as shown in figure 3.1. Cells in the G2/M phases (tetraploid) of the cell cycle have twice the DNA content of those in G0/G1 (diploid), with S phase having the intermediate content. DNA contents were determined by DAPI staining, as per the profile in figure 3.3. The percentages of LSK cells in S/G2/M phases of the cell cycle were measured and compared among all chromosome 3 congenic lines and their respective background strains. Data shown in above table are the mean ± SEM that were pooled from at least 3 independent experiments, with at least 2 mice per experiment.

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Table 3.2 Apoptotic analysis of LSK HSCs by Annexin-V and 7-AAD staining in chromosome 3 congenic and their background mouse strains.

Mouse strains	Percentage of apoptotic LSK cells (% ± 1 SEM)
B6	2.67 ± 0.42 %
B.D Chr3	0.55 ± 0.1 % *
D2	0.86 ± 0.2 % *
D.B Chr3	0.29 ± 0.03 % *

Bone marrow LSK stem cells were identified as shown in figure 3.1. Apoptotic cells were identified by Annexin V and 7AAD staining and the staining profiles were shown in figure 3.4. The percentages of apoptotic LSK cells were measured and compared among all chromosome 3 congenic and their respective background strains. Data shown are the mean ± SEM that were pooled from at least 3 independent experiments, with at least 2 mice per experiments. * denotes a statistical significance ($p<0.001$) in any pair comparison of all 4 strains.

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CHAPTER FOUR

Identification of quantitative trait gene responsible for HSC number regulation

Summary

In order to identify the gene within chromosome 3 QTL region that are responsible for HSC number variations between B6 and D2 mouse strains, a microarray-based candidate gene approach was performed. By measuring mRNA expression profiles in purified HSCs (LSK cells) using Affymetrix gene chips (MGU74Av2), I found that 17 genes were differentially expressed among chromosome 3 congenic and their respective background mice. However, only one gene, which encodes latexin, was located within the chromosome 3 QTL interval that was overlapping in both congenic strains. Latexin mRNA was found to be highly expressed in D.B Chr3 and B6 LSK cells compared to B.D Chr3 and D2 LSK cells, and these differential expressions were validated by quantitative real-time PCR. Surprisingly, I also found the identical differential latexin mRNA expressions in all downstream progeny of LSK cells, including bone marrow Lin- and nucleated cells, and mature peripheral blood cells. Moreover, Western blot using polyclonal anti-latexin Ig-G antibody demonstrated that the differential expression pattern of latexin protein was consistent with that of its mRNA in bone marrow Lin- and nucleated cells. Altogether, our results suggest that B6 alleles

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in chromosome 3 QTL up-regulated, whereas D2 alleles down-regulated the expression of latexin mRNA and proteins. In order to identify the relationship between latexin expression level and HSC number changes, I constructed recombinant retroviral vector containing latexin cDNA and overexpressed this gene in D2 and D.B Chr3 congenic bone marrow cells. CAFC and CRU functional analyses of latexin-transduced cells were performed to investigate the regulatory role of latexin on HSC numbers. However, the results were not available at the time when the dissertation was under preparation. Our data is the first to report that latexin is expressed in HSCs; it also provides strong evidence concerning the reverse relationship between latexin expression level and the changes of murine HSC numbers. These findings may be useful for developing effective stem cell expansion strategies in the clinic. Moreover, understanding the underlying mechanisms and signaling pathways involving latexin will probably reveal further not appreciated as regulators of stem cell function.

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Introduction

Genetic complex traits (also called quantitative traits) are usually controlled by multiple genes, which pose special challenges that make gene discovery more difficult (Biola et al. 2003; Darvasi and Pisante-Shalom 2002). Current strategies aimed at the identification of quantitative genes typically employ a combination of positional gene mapping, candidate gene testing, and bioinformatics, as depicted in Figure 1.1. Most importantly, having congenic strains that display the phenotype of interest is a critical jumping off point for both a candidate gene approach and a positional cloning approach leading to gene discovery (Glazier et al. 2002).

With the development of an extensive array of genomic resources and technologies, the success for identifying “stemness” genes have improved markedly. Four comprehensive studies have recently been published describing stem cell-specific genes. Three of them used similar approaches by generating profiles of genes expressed in embryonic stem cells, neural stem cells, and hematopoietic stem cells. After reductionist comparisons of the profiles, all studies showed around 200 to 300 genes were commonly expressed in all three categories of stem cells, although the genes that overlapped among these studies were very few (Fortunel et al. 2003; Ivanova et al. 2002; Ramalho-Santos et al. 2002). Nevertheless, the commonly expressed genes fell into several functional categories, including those encoding transcription factors, membrane proteins, protein binding nucleic acids, and proteins involved in cell-cycle control

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and apoptosis. In a fourth study, Park et al. used substractive hybridization and gene array analyses to screen and identify the genes specifically regulating HSC functions (Park et al. 2002). The four studies collectively turned to so many stem cell-specific genes that the task of unraveling the mechanistic role of the genes in stem cell functions is demanding. In contrast, gene discovery in forward genetics does not have the problem of determining phenotype because a measurable difference in stem-cell function is the starting point of analysis. In my study, although I also performed microarray-based candidate gene approach to identify genes that regulate an already-known phenotype (HSC numbers) (Tabor et al. 2002), congenic strains displaying the phenotype was a great advantage to reduce the number of candidate genes to a level feasible enough that functional study of individual genes could be undertaken.

Because of extensively documented differences between the hematopoietic systems of C57BL/6 and DBA/2 mice and the powerful genetic tools afforded by the recombinant inbred strains by crossing them, several genetic studies using forward genetic approach have begun to shed light on loci in this genetic context that account for the strain-specific variations in hematopoietic stem cell (HSC) properties. The natural variations of HSC numbers, the changes of its number with age and self-renewal capabilities of HSCs have been reported to link to several QTL (Chen et al. 2000; de Haan and Van Zant 1997; Geiger et al. 2001; Kamminga et al. 2000; Muller-Sieburg et al. 2000; Muller-Sieburg and Riblet 1996; Phillips et al. 1992). Moreover, other QTL responsible for HPC mobilization and their sensitivity to the genotoxic effects of

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the cell cycle-specific drug hydroxyurea have been also mapped (de Haan and Van Zant 1999; Geiger et al. 2004). In addition to these cell-intrinsic properties, the responses of HSCs to extracellular cueswere reported to link to specific QTL (Henckaerts et al. 2002; Langer et al. 2004). Furthermore, de Haan et al. used a combination of linkage analysis, a congenic mouse model, substractive hybridization, and gene array technologies to reveal gene clusters on chromosome 11 associated with QTL that were linked with the sensitivity of HPCs to hydroxyurea (de Haan et al. 2002). Although some QTL have been confirmed in congenic mouse models and some even provide evidence for the underlying molecular basis of the QTL, none of them have successfully identified the specific gene (also called quantitative trait genes) that are directly associated with the corresponding quantitative traits. However, in our present study, I have for the first time identified latexin, a gene near the chromsome 3 QTL, and have shown that it is potentially responsible for strain-specific variation in HSC numbers. By performing gene array hybridization, quantitative real-time PCR, and Western blotting, I found that latexin, which encodes a carboxypeptidase A inhibitor (Normant et al. 1995), was differentially expressed in hematopoietic cells at both the mRNA and protein levels among chromosome 3 congenic lines and their respective background strains. Upregulated latexin expression was associated with the decreased HSC numbers, and vice versa. These promising results not only show the potential role of latexin in regulating HSC number, but also demonstrate the feasibility of a forward genetic approach for gene discovery.

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Materials and Methods

Animals

Background strains: C57BL/6J (B6) (Ptprc^b [Ly-5.2]), DBA/2J (D2) mice were purchased from the Jackson Laboratories (Bar Harbor, ME).

Congenic stains: Chromosome 3 congenic mice were generated by Geiger *et al.* as described previously (Geiger et al. 2001). Genotypes of congenic mice were validated every 6 months by screening genetic markers flanking the congenic interval and located throughout the genome. All primers for simple sequence repeat (SSR) genetic markers were bought from Research Genetics, Huntsville, AL and genotyping was carried out by PCR. Two strains of chromosome 3 congenic mice were generated and maintained: B.D Chr3 (14cM~33cM) congenics with D2 QTL being introgressed onto B6 background, and their reciprocal congenic strains D.B Chr3 (19cM~60cM) with B6 QTL onto D2 background. The overlapping congenic interval in the two strains spanned 14cM that is from 19~33cM.

Cell preparations

All cell populations were prepared from the above 4 strains and at least 3 independent biological samples were used in each analysis.

1. ***Enrichment of hematopoietic stem cells***

Bone marrow cells from the femora and tibiae were flushed into Hank's balanced salt solution (HBSS, Gibco, Grand Island, NY) containing 2% heat

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inactivated fetal calf serum (FCS, Life Technologies). The pooled marrow was layered over a Ficoll-Paque PLUS (Amersham Biosciences, Uppsala sweden) gradient and after centrifugation the mononuclear cell layer was removed. Mononuclear cells were subsequently stained with a panel of biotinylated antibodies specific to lineage mature blood cells, including CD5 (clone 53-7.3), CD8a (clone 53-6.7), CD45R/B220 (clone RA3-6B2), CD11b/Mac-1 (clone M1/70), Ly-6G/Gr-1 (clone RB6-8C5), and TER119/Ly-76 (clone TER-119)]. The labeled cells were then incubated with goat antirat IgG paramagnetic beads (Dynal, Lake Shearer, NY) at a bead:cell ratio of 4:1, and cells bound to lineage-specific antibodies were removed in a strong magnetic field. The lineage-depleted cells were blocked with anti-CD16/32 (clone 2.4G2), and stained with streptavidin-FITC, phycoerythrin (PE)-anti-Ly-6A/E (Sca-1; clone E13-161.7) and allophycocyanin (APC)-anti-CD117 (c-kit; clone2B8). The labeled cells were resuspended into PBS containing 5ug/mL propidium (PI) solution to distinguish viable and dead cells. PI-, Lin-, Sca-1+ and c-kit+ (LSK) cells were sorted on a Becton Dickinson FACS Vantage flow cytometer.

2. *Purification of hematopoietic progenitor-enriched cell population*

The entire procedure was similar to LSK cell sorting except that at the final step the blocked lineage-depleted cells were stained only with streptavidin-FITC and PI. The cell suspensions were subsequently sorted for the Lin- population, in which almost all mature blood cells were eliminated and progenitor cells were enriched.

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3. Preparation of peripheral blood and bone marrow nucleated cells

Mice were bled from the retro-orbital venous plexus and bone marrow cells from 1 femur per mouse were flushed into medium containing Hank's balanced salt solution (HBSS, Gibco, Grand Island, NY) with 2% heat inactivated fetal calf serum (FCS, Life Technologies). Erythrocytes in peripheral blood and bone marrow cell suspensions were eliminated by hypototic lysis. The remaining cells were washed with medium twice and cells were counted by cell coulter. A precised number of cells were used for analyses.

Total RNA isolation

Total RNA was extracted from a prescribed number of cells by using RNeasy® Kit (QIAGEN, Valencia, CA). Briefly, cells were centrifuged to obtain a cell pellet, which was disrupted with guanidine isothiocyanate - containing buffer (Buffer RLT) with β-mercapitoethanol and homogenized by vortexing for 1 minute (homogenized cell lysate could be frozen in -80°C and used later). An equal volume of 70% ethanol was added to adjust conditions suitable for the binding of RNA to the membrane. Samples were applied to an RNase spin column for adsorption of RNA to membrane. The contaminants were removed with wash spins (Buffer RW1 and RPE provided by kit). The total RNA was then eluted into RNase-free water and quantified by spectrophotometric analysis. The extracted total RNA was used for either microarray analysis on the Mouse Genome U74Av2 chip or quantitative real-time PCR analysis.

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Microarray analysis of LSK cells

Oligonucleotide arrays of LSK cells were performed by the Microarray Facility Center at the University of Kentucky. Briefly, for each of 4 mouse strains, total RNA were extracted from at least 550,000 LSK cells from a minimum of 30 mice in order to obtain sufficient RNA for probing Affychips. The extracted RNA was then reverse transcribed into cDNA, which was subsequently used to generate biotin-labeled cRNA. Fragmentation and hybridization of labeled cRNA to the Mouse Genome U74Av2 chips (MGUAv2, Affymetrix, Santa Clara, CA) were then performed. The genechips were then washed and stained and subsequently scanned for quantification of gene expression. Three independent biological samples were obtained for each strain and each was run on an individual chip. The gene expression levels were compared among all 4 strains by using one way-ANOVA with a statistical cutoff of $p<0.05$. The genes that were differentially expressed were screened for genomic location and function of the gene products.

Quantitative real-time PCR

Identical numbers (200,000) of cells were used for total RNA extraction in each type of cell population (peripheral blood, bone marrow, Lin- and LSK cells) isolated from each mouse strain. Isolated total RNA was reverse transcribed into cDNA using random hexamers in a TaqMan® reverse transcription solution (PN N8080234) and stored at -20ºC. Quantitative real-time PCR analyses of selected genes were performed in single reporter assays with an ABI PRISM 7700

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sequence detection system (PE Biosystems, Foster city, CA, USA). PCR reactions were set up according to manufacturer's instructions using TaqMan® universal PCR master mix (PN 4304437), and the primer and probe mixes for each tested gene were purchased from Applied Biosystems (Foster city, CA, USA). TaqMan® rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH (PN 4308313) served as an endogenous control to normalize gene expression. The analysis of gene expression was performed by the relative standard curve method. In brief, standard curves were prepared for the GAPDH control and for each of tested genes, and the amount of GAPDH control and test gene were determined from the standard curve. A normalized test gene value was determined relative to GAPDH expression. Each biological sample was obtained from at least 5 mice, and each sample was run with at least 5 replicates within an experiment. Three replicate biological experiments were performed in each strain.

Immunoblots

Cell samples were lysed at a concentration of $2x10^7$ cells/ml in protein lysis buffer containing: 10mM tris pH7.5, 50mM NaCl, 30mM sodium pyrophosphate, 50mM NaF, 5μM $ZnCl_2$ and 1 % Triton X-100, 2.8ug/ml aprotinin (Sigma), 1mM phenylmethylsulfonyl fluoride (Sigma), 1mM sodium vanadate (Na_3VO_4) 1ug/ml pepstatin, and 1μg/ml leupeptin (Oncogene Research, MA, USA). Lysate was incubated on ice for 30min, and then centrifuged at 15,000 x g for 10 minutes to remove debris. The resulting supernatant was then aliquoted

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and stored at -80°C. For immunoblot analysis, protein lysates were thawed and mixed with running buffer and a reducing agent (Novex, San Diego, CA, USA, per manufacturer's instruction) and heated at 95°C for 5 minutes. Samples were then analyzed by denaturing PAGE (Novex, 10% Bis-Tris gel) using the equivalent of $4x10^5$ cells per lane. Following electrophoresis, samples were electro-transferred onto immunobilon-P membranes (Millipore, Bedford, MA, USA), which were subsequently blocked and probed with polyclonal rabbit anti-latexin Ig-G antibody at a 1:3000 dilution. This polyclonal rabbit anti-latexin Ig-G antibody was generated against an amino acid sequence of mouse latexin protein that I selected, CKHNSRLPKEGQAE, and was produced by Bethyl Laboratories, Inc (Montgomery, TX). Primary antibodies were detected using alkaline phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology) and electro-chemifluorescent (ECF) reagent (Pharmacia Biotech) according to the manufacturer's instructions. Blots were visualized using a Molecular Dynamics STORM 860 system and Imagequant Software. Following the detection and quantification of anti-latexin antibody, immunobilon-P membrane was sequentially stripped in 40% methanol and buffer containing 100mm ß-mercaptoethanol, 2% sodium dodecyl sulpahate and 62.4mM Tris-HCL to remove ECF reaction product and antibodies, respectively. The stripped membrane was reprobed with anti-actin antibody (Sigma) at 1:500,000 dilution and detected as previously described.

Retroviral vectors

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Retroviral vector, Sfbeta 91 (a gift from Dr. Hartmut Geiger, Cincinnatti Children's Hospital), served as both control and the backbone for cloning of latexin (Lxn) cDNA. It contained the 5'-long terminal repeat (LTR) derived from myeloproliferative sarcoma virus (MPSV) and a 3'-LTR that is derived from spleen focus forming virus (SFFV). The internal ribosomal entry site (IRES) sequence derived from the encephalomyocarditis virus was used for simultaneous translation of gene insert and the gene for enhanced green fluorescent protein (GFP). The latexin cDNA sequence was cloned upstream of the IRES of the Sfbeta91 vector (MPSV-IRES-GFP-SFFV) to create a recombinant latexin-carrying vector (MPSV-Lxn-IRES-GFP-SFFV). Production of high-titer helper-free retrovirus was carried out by standard procedure in ectotropic Phoenix packaging cells.

Infection of primary murine bone marrow cells

Primary mouse bone marrow cells were transduced as previously described with modifications specified below (Sauvageau et al. 1995). Briefly, bone marrow cells were extracted from mice treated 4 days previously with 150 mg/kg body weight 5-fluorouracil (5-FU) (Sigma, St. Louis, MO) and cultured for 24 hours in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 50 ng/mL recombinant mouse stem cell factor (mSCF), 10ng/mL mouse interleukin 6 (mIL-6), and 10ng/mL mouse interleukin 3 (mIL-3) (R&D Systems, Minneapolis, MN). Media and serum were purchased from Gibco Technologies (Carlsbad, CA). The cells were then

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harvested and slowly spread on the top surface of the membrane of a Transwell insert (Corning Incorporated Life Sciences, Acton, MA) at a density of $2x10^6$ cells per well. The viral supernatants were added to the Transwell inserts along with 4 µg/ml of polybrene to enhance adherence of viral particle to cells. The membrane provided a platform to enhance infection of cells by viral particles (Liu et al. 2000a). The cells were co-cultured with viral supernatants for a further 48 hours and the viral supernatant was changed 3 times during this period of time. The recovered cells were then transplanted into sub-lethally irradiated (600 Rad) recipient mice at a dose of at least $1x10^6$ cells per mouse. Around 12 weeks after transplantation, retrovirally-transduced, i.e. GFP positive, bone marrow cells of the recipients were flow cytometrically sorted using a FACSVantage (Becton-Dickinson) and subject to functional analysis of HSCs (CAFC and CRU assays).

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Results

Identification of differentially-expressed gene in chromosome 3 congenic HSCs by microarray analysis

We have found that a quantitative trait locus (QTL) on mouse chromosome 3 affects the number of bone marrow HSCs as well as their cycling, apoptotic and self-renewal states. In order to identify the quantitative trait gene responsible for these effects, a candidate gene approach was pursued as indicated in the upper path of the schema in Figure 1.2. To this end, gene expression levels of LSK stem cells from all 4 mice strains (B.D Chr3 and D.B Chr3 reciprocal congenics and their respective background B6 and D2 strains) were measured by microarray analysis using Affymetrix chips (Mouse Genome U74Av2 set). The genes that were differentially expressed were screened according to a statistical cutoff of $p<0.05$ when one-way ANOVA analysis was performed. Ninety six of the ~12,000 genes on the Affychip were differentially expressed, either up- or down-regulated, in the congenic B.D Chr3 LSK cells relative to B6 background cells. Comparable comparison of D.B Chr3 reciprocal congenic and its D2 background strains showed 84 differentially expressed genes. However, only 17 genes overlapped in these two comparison groups and 3 of them were located on chromosome 3 (Table 4.1), with the rest scattered throughout the genome. These 3 genes were latexin, S100a6 and S100a11, whose genomic locations, relative to the congenic interval and QTL marker, are shown in Figure 4.1. The overlapped congenic interval in B.D Chr3 and D.B Chr3

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mice ranges from 19cM to 33cM, and the marker of highest linkage to the trait (HSC number) is D3Mit5 at 25cM. As indicated in the figure, the only gene located in the overlapping region is latexin at the genomic position of 31.6cM, whereas the other two are 20cM telomeric to D3Mit5. Although latexin apparently lies almost 6.6cM downstream from D3mit5 and outside the 95% confidence limits of the mapping statistics, the results suggested that latexin was the cis-regulated quantitative gene responsible for the stem cell frequency trait under study (Bystrykh et al. 2005). The lack of gene and genetic markers (single nucleotide polymorphisms, SNPs) between D3Mit5 and the start codon for latexin suggests that the distance may be considerably less in operational terms, although it is also possible that an important latexin regulatory site may exist such a long distance upstream (Cowles et al. 2002). Both scenarios are possible and have precedents. Moreover, the RNA expression levels of latexin in LSK cells from all 4 mouse strains showed that latexin was highly expressed in D.B Chr3 and B6 LSK cells compared to B.D Chr3 and D2 cells, indicating that B6 alleles may up-regulate whereas D2 alleles down-regulate latexin expression. Considering the phenotypic differences in chromosome 3 congenic and background mice, it is therefore hypothesized that B6 alleles might be responsible for the higher expression of latexin which subsequently results in decreased HSC number, whereas D2 alleles decrease latexin expression and thereby increase HSC number.

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Latexin expression in hematopoietic cells at different differentiation stages

Latexin, a candidate gene for regulating HSC numbers, was first identified in neurons of the lateral neocortex of rat with immunohistochemistry employing a monoclonal antibody PC3.1 in 1992 (Arimatsu et al. 1992). It is also detected in non-neural tissues in rat and human, including lung, spleen, kidney, heart, digestive tracts, ovary and prostate. (Hatanaka et al. 1994; Liu et al. 2000b) Further studies have shown that its protein product, a 29kD molecule with 222-amino-acid residues, contains two potential Ca2+ /calmodulin-dependent protein kinase sites and one cGMP-dependent protein kinase phosphorylation site (Callahan 1999; Takiguchi-Hayashi and Arimatsu 1995). Although the functions of latexin haven't been well studied, Normant et al. showed that it functioned as a carboxypeptidase A inhibitor and was involved in protein degradation (Normant et al. 1995). Studies from Takiguchi-Hayashi et al. also suggested that it might play a role in cortical regional specification of the brain (Takiguchi-Hayashi and Arimatsu 1995). All of the above results suggest that latexin is mainly expressed in differentiated cells at least in the nervous system.

Since my microarray data for the first time showed that HSCs express latexin, I further investigated whether hematopoietic cells at other differentiation stages also expressed the gene. To this end, quantitative real-time PCR was performed on populations of peripheral blood nucleated cells (PB), bone marrow nucleated cells (BM), progenitor-enriched Lin- cells, and LSK HSCs. As shown in Figure 4.2, a very low level of latexin was expressed in mature blood cells and its expression gradually increased with the enrichment of HSCs, reaching the

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highest level in primitive progenitor and stem cell populations. Moreover, the data for LSK cells also confirm, both qualitatively and quantitatively, the results obtained with microarrays. Most interestingly, cells that had B6 alleles had significantly higher levels of latexin transcripts than cells containing D2 alleles in each cell type. Therefore, these results indicate that latexin is specifically expressed in bone marrow primitive hematopoietic cells, and high expression is associated with B6 alleles that have been shown to contribute to a smaller HSC population size. If latexin functions as a carboxypeptidase inhibitor in HSC population size regulation, it may imply that protein target of carboxypeptidase may be associated with increasing HSC cycling, decreasing HSC apoptosis, or both.

Latexin is differentially expressed at the protein level in congenic cells

Differential mRNA expression patterns of latexin have been demonstrated by both microarray and real-time PCR. These differences are also associated with the strain-specific genotypes (B6 vs D2 alleles) and phenotypes (low vs high number of HSCs). We next measured latexin protein expression by Western blotting. Mouse anti-latexin antibody is not commercially available, polyclonal rabbit anti-latexin Ig-G antibody was therefore specifically produced against an amino acid sequence of mouse latexin protein that I specified: CKHNSRLPKEGQAE. To test the validity and specificity of this antibody, we performed Western blot using this antibody on B6 BM nucleated cells as well as on human cord blood, peripheral blood cells and adult BM cells from subject of

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different ages. The results (Figure 4.3) showed that a 29 kD protein, which is consistent with the size of latexin protein reported previously (Arimatsu et al. 1992; Hatanaka et al. 1994), was detected in mouse BM cells by this antibody. However, this antibody failed to demonstrate the presence of latexin protein in human-derived biological samples. These results suggest that the antibody specifically bound to mouse latexin protein without cross-reacting with the human species.

In order to test the hypothesis whether or not the differential expression pattern of latexin transcripts is reflected at the protein level, Western blots of two populations of cells were performed: bone marrow (BM) and Lin- cells. Because LSK stem cells are so rare, insufficient numbers could be obtained to perform this analysis on this population. As shown in figure 4.4, latexin protein expression is much higher in Lin- cells than in BM cells. Moreover, consistent with the microarray and real-time PCR results, both D.B Chr3 and B6 cells in each population have a higher content of latexin protein compared to the reciprocal B.D Chr3 and D2 cells. Therefore, the differential expression patterns of latexin among all 4 stains are identical at both mRNA and protein level. It is reasonable to speculate that this expression pattern for latexin protein might also exist in LSK stem cell populations, albeit at even higher expression level.

Model of latexin overexpression in bone marrow cells

Since I have shown that D2 alleles on chromosome 3 QTL are associated with downregulated expression of latexin as well as increased HSC number

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(CAFC day35), I next investigated the effects of overexpressing this gene on HSC functions. To this end, recombinant retroviral vectors containing latexin cDNA were constructed and transduced into D2 and B.D Chr3 congenic bone marrow cells. Isolated cells were subsequently transplanted into sublethally irradiated recipient mice of the same genetic background, and the GFP+ cells will be selected at least 12 weeks post-transplant. At the time of dissertation writing, these data haven't been available yet. It is hypothesized that overexpression of latexin will lead to the decreased HSC numbers in D2 and B.D Chr3 congenic mice. In other words, overexpression of latexin may confer a B6-like phenotype to D2 HSCs.

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Discussion

It has been very difficult to identify quantitative trait (QT) genes underlying QTL that are linked to quantitative traits, especially those related to steady-state hematopoiesis (Biola et al. 2003; Darvasi and Pisante-Shalom 2002; Korstanje and Paigen 2002). The prospects for successful gene discovery have improved markedly, however, with the recent development of gene array technology and the sequencing of mouse strain genomes (Glazier et al. 2002). In my studies, I took advantage of the former technology and have been the first demonstrate that the latexin gene, located in the chromosome 3 QTL region, was differentially expressed in C57BL/6 (B6), DBA/2 (D2) and Chr3 congenic HSCs. The differential expression pattern was further confirmed by quantitative real-time PCR and by Western blot analyses. Moreover, down-regulated latexin expression is associated increased HSC numbers, and vice versa. Retroviral gene transfer and bone marrow transplantation results will provide further evidence for the potential roles of this gene in the hematopoietic system.

Compared to previous forward genetic studies of hematopoietic cells, several novel features have been added in my studies. First, we used unique congenic mice, whose genome differs only in the genomic region of the chromosome 3 QTL from background strain mice. Since the mutually inclusive interval in the reciprocal congenics is 14cM (19~33cM, figure 4.1), this represents only around 1% of the entire mouse genome assuming the total length of mouse genome is 1300cM. This 1% genomic difference greatly reduces the number of

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polymorphic loci between congenic and background mice. The microarray results underscored this. Only 90 genes were differentially expressed ($p<0.05$) between congenic and background LSK HSCs whereas 940 genes were differentially expressed between D2 and B6 cells. A second feature of the microarray approach was that I used two reciprocal congenic strains. A comparison of differential gene expression between strains with overlapping congenic intervals provided not only corroborating evidence for a particular candidate, for example if it were up-regulated in one congenic and down-regulated in the reciprocal, but it provided evidence and identities of modifying genes in the respective genetic backgrounds (Nadeau 2001; Nadeau 2003). Thirdly, unlike most studies, cDNA amplification was not used. Due to the limited numbers of HSCs in BM, most, if not all, other stem cell studies using gene arrays were performed by hybridizing amplified RNA samples onto gene chips (Bystrykh et al. 2005; Ivanova et al. 2002; Ramalho-Santos et al. 2002). Amplification may cause representational skewing of low abundance RNA species. In order to avoid this problem, we made great efforts to obtain sufficient LSK cells and RNA for probing Affychips without amplification. Usually more than 550,000 LSK from a minimum of 30 mice of each strain were needed for a single chip. Moreover, three independent biological samples were obtained for each strain and each was run on an individual chip. Thus, our gene expression profiles of LSK cells are more reliable and the results of differentially expressed genes are more convincing. Most importantly, the microarray results reveal the relationship of differential

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expression of a single candidate gene, latexin, to the HSC number changes (Wayne and McIntyre 2002).

Validation of the candidate gene, latexin, identified by microarray was accomplished by quantitative real-time PCR. This method is substantially more quantitative than microarrays and reliably detects small expression differences (Bustin 2000; Chuaqui et al. 2002). Consistent with the microarray data, the quantification of latexin in LSK stem cells demonstrated that B6 alleles on chromosome 3 QTL increased whereas D2 alleles decreased latexin expression by around 2-fold. These results are in quantitative agreement with my microarray results. Differential expression patterns were also reproduced at the protein level, indicating that latexin is mechanistically involved in our quantitative trait loci and trait. However, its location 6.6cM telomeric to the markers of highest linkage to our trait, presents problems in interpreting these results. Perhaps the simplest explanation is that too few BXD strains, or a lack of microsatellite markers used for linkage analysis, led to discrepant mapping. However, two recent studies identifying the regulatory QTL that affect gene expression in HSCs and brain cells demonstrated that most of genes were regulated through cis-acting QTL that mapped within 10cM of the gene itself. A regulatory site may therefore exist a long distance upstream of latexin start codon that is involved in the latexin expression (Bystrykh et al. 2005; Chesler et al. 2005).

In order to add to the veracity of latexin as a candidate, we used recombinant retrovirus containing latexin cDNA and overexpressed latexin in D2 and B.D Chr3 congenic bone marrow cells. Although data in these studies

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haven't been available at the time of dissertation writing, and further studies, such as knockdown latexin in B6-derived cells using small interfering RNAs and in vivo functional assay of latexin-trasnduced cells, are needed, our current findings demonstrate the reverse relationship between latexin expression and hematopoietic cell population size. These findings also suggest the potential role of latexin in the regulation of HSC numbers.

I next asked what mechanisms may contribute to the differential expression of latexin in different mouse strains. The level of a given mRNA transcript is controlled both by cis-acting factors (such as DNA polymorphisms and methylation) in the regulatory DNA sequence of the gene and trans-acting modulators (such as transcription factors) that are themselves regulated by other genetic and environment factors (Cheung and Spielman 2002; Hubner et al. 2005; Pastinen and Hudson 2004)). Cis-regulatory polymorphisms are DNA elements and are distributed over long distances upstream and downstream of a gene, but on the same linkage group. Introns as well as the 5' and 3' untranslated regions have also been shown to harbor regulatory elements. Therefore, I hypothesized that the cis-acting variations might explain the different expression level of latexin transcript in B6 and D2 HSCs. Celera Discovery System (CDS) (http: www.celeradiscoverysystem.com) is a commercially available database in which single nucleotide polymorphisms (SNPs) of a given gene have been detected among different mouse strains and species. I thus searched for SNPs in and around the latexin open reading frame in B6 and D2 mouse stains. Only 4 SNPs were shown in both strains and all of them were

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located in intronic regions. It is possible that some of these SNPs may modify latexin expression, but more experimental evidence is needed to test their effects, such as site-directed mutagenesis (Mackay 2001). Moreover, more SNPs may also be discovered with additional repeated coverage and scrutiny of longer distance of upstream flanking regions (Brent 2000).

As for the regulatory roles of trans-acting factors in the differential expression of latexin, another useful database (www.WebQTL.org) was used (Chesler et al. 2004; Wang et al. 2003). WebQTL is a database of quantitative gene expression in LSK stem cells of all of the BXD strains currently available, and an important feature is that it is linked to a mapping program. Thus one can map the location of genes or QTL that modify the expression of other LSK genes (Bystrykh et al. 2005; Chesler et al. 2005). When modifiers of latexin expression were queried using such an approach, highly suggestive linkage (LRS=11) was obtained to two markers at 29.5cM and 33cM, which are very close to genomic locations of D3Mit5 (25cM) and latexin (31.6cM) (Figure 4.6). Moreover, the negative additive effects for both markers suggest that the B6 allele increases the trait values, i.e., latexin expression, which is precisely consistent with our results from microarray, real-time PCR, and Western blot analyses. Therefore, these results not only suggest existence of regulatory factor in the upstream and downstream region of the latexin sequence, but also corroborate D3Mit5 as a marker, presumably the primary one, for a QTL directly or indirectly responsible for the phenotypes conferred by the chromosome 3 QTL.

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Latexin is expressed in specific neurons in both the central and peripheral nervous system in the rat. It is mainly used as a molecular marker for the regional specification of the neocortex (Arimatsu et al. 1999a; Arimatsu et al. 1999b). Contrary to the expression pattern in the nervous system, we found that latexin was highly expressed in more primitive hematopoietic cells (Lin- progenitor and LSK stem cells) rather than differentiated blood cells. Nevertheless, the fact that latexin is expressed in both hematopoietic and neuropoietic cells is in line with the recent demonstration of the overlapping gene expression profiles between these two systems (Goolsby et al. 2003; Li et al. 2004; Steidl et al. 2004; Terskikh et al. 2001). More and more genes that are primarily assigned to the nervous system have been found in primitive hematopoietic cells (HSCs and HPCs) in both mice and humans. Recently, two comprehensive studies from the Melton and Lemischka laboratories detected gene expression profiles in embryonic stem cells (ESCs), neural stem cells (NSCs) and HSCs by using microarray techniques (Ivanova et al. 2002; Ramalho-Santos et al. 2002). 432 genes were found to be commonly expressed in NSCs and HSCs in the former study and 644 genes in the latter. Latexin, however, is not included in both gene lists. To our knowledge, the only evidence for the existence of latexin in NSCs comes from the supplementary database published online in the Melton study (Ramalho-Santos et al. 2002). According to the concept of adult stem cell plasticity and the fact of genetic overlapping between NSCs and HSCs (Terskikh et al. 2001; Wagers and Weissman 2004), our results showing expression of latexin as well as its potential regulatory roles

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in HSCs may also apply to NSCs. Studies aimed at addressing these questions are currently being performed in collaboration with the lab of Dr. James Geddes and the results could provide potential implications in areas of transplantation and tissue regeneration.

Although we provide strong evidence that the potential association of latexin with the regulation of HSC numbers, very little information is available to help us to understand the underlying molecular mechanisms. Latexin has been used to elucidate the mechanism of cortical regional specification and modulation of sensory perception in the nervous system (Arimatsu et al. 1999a). Studies by Normant et al. demonstrated that latexin had carboxypepsidase A inhibitor activity (Normant et al. 1995). Recent findings concerning latexin expression in rat mast cells and mouse macrophages indicated it might also be involved in inflammation. Moreover, studies of the latexin protein structure showed that latexin protein had a similar fold architecture as cystatin (cysteine proteases inhibitor) and had a motif similar to TIG1 (human putative tumor suppressor protein), indicating other potential roles of latexin (Aagaard et al. 2005). Studies by Callahan et al. revealed that the absence of a candidate gene, whose protein product had 85% identity of the mouse latexin protein, was associated with increased incidence of ovarian cancer. In addition, induced expression of this gene by 5-aza-2'-deoxycytidine implicated methylation as one of the mechanisms for its inactivation. Latexin was also found to contain two potential Ca2+/calmodulin-dependent protein kinase site, one c-GMP-dependent protein kinase phosphorylation site, and three glycosylation sites (Callahan 1999).

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Therefore, G protein-coupled receptor and/or calcium/calmodulin singnaling pathways might also be involved in the latexin-mediated regulation of HSCs. This hypothesis is supported to a certain extent by our observations of differential expression of S100a6 and S100a11 (Table 4.1), a cluster of calcium binding protein genes, between both sets of congenics and their respective background strains. Therefore, we propose that genes, or gene clusters, at these several locations in and around the chromosome 3 congenic interval may have interrelated roles in stem cell function.

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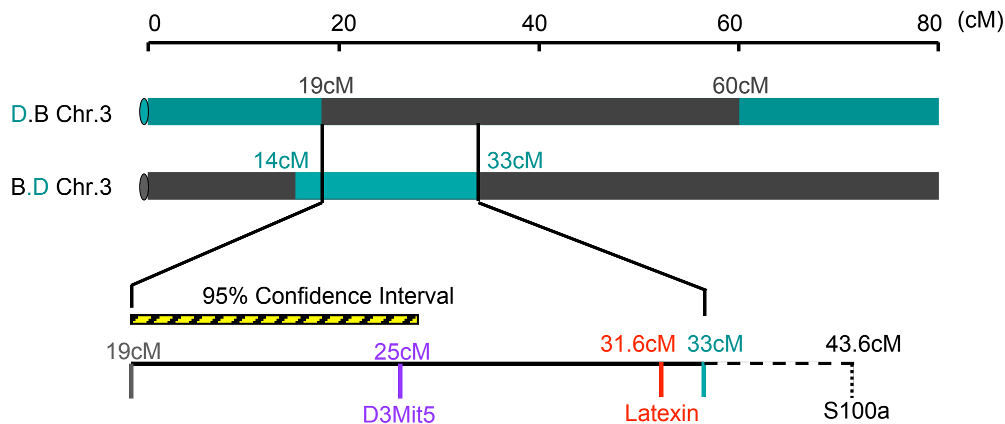

Figure 4.1 Schematic illustration of chromosome 3 congenic map and genomic location of candidate quantitative genes. D.B Chr3 congenic mice contain a B6-derived genomic interval from 19cM to 60cM, while the reciprocal B.D Chr3 mice have a D2-derived interval between 14cM and 33cM. The consensus congenic region between both strains is from 19cM to 33cM on chromosome 3 (80cM of total length), which contains the marker of tightest linkage to the trait, D3Mit5, at 25cM. 3 out of the 17 genes, which are differentially expressed among Chr 3 congenic and their respective background strains, are located in chromosome 3. One of them is latexin, which is in the consensus congenic interval at 31.6cM. The other two are members of S100a family, S100a6 and S100a11, which are in D.B Chr3 congenic region, but not in B.D Chr3 interval. Latexin is the only one of the genes to be up-regulated in one congenic strain (D.B Chr3) and down-regulated in the reciprocal congenic strain (B.D Chr3)

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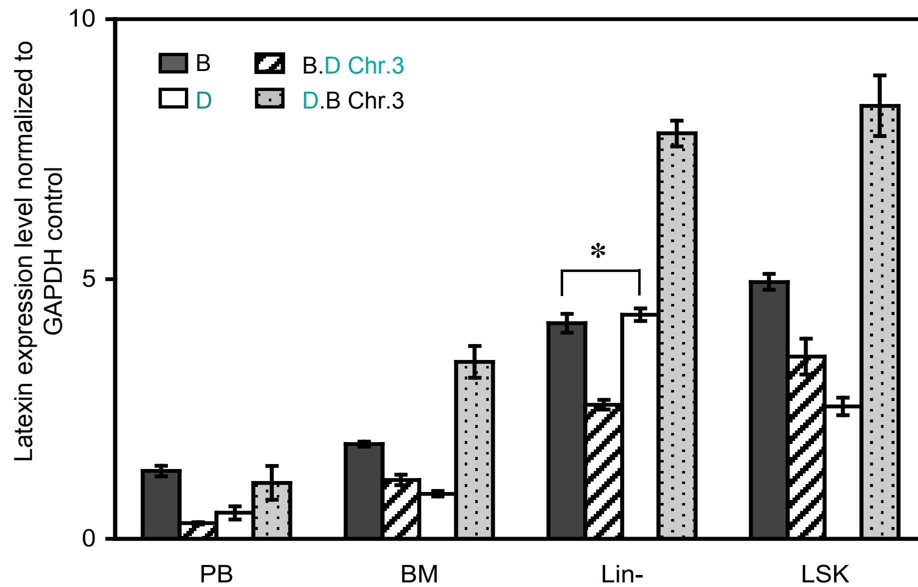

Figure 4.2 Development-dependent differential expression of latexin mRNA in chromosome 3 congenic mice. Identical numbers (200,000) of peripheral blood (PB), bone marrow (BM), lineage-depleted, progenitor-enriched bone marrow cells (Lin-), and LSK stem cells were obtained from chromosome 3 congenic (B.D Chr3 and D.B Chr3) and their respective background mice (B6 designated as B and D2 as D). Quantitative real-time PCR was performed to measure latexin mRNA expression in these cell populations. The latexin expression level represented in Y axis is the mean value (±SEM) of 3 separate experiments performed on 3 independent biological samples. With one exception (B and D Lin- population as signed *), all B-derived alleles significantly ($p<0.05$) up-regulate latexin expression whereas D alleles down-regulate latexin expression.

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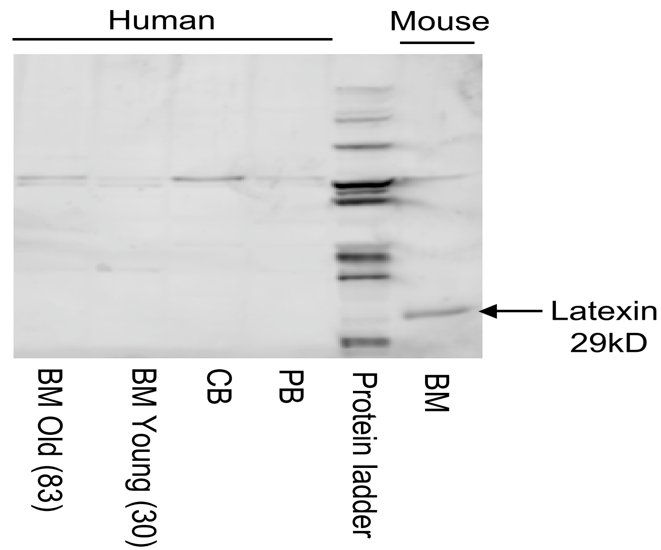

Figure 4.3 Latexin protein expression in mouse and human cells. Western blot was performed using mouse bone marrow (BM) cells and human peripheral blood (PB), cord blood (CB) and BM (young and old adults, 30 and 83 years old respectively) cells as described in Methods. Blots were probed with polyclonal rabbit anti-mouse latexin Ig-G antibody. Latexin protein was detected as a 29kD molecule in mouse BM cells.

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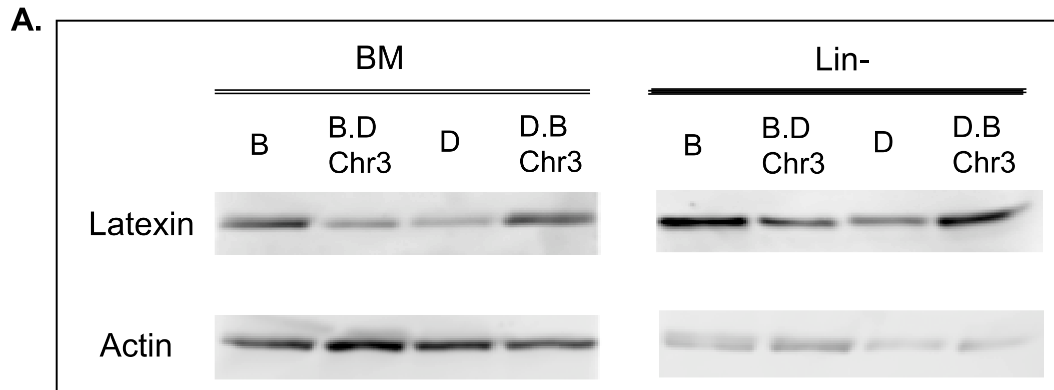

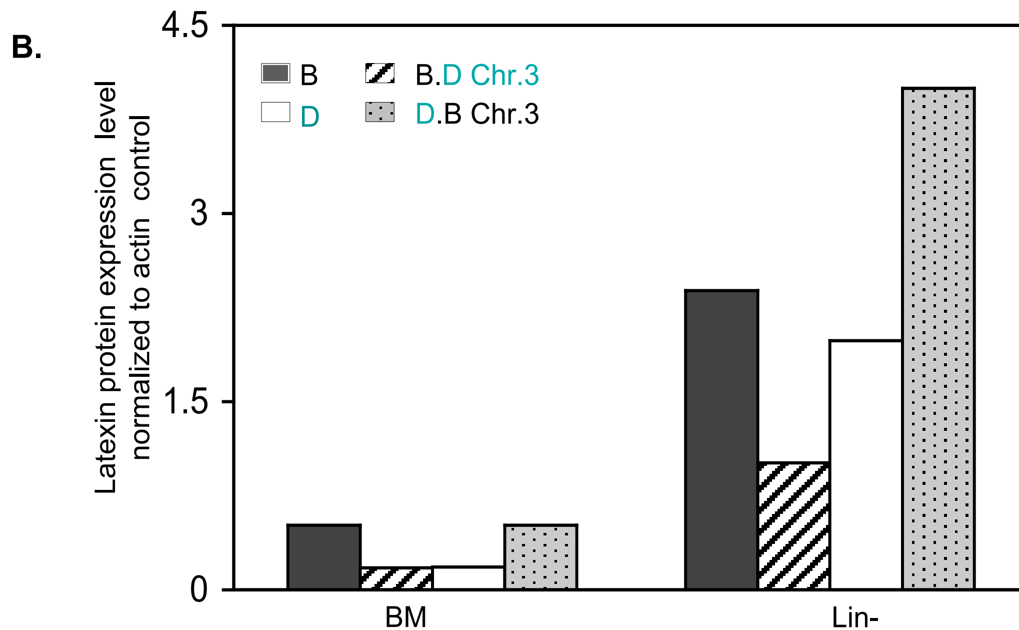

Figure 4.4 Development-dependent differential expression of latexin protein in chromosome 3 congenic mice. Western blot was performed to quantify latexin protein level in bone marrow (BM) nucleated cells, lineage-depleted and progenitor-enriched bone marrow cells (Lin-) from chromosome 3 congenic (B.D Chr3 and D.B Chr3) and their respective background mice (B6 designated as B and D2 as D). Panel A is a representative Western blot profile of latexin protein;

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actin was used as the control. In each cell population and each strain, cell lysate derived from equal numbers of cells were probed with polyclonal rabbit anti-mouse latexin antibody and detected as described in Methods. Panel B shows corresponding quantification of latexin protein level normalized to control actin level. Two separate experiments were performed with 2 independent biological samples.

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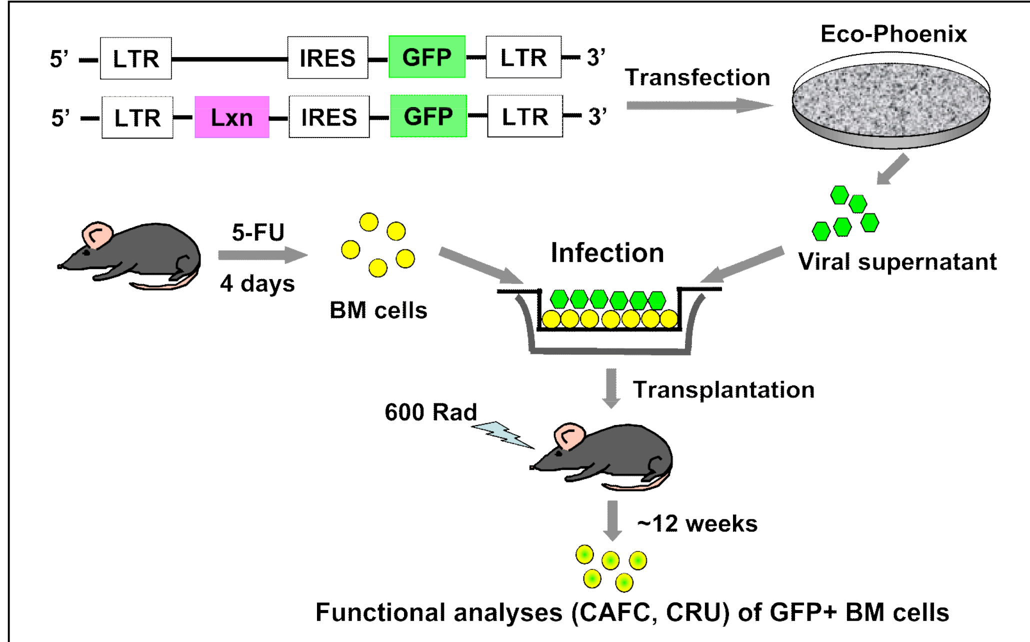

Figure 4.5 Experimental design for latexin overexpression model. The Sfbeta 91 recombinant retroviral vector was used as control and backbone to construct latexin (Lxn) cDNA-containing vector. High-titer viral supernatant was generated in the ectotropic Phoenix (Eco-Phoenix) packaging cell line as described in Methods. Bone marrow cells were obtained from D2 mice that had 4 days previously received an intravenous injection of 150mg/kg body weight of 5-fluorouracil (5-FU) and co-cultured with viral supernatant in a Transwell insert for 2 days. The transduced cells were recovered and transplanted into sub-lethally irradiated (600Rad) D2 recipient mice. Around 12 weeks after transplantation, transduced cells (GFP+) were selected for functional analyses of HSCs (CAFC and CRU assays)

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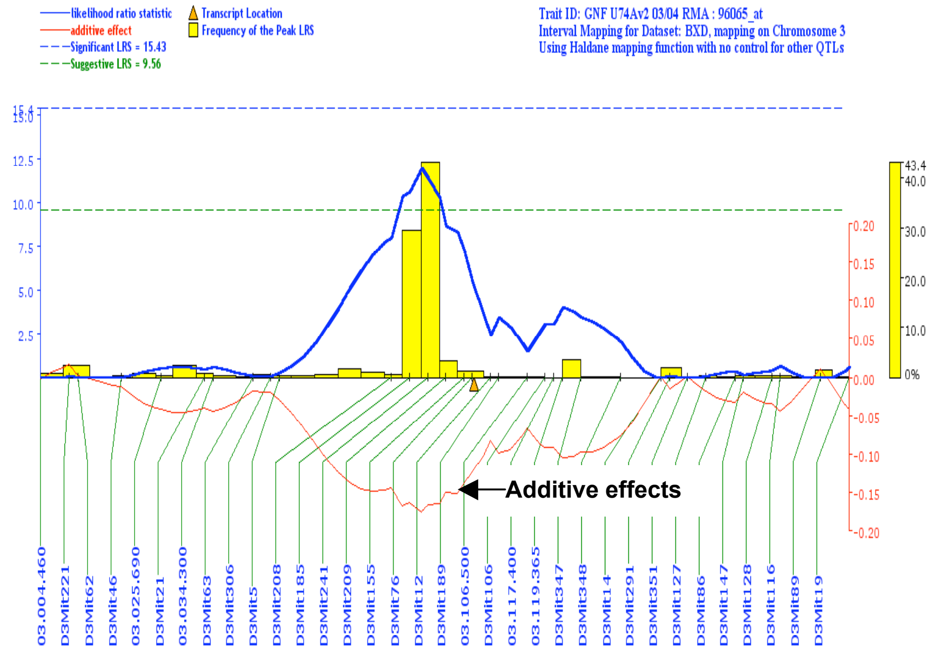

Figure 4.6 Identification of QTL regulating latexin expression. WebQTL was used to identify loci that regulate latexin expression in LSK stem cells. This database is based on the gene expression profile of LSK cells in all BXD recombinant strains and linked to mapping program. Two QTL on chromosome 3 were linked to latexin expression with a suggestive significance, D3Mit185 (29.5cM) and D3Mit241 (33cM). The additive values, as indicated in the figure, for both markers are negative, indicating that the B allele increases the trait whereas the D allele decreases the trait.

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Table 4.1 Differentially expressed genes in both chromosome 3 congenic and their respective background mice.

Gene symbol	Gene title	Location	Functional annotation
Fcer1a	Fc receptor, IgE, high affinity I, alpha polypeptide	Chr1, 94.2cM	Signal transduction
Gfi1b	Growth factor independent 1B	Chr2, 28.9Mb	ATP binding
Lxn	Latexin	Chr3, 31.6cM	
S100a6	S100 calcium binding protein A6 (calcyclin)	Chr3, 43.6cM	Calcium ion binding Growth factor activity Regulation of cell cycle and cell proliferation
S100a11	S100 calcium binding protein A11(calizzarin)	Chr3, 93.7Mb	Cytokine activity Calcium ion binding
C730036B01Rik	RIKEN cDNA C730036B01 gene	Chr3, 121Mb	Not available
Gtf3a	General transcription factor IIIA	Chr5, 144Mb	DNA binding

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2610507 L03Rik	RIKEN cDNA 2610507L03 gene	Chr7 23.8Mb	Not available
9130011 J15Rik	RIKEN cDNA 9130011J15 gene	Chr8, 71.9Mb	Not available
Aplp2	Amyloid beta (A4) precursor-like protein 2	Chr9, 13.0cM	Endopeptidase inhibitor activity Serine protease inhibitor activity DNA binding
Pttg1	Pituitary tumor-transforming 1	Chr11, 29.4Mb	Cell growth and/or maintenance DNA repair Chromosome segregation Mitosis
Mybbp1 a	MYB binding protein (P160) 1a	Chr11, 40.0cM	
Nptx1	Neuronal pentraxin	Chr11, 75cM	
Mapk8	Mitogen activated protein kinase 8	Chr14, 29.4Mb	Signal transduction
Tuba1	Tubulin, alpha1	Chr15, 60.4cM	Microtubule-based movement and process
Rasgrp2	RAS,guanyl releasing protein 2	Chr19, 6.1Mb	Calcium ion binding kinase activity Guanyl-nucleotide exchange facor activity

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			Small GTPase mediated signal transduction
0710001 O03Rik	RIKEN cDNA 0710001O03 gene	Chr19, 9.5Mb	Not available

The genes are listed in the sequence of chromosome location. Some locations are represented as centimorgan (cM), others are megabases (Mb). 1cM is theoretically equal to 2Mb.

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CHAPTER FIVE

Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells

Summary

To test the hypothesis that aging has negative effects on stem cell homing and engraftment, young or old C57BL/6 BM cells were injected, using a limiting-dilution, competitive transplant method, into old or young Ly5 congenic mice. Numbers of hematopoietic stem cells (HSCs) and progenitor cells (HPCs) recovered from BM or spleen were measured and compared to the numbers initially transplanted. Although the frequency of marrow competitive repopulation units (CRU) increased ~2-fold from 2 months to 2 years of age, the BM homing efficiency of old CRU was ~3-fold lower than that of young CRU. Surprisingly, the overall size of individual stem cell clones generated in recipients transplanted with a single CRU was not affected by donor age. However, increased age of both HSC donors and transplant recipients caused a marked skewing of the pattern of engraftment toward the myeloid lineage, indicating that both HSC-intrinsic and HSC-extrinsic (microenvironmental) age-related changes favor myelopoiesis. This correlated with changes post-transplant in the rate of recovery of circulating leukocytes, erythrocytes, and platelets. Recovery of the latter was especially blunted in aged recipients. Collectively, these findings have important

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implications for clinical HSC transplantation where older individuals increasingly serve as donors for elderly patients.

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Introduction

Hematopoiesis is maintained throughout life by self-renewing stem cells with a high potential for proliferation and multilineage differentiation (Szilvassy 2003). Accumulating evidence indicates that as animals age, both the number and functional properties of HSCs are altered (Geiger and Van Zant 2002; Van Zant and Liang 2003). However, these effects of aging on stem cells and their BM microenvironment are not clearly defined. For example, marrow from old C57BL/6 mice contains more HSCs (measured by cobblestone area formation, primitive phenotype, and competitive repopulating ability) than BM from young mice (de Haan and Van Zant 1999; Geiger et al. 2001; Henckaerts et al. 2004; Morrison et al. 1996; Sudo et al. 2000). In contrast, the stem cell pool from DBA/2 and all other mouse strains studied contracts in size during aging (de Haan and Van Zant 1999; Harrison et al. 1989). There is compelling evidence that strain-specific variation in this and other stem/progenitor cell parameters is regulated by cell-intrinsic mechanisms, and is affected by several quantitative trait loci (QTL) (Chen et al. 2000; Henckaerts et al. 2002; Henckaerts et al. 2004; Kamminga et al. 2000; Muller-Sieburg and Riblet 1996; Van Zant et al. 1990). In serial transplantation experiments, marrow from old animals was less able to engraft later passage recipients than young BM cells (Ogden and Micklem 1976). Moreover, old HSCs exhibit a differentiation pattern skewed toward the myeloid lineage at the expense of lymphopoiesis (Kim et al. 2003; Sudo et al. 2000). Further evidence of age-related changes in stem cells include the finding that a

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higher proportion of Thy-1^{lo}Sca-1^{+}Lin^{-}Mac-1^{-}CD4^{-}c-kit^{+} cells from old mice are in $S/G_2/M$ phases of the cell cycle (Morrison et al. 1996), and the results of Henckaerts et al. who showed that the proliferative response of Lin^{-}Sca-1^{+}c-kit^{+} marrow cells to the early-acting cytokines KL, Flt3L and TPO, decreased dramatically with age (Henckaerts et al. 2004).

In addition to such quantitative and functional changes of HSCs with aging, the density or activity of several cell surface antigens and membrane transporters that facilitate their identification and isolation fluctuates during ontogeny and throughout adulthood (Ito et al. 2000; Matsuoka et al. 2001; Ogawa 2002). Therefore, functional measurements of HSC properties may actually reflect the effects of aging on this important population. The competitive repopulation assay is currently the most rigorous test for defining HSCs by their capacity for long-term reconstitution of the lympho-hematopoietic system (Harrison 1980; Szilvassy et al. 1990). When combined with a limiting-dilution design, this assay enables measurement of HSC numbers in vivo (Szilvassy et al. 1990). In the present study, graded numbers of “test” BM cells were co-transplanted into myeloablated mice together with competitor BM cells containing an allelic variant of the hematopoietic cell-specific marker, Ly-5 (also known as CD45 and Ptprc). The frequency of HSCs in the test population, measured as competitive repopulating units or CRU, was then determined by applying maximum likelihood analysis and Poisson statistics. (Szilvassy et al. 1990). Because this method also identifies animals engrafted by a single HSC, it has the further advantage of determining the proliferation and differentiation status of HSCs at the individual

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cell level by permitting measurements of clone size and composition. Competitive repopulating advantages of young or old HSCs can then be ascribed to qualitative properties such as higher proliferative state, or to quantitative variation in population size (Szilvassy et al. 2003).

In all experimental and clinical stem cell transplants, the critical first step leading to successful engraftment is homing of stem cells to the BM. Szilvassy et al. previously showed that CRU from fetal liver (FL) and young adult BM had roughly the same seeding efficiency to the marrow of lethally irradiated young recipients when measured 24 hours after intravenous transplantation (Szilvassy et al. 2003). However, Morrison et al. found that old stem cells have only about one-fourth the competitive repopulating activity of young stem cells (Morrison et al. 1996). As more and older patients become candidates for transplantation in the treatment of hematological malignancies and non-hematological diseases, the effects of aging on homing of stem/progenitor cells are clinically relevant, yet remain largely unexplored.

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Materials and Methods

Animals

Female C57BL/6J (B6) mice (Ptprc^b [Ly-5.2]) were used as BM donors, and congenic female B6.SJL(BoyJ) mice (Ptprc^a [Ly-5.1]) were used as recipients. Young mice (6 to 8 weeks) were purchased from Charles River Laboratories (Frederick, MD) via the National Cancer Institute Animal Program. Old mice (22-25 months) were either purchased from Harlan (Indianapolis, IN) via the National Institute on Aging Animal Program, or aged at our own facilities. Mice were maintained under specific pathogen-free conditions in the animal facility of the University of Kentucky Chandler Medical Center.

Homing assay for competitive repopulating units (CRU)

Young or old B6.SJL mice were exposed to 9.0 Gy total body irradiation administered in two doses of 4.5 Gy, ~3 hours apart. Later the same day, 6 irradiated mice (primary recipients) were injected intravenously with 3 x 10^6 B6 BM cells and, 24 hours later, cells from pooled femora and tibiae were harvested into 1.5 ml of medium. Assuming that the 4 long bones represent 25% of the total marrow mass of the mouse, this cell suspension thus contained 1.5 primary BM equivalents (3.0 equivalents analyzed in total for the 2 replicates of this experiment that were performed in Tables 5.1 and 5.2) at a concentration of 1 “homed” BM equivalent/ml. To measure the B6 HSC content of this homed suspension, 4 groups of secondary, irradiated B6.SJL mice (6-8 weeks of age) were injected with graded numbers of primary BM cells (0.5%-12.5% homed BM

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equivalents/mouse), together with 2 x 10^5 B6.SJL competitor BM cells (6-8 weeks of age). The number of CRU in the original B6 cell suspensions (fresh BM cells) was determined by limiting-dilution assays in separate sets of irradiated B6.SJL animals (6-8 weeks of age). Four groups of mice were each injected with 2 x 10^3 to 6 x 10^4 BM cells admixed with 2 x 10^5 B6.SJL competitor BM cells as previously described (Szilvassy et al. 2001a; Szilvassy et al. 2003). Recipients of fresh or homed BM cells were bled from the retro-orbital sinus at 5, 10, 17 and 26 weeks after transplantation. Erythrocytes were depleted from each peripheral blood (PB) sample by hypotonic lysis, and the remaining leukocytes then stained in triplicate with a donor (B6)-specific anti-Ly-5.2 monoclonal antibody (mAb) conjugated with fluorescein isothiocyanate (FITC) (clone ALI4A2; purified from hybridoma supernatant and conjugated in our laboratory) and phycoerythrin (PE)-conjugated mAbs (Becton-Dickinson-PharMingen, San Diego, CA) specific for either B (anti-CD45R/B220; clone RA3-6B2) or T lymphocytes (anti-Thy-1.2; clone 30H12), or granulocytes (anti-Ly6G/Gr-1; clone RB6-8C5) and macrophages (anti-CD11b/Mac-1; clone M1/70). Samples were analyzed using a FACScan instrument (Becton-Dickinson Immunocytometry Systems, San Jose, CA). The frequencies of CRU in the initial B6 BM suspensions, and in the BM of primary mice 24 hours after homing, were calculated from the proportions of negative recipients (in which <5% of the circulating B, T and myeloid cells were regenerated by Ly-5.2$^+$ stem cells) in each cell dose group using L-Calc software (StemCell Technologies Inc., Vancouver, BC). The seeding efficiency of CRU was then calculated by dividing the number of stem cells recovered in the BM of

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primary recipients by the number initially transplanted (x100%). Mice engrafted by single HSCs were retrospectively identified by two criteria: (i) they were determined by limiting-dilution analysis to be transplanted with $\leq$ 0.3 CRU, and (ii), they contained >5% donor (B6)-derived PB cells detectable among B, T, and myeloid lineages (Szilvassy et al. 2003). Although experiments to measure the homing of young CRU to young BM were reported previously in part these were performed contemporaneously with the present studies using animals of varying ages.

Homing assay for HPC

Young or old B6 BM cells were injected into lethally irradiated old or young B6.SJL recipients (1 x 10^7 cells/mouse). The number of clonogenic progenitors in these BM suspensions and in the BM and spleen of transplanted mice 3 hours after homing was determined by plating cells in duplicate 35-mm culture dishes (3 x 10^4 to 1x10^5 cells/dish) containing Methocult medium (StemCell Technologies) as previously described (Szilvassy et al. 2001a). BM and spleen cells from irradiated but untransplanted mice served as negative controls and did not generate any colonies in this assay. The total number of HPCs that had homed to the BM and spleen was calculated by multiplying progenitor cell frequencies by total organ cellularities (in the case of the marrow assuming that 2 femurs and 2 tibiae represent 25% of the total marrow mass of the mouse) (Boggs 1984). The seeding efficiency of HPC was then calculated by dividing progenitor cells that were recovered in the BM or spleen of primary recipients by

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the total number initially transplanted (x100%).

Analysis of lineage-specific engraftment kinetics

Young or old B6.SJL(BoyJ) mice were irradiated as described above and injected intravenously with 2 x 10^6 old, or 3 x 10^6 young nucleated BM cells, respectively. PB was collected from the retro-orbital sinus 6, 9,12, 15, 18, 25, 32, 42, 56, and 120 days after transplantation. Until day 25, only half of the mice in each cohort were analyzed alternately at each time-point so that no individual animal was bled more frequently than every 7 days. Circulating leukocyte, erythrocyte, and platelet counts were measured by analysis of 40 µL blood using a System 9118^+ Hematology Series Cell Counter (Biochem Immunosystems, Allentown, PA).

Statistical analysis

All values represent the mean ± SEM. Statistical differences between means were assessed using the two-tailed *t*-test assuming unequal variances and ANOVA with two factors.

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Results

The homing efficiency of HPCs declines with donor and recipient age

Compared to young adult BM progenitors, ~10-fold fewer fetal liver HPCs homed to the BM in 3 hours after intravenous injection and a similar trend toward reduced BM homing of FL HSCs has been reported (Szilvassy et al. 2001a; Szilvassy et al. 2003). To determine whether aging of the BM also influenced homing, lethally irradiated young (6 to 8 weeks) or old (22 to 25 months) Ly-5.1 mice were transplanted with young or old Ly-5.2 BM cells, and 3 hours later recipient BM and spleen cells were assayed for HPCs. As shown in Figure 5.1, advanced donor or recipient age reduced the homing of HPCs to the BM by about one-third (from ~9% to ~6%, $P<0.01$). The age-related intrinsic (i.e. progenitor-dependent) and extrinsic (i.e. microenvironment-dependent) mechanisms contributing to decreased BM seeding efficiency were similar in magnitude. The effects of age on splenic homing of HPCs were even more dramatic. HPC recovery declined from ~6% for the young into young donor/host combination to ~1% for the young into old, or old into young combinations ($P<0.001$).

The homing efficiency of HSCs declines with donor and recipient age

We hypothesized that aging had similar effects on the homing of more primitive HSCs that regenerate and maintain lymphohematopoiesis for up to 6 months after transplantation. To address this question, we used a limiting-

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dilution competitive repopulation assay to measure the 24-hour BM homing efficiencies of young adult BM cells transplanted into old recipients and old BM cells transplanted into young recipients. The design of this assay is depicted in Figure 5.2. BM cells isolated from young or old Ly-5.2 mice were injected into lethally irradiated, old or young Ly-5 congenic mice (3×10^6 c/mouse). Twenty-four hours later, BM from these primary recipients was harvested and competitively transplanted into Ly-5.1 secondary recipients to measure the number of Ly-5.2$^+$ CRU that had "homed" to this organ. Simultaneously, the number of CRU that were present in the original young or old B6 BM cell suspensions was measured by limiting-dilution competitive repopulation assays in a separate set of irradiated Ly-5.1 mice.

The results from the experiments performed with young BM cells transplanted into old recipients are given in Table 1 and the results of old BM cells transplanted into young recipients are in Table 2. Comparison of the upper half of the two tables shows that the frequency of both early (5 and 10-week time points) and long-term (17 and 26-week time-points) CRU increased by ~2-fold from early adulthood to old age. For example, CRUs that contribute to hematopoiesis for at least 10 weeks post-transplant represent 1 per 20,000 young BM cells and 1 per 10,000 old BM cells. As shown previously, CRU contributing to early engraftment were somewhat more prevalent (one 5-week CRU per ~15,000 young BM cells) than those detectable at later times (one 26-week CRU per 7,000 old BM cells), consistent with the hierarchical organization of the stem cell compartment (Morrison et al. 1995; Rosendaal et al. 1979).

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Furthermore, the HSC frequencies obtained in this study using a rigorous functional assay are similar to those determined previously using phenotypic or surrogate in vitro measures (such as cobblestone area-formation) of stem cells and confirm that HSC in B6 mice increase significantly with age (Chen et al. 2000; de Haan and Van Zant 1999; Morrison et al. 1996; Szilvassy et al. 2003).

The seeding efficiency of young or old CRU in old or young recipients, respectively, was determined by dividing the total number of CRU recovered in the BM of primary recipients 24 hours after homing (as shown in the lower halves of each Table) by the absolute number of CRU originally injected (calculated from the CRU frequencies in freshly isolated BM in the upper halves of Tables 5.1 and 5.2). These data are summarized in Figure 5.3 and compared to previous studies begun contemporaneously with the present studies in which young BM CRU were transplanted into young recipients (Szilvassy et al. 2003). Twice as many young (6% to7%) than old (2% to 3%) HSCs homed to the BM of young recipients in the first day after transplantation. Moreover, CRU that generated lymphoid and myeloid progeny at 5 weeks exhibited a greater decline in homing as they aged than CRU that are detectable at later times. Of equal importance, advanced recipient age also resulted in significantly reduced homing of HSCs (from 6% to 7% to 2% to 3%) compared to young CRU transplanted into young hosts. Thus, the effects of donor or recipient aging are similar, each leading individually to a 50% to 60% decline in HSC homing compared to their young counterparts.

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Effects of aging and prior transplantation on the proliferation potential of HSCs

One advantage of the CRU assay is that it enables the retrospective identification of recipient mice that were repopulated by a single HSC. Circulating blood cells in such mice, identified using criteria described previously (Szilvassy et al. 2003) and in the Methods, were then analyzed in more detail to characterize the proliferation and differentiation potentials of individual CRU in vivo. To assess the impact of aging on proliferation potential, we compared the size of clones generated by single young or old CRU (expressed as the percentage of donor-derived PB leukocytes) in recipients of varying age. The average clone sizes of freshly isolated young CRU in young recipients differed for stem cells whose progeny were detected at early or late times after transplantation (Figure 5.4A); ~9% for 5- and 10-week CRU vs. 25% to 28% for 17- and 26-week CRU, $P<0.05$). This finding is consistent with the empirical use of longer assay times in repopulation assays to identify more primitive HSCs with a higher proliferation potential. Perhaps surprisingly, no difference was observed in the size of clones generated by young and old CRU (Figure 5.4A and 5.4C). However, both young and old long-term CRU (17- and 26-week) that were selected by prior homing to either young or old BM generally produced smaller clones than those generated by freshly isolated HSCs. The sole exception was the larger clone generated by "homed" old CRU at the 26-week time point, but because this value was derived from a single clone in a single animal, we view it with caution. Interestingly, young CRU that previously "homed" to either young

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or old BM did not differ in their subsequent proliferation in young secondary recipients (compare panels A and B for homed HSCs). In aggregate, these data indicate that the proliferation potential of long-term repopulating HSCs is reduced significantly by prior transplantation.

Effects of aging and prior transplantation on the differentiation potential of HSCs

We next compared the proportion of B and T lymphocytes, and myeloid cells, in the PB of young or old animals engrafted with single, freshly isolated or "homed" CRU that were isolated initially from young or old donor mice. As shown in Figure 5.5, clones generated by fresh old CRU contained ~5-fold more myeloid cells (40% to 60% of donor-derived cells), and ~2.5-fold fewer T cells (15% to 20% of donor-derived cells) than those produced by young CRU (compare panels A and C). This skewing of the differentiation potential of old HSCs toward myelopoiesis, at the expense of T lymphocyte production, was also evident among old CRU that had been subjected to prior homing (Figure 5.4C). Myeloid skewing of older HSCs has been reported previously (Offner et al. 1999; Sudo et al. 2000) and suggests an age-related alteration of their proportional production of various blood cell lineages relative to that normally observed in early adulthood.

Aging of the recipient BM microenvironment had similar effects on HSC differentiation. Young CRU "homed" in old recipients also produced ~3.5-fold more myeloid cells and ~2-fold fewer T cells than young CRU homed to young

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BM, or than freshly isolated CRU (compare Figures 5.5A and 5.5B; $P<0.05$). There were no significant changes in the average proportional representation of B-lineage cells in the clones produced by freshly isolated vs. "homed" HSCs in young or old recipients. These data suggest that HSC differentiation potential changes not only as a function of cell-autonomous, age-related mechanisms, but also in response to extrinsic signals from the marrow microenvironment that are altered by aging.

Finally, we compared the contributions that individual CRU made to all PB cells of the myeloid, B or T lineages (Figure 5.6). In general, "homed" CRU recovered from the BM of primary recipients 24 hours after transplantation made significantly smaller contributions to all three lineages in secondary mice than did fresh CRU that were allowed to engraft primary hosts long-term. Once again, donor aging resulted in increased myeloid contribution, and the total number of myeloid and B cells produced by young CRU that had homed to old BM were 2 to 3-fold higher than those generated from young CRU homed to young BM (compare panels A and B).

Differential engraftment kinetics of young and old BM cells in recipients of variable ages

We hypothesized that age-related variation in the homing efficiency of HSCs and HPCs would translate into differences in the subsequent rate of hematopoietic reconstitution. To address this question, young or old BM cells were injected into old or young Ly-5 congenic recipients, and the recovery of

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circulating blood cells was measured over 4 months after transplantation. Until day 12, the rate of leukocyte recovery was indistinguishable among the 3 groups (young into young, young into old, and old into young) (Figure 5.7A). Thereafter, the rate of leukocyte recovery in old mice that were transplanted with young BM cells began to surpass the other two groups. In young recipients that were engrafted with old BM, leukocyte production accelerated sharply after ~1 month and reached normal levels shortly after the young into old pairing. White cell recovery in young mice injected with young BM cells lagged behind the other two groups and failed to reach normal levels even after 4 months. This finding was consistent with the myeloid skewing of old CRU, or as a result of recipient aging, that was observed in Figure 5.5. Young and old BM cells were similarly effective in preventing anemia following transplantation into young recipients (Figure 5.7B). In contrast, old mice that were transplanted with young BM cells became severely anemic early after transplant, and erythrocytes recovered at a slow rate until reaching normal levels after 6 weeks.

Figure 5.7C depicts the dramatic age-related differences observed in platelet recovery kinetics. Young BM cells required nearly a month to regenerate near normal levels of platelets following transplantation into young recipients. In contrast, transplantation of old marrow into young mice led to normal platelet counts in ~15 days. Profound thrombocytopenia was still not resolved by 4 months after the transplant of young marrow into old mice. These data illustrate both the strong intrinsic bias of old BM cells toward platelet production and the

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failure of the old marrow microenvironment to foster platelet recovery when seeded with young marrow.

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Discussion

Homing of primitive stem/progenitor cells to the BM represents the crucial first step to successful engraftment after transplantation. However, the use of the term 'homing' in the literature has been confusing and duplicitous, referring both to (a) the initial events following hematopoietic cell infusion in which primitive cells lodge in supportive niches, and (b) extending erroneously to the subsequent engraftment process, which is characterized by differentiated blood cell formation over many weeks and months. Experiments conducted in the 1960's to measure the 24-hour seeding efficiency of spleen colony-forming units (CFU-S), termed 'f' for factor (Siminovitch et al. 1963), yielded estimates of 10% to 20% (Fred and Smith 1968; Lord 1971; Siminovitch et al. 1963). Subsequent studies to determine the 'f' factor of HSCs for BM seeding yielded estimates of ~1% per femur, or ~12% for the entire marrow mass of the mouse (Lord 1997; Lord et al. 1975). As rigorous functional assays for HSCs have been refined, a similar value of ~10% was obtained for CRU derived from murine BM and FL and for human CB and FL (Szilvassy et al. 2003) repopulating cells assessed in a xenotransplant model (Cashman and Eaves 2000).

These findings, and those from clinical experience that demonstrate graft failure if too few stem cells are transplanted (Bender et al. 1992; Bensinger et al. 1995), beg reconciliation with experiments demonstrating engraftment with only a few, or even one, purified HSCs (Benveniste et al. 2003; Matsuzaki et al. 2004; Osawa et al. 1996). In many such studies claiming highly efficient seeding, it was

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long-term engraftment that was assessed, not homing of HSCs or HPCs within the first day after infusion. Small numbers of (or even single) HSCs will engraft if they fortuitously lodge in a receptive niche in either the BM or spleen. Cao et al. used bioluminescent imaging to monitor engraftment derived from single luciferase-labeled HSCs. They showed that foci of hematopoiesis are detectable within the first few weeks post-transplant in the spleen and in widely dispersed BM sites throughout the skeleton, with no apparent preference for one site or another (Cao et al. 2004). Primitive hematopoietic cells emanating from the primary focus then initiated additional foci that nearly completely engrafted all hematopoietic sites over the following several weeks. These results are consistent with a dynamic series of events initiating widespread hematopoiesis involving multiple progeny of transplanted HSCs.

Despite increasing evidence that aging causes quantitative and qualitative changes in HSCs directly, or indirectly through BM stromal cells (Geiger and Van Zant 2002), the effects of age on homing of primitive hematopoietic cells remain poorly understood. In the present study, we provide the first measurement of the absolute homing efficiency of old HSCs to BM 24 hours after intravenous injection. Only 2% to 3 % of transplanted CRUs were recovered at this time from the BM of young recipients. This value is 2 to 3-fold lower than that for young HSCs infused into young recipients (7% to 10%), as shown here and in previous studies measuring homing of hematopoietic cells labeled with a vital fluorescent dye (Hendrikx et al. 1996; Szilvassy et al. 1999). This reduced seeding efficiency for old CRU is consistent with a report by Morrison *et al*. who found that a smaller

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proportion of young recipients were engrafted long-term when transplanted with limiting numbers of phenotypically identical old vs. young HSCs (Morrison et al. 1996).

Several mechanisms may be involved in the intrinsic, age-associated decline of HSC homing. First, impaired homing may reflect changes in the levels or binding activity of receptors, extracellular matrix or adhesion molecules during aging. Indirect evidence indicates that CXCR4 expression on HSCs may change with age (Martin et al. 2003) and result in their altered ability to enter BM "niches". Possible modulation of CD26, a peptidase expressed on engrafting hematopoietic cells and which negatively regulates homing and engraftment potential (Christopherson et al. 2004), during aging also deserves study. Second, changes in the cycling status of HSCs during aging may alter their homing. HSCs that are in G_0 phase of the cell cycle at the time of transplantation promote higher levels of engraftment than HSCs in G_1 (Gothot et al. 1998; Habibian et al. 1998) and specifically in S-phase (Monette and DeMello 1979; Orschell-Traycoff et al. 2000). A greater proportion of HSCs in old mice are cycling (Morrison et al. 1996) so this difference may underlie their diminished capability for homing. Third, it is possible that there is an age-related change in radiosensitivity of HSCs manifested in the present experiments by differential ablation of host HSCs, and thus a differential probability with which transplanted HSCs may find unoccupied niches. In addition, there may be an age-related change in the radiosensitivity of the BM microenvironment such that hematopoietic support for stem cels that do home to old BM is impaired. To our knowledge, neither the effect of age on the

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radiosensitivity of HSC nor the microenvironment has been tested experimentally, but this important issue deserves further study.

In the present study, we have for the first time quantitatively determined that recipient age has a dramatic influence on HSCs homing; the seeding efficiency of young HSCs in the BM of old mice is only one-third to half (2% to 3%) that measured in young mice. This striking difference points to a decline in the capacity of the marrow stroma to capture or retain, or both, stem cells in old age. Stromal cells regulate HSCs by secreting various growth factors and providing appropriate cell contacts that define the BM “niche”. Aged stromal cells produce lower amounts of interleukin (IL)-7 and are less capable of supporting the proliferation of B lymphocytes in vitro (Stephan et al. 1998). Although no studies have demonstrated unequivocally that IL-7 is involved in homing of HSCs, it and other stromal cell-derived chemokines, such as SDF-1, may be down-regulated during the aging process, leading to defects in the ability of HSCs to maintain associations with the stroma (Petit et al. 2002).

In assays to measure the homing of HPCs, we also observed a dramatic (~80%) decline in homing to the spleen with donor and recipient age. Reduced splenic seeding was not due to a decrease in organ size or cellularity with age (data not shown). In these studies, homing of progenitors was determined after 3 hours rather than the 24-hour period used for HSCs to preclude unpredictable population size changes (either losses or gains) in HPCs that resulted from proliferation or differentiation initiated 1 day after transplantation. Because HPCs mediate short-term engraftment and ameliorate myelosuppression immediately

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following ablative conditioning, the present findings bear consideration when transplanting older patients and indicate that both donor and recipient age might affect outcome.

Analysis of mice that were transplanted at limiting-dilution with <0.3 CRU, which when engrafted were ascertained with 95% statistical confidence to be repopulated by a single HSC, revealed important age-related differences in stem cell developmental potential. No difference was observed in the sizes of clones generated by young and old CRU, indicating that HSC proliferation potential is not affected by donor age. In contrast, young CRU that were recovered from the BM of primary mice 24 hours after homing exhibited diminished proliferation in secondary recipients, regardless of the age of primary host. Donor and recipient aging was associated with enhanced myelopoiesis at the expense of lymphopoiesis and further amplified in HSCs selected by prior homing. Our findings corroborate and extend several previous reports of the link between aging, transplantation, and this characteristic pattern of lineage skewing (Kim et al. 2003; Spangrude et al. 1995; Sudo et al. 2000). Interestingly, in contrast with these findings of diminished differentiation potential of previously homed BM, Lanzkron et al. and Krause et al. found lineage-negative and quiescent BM cells that were re-isolated from the BM 48 hours following transplantation not only contained long-term repopulating HSCs (Lanzkron et al. 1998) but were also capable of differentiation into non-hematopoietic lineages (Krause et al. 2001). There are clear methodological differences between their findings and our study that likely account for this discrepancy: (i) we did not enrich stem cells from

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either the initial marrow source or the BM that was re-isolated for secondary transplantation, and (ii) we recovered homed CRU after 24 hours rather than 48 hours. Nevertheless, our finding that age-related HSC changes were exacerbated by prior homing is consistent with Harrison's early finding that transplantation itself induced decrements in HSC function (Harrison et al. 1978). However, unlike Harrison et al. who attributed the erosion of HSC function to engraftment history rather than aging, we found that both donor and recipient age as well as prior homing are important.

Differences in the recovery rates of PB cells following BM transplant (Figure 5.7) underscored the theme that old HSCs and an old marrow microenvironment both predispose HSC differentiation to myelopoiesis. Young HSC engrafting in old recipients dramatically enhanced leukocyte recovery at the expense especially of platelet recovery. Interestingly, anemia resulted in old recipients transplanted with young marrow but not in young recipients transplanted with old marrow. This suggests that the old microenvironment plays a more dominant role in determining the numbers of various lineages of terminally differentiated cells in the circulation early after transplantation than the age of the HSC. This is not surprising given the important role the microenvironment plays in supporting hematopoiesis through cell:cell interactions and especially cytokine production, the profile of which changes during aging *(e.g.* IL-7 and B cell production). Interestingly, the age of the transplanted HSC has meaningful effects on platelet formation during the first two weeks following transplant and on leukocyte generation after the first month of engraftment. Old

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HSCs, in both cases, have enhanced generative capacity compared to young HSC when engrafted into young recipients. Thus, the diminished homing ability of old stem cells may be more than offset by the increase with age in CRU numbers in C57BL6 mice as shown here and/or by the increased numbers of progenitors we showed previously. The enhancement of platelet recovery by old HSC is noteworthy because in clinical transplantation prolonged thrombocytopenia is a consequential issue. Unfortunately, in the clinical setting the recipient, not the donor, is more likely to be older, and as can be seen in Figure 5.6, this age combination gives, by far, the poorest platelet recovery.

A final important result of this study is that decrements in the seeding efficiency of HSCs caused by (a) procurement from old donors and (b) the marrow microenvironment of old recipients, individually and in concert, will affect calculations of stem frequency in experimental transplantation assays. For example, the measured frequency of BM CRU from young C57BL/6 donors (1 in ~20,000) is about half that of HSCs taken from old mice (1 in ~10,000. However, because the marrow seeding efficiency (in young recipients) of young HSCs is 7% and that of old HSCs is 2% to 3%, the actual difference in stem cell numbers may be twice again as large, or 4-fold overall between young and old C57BL/6 mice. It might be argued that a distinguishing characteristic of stem cells is their ability to home to hematopoietic sites, and the decline in seeding efficiency with HSC age may represent an age-related decline in function. If this argument is accepted, an adjustment in stem cell frequency on the basis of a lower seeding efficiency would be unwarranted. However, Table 5.2 shows that the seeding

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efficiency in old recipients of young HSCs is 2-fold less than the same cells assayed in young recipients. In this case because the alteration in seeding efficiency is extrinsic to the HSC, an adjustment in measured stem cell frequency would be justified.

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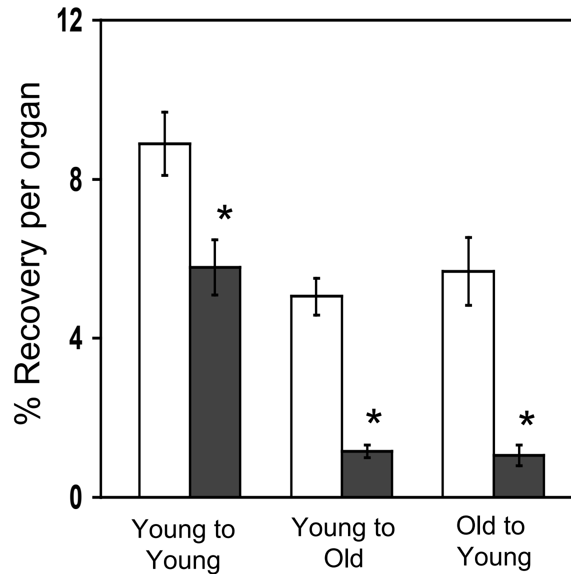

Figure 5.1 Reduced homing capacity of HPCs with donor and recipient age. Lethally irradiated, young or old Ly-5.1 mice were injected with 1 x 10^7 old or young Ly-5.2 donor cells. Three hours later, HPCs that had homed to the BM (white bars) or spleen (black bars) were measured by hematopoietic colony-formation in vitro. Shown are the mean (± SEM) percent of donor-derived HPCs recovered per organ, relative to numbers injected. Data are pooled from 3 independent experiments with 3-5 mice per group. Differences between young cells injected into young recipients, and either young cells transplanted into old recipients, or old cells transplanted into young recipients are significant (* represents $P<0.05$).

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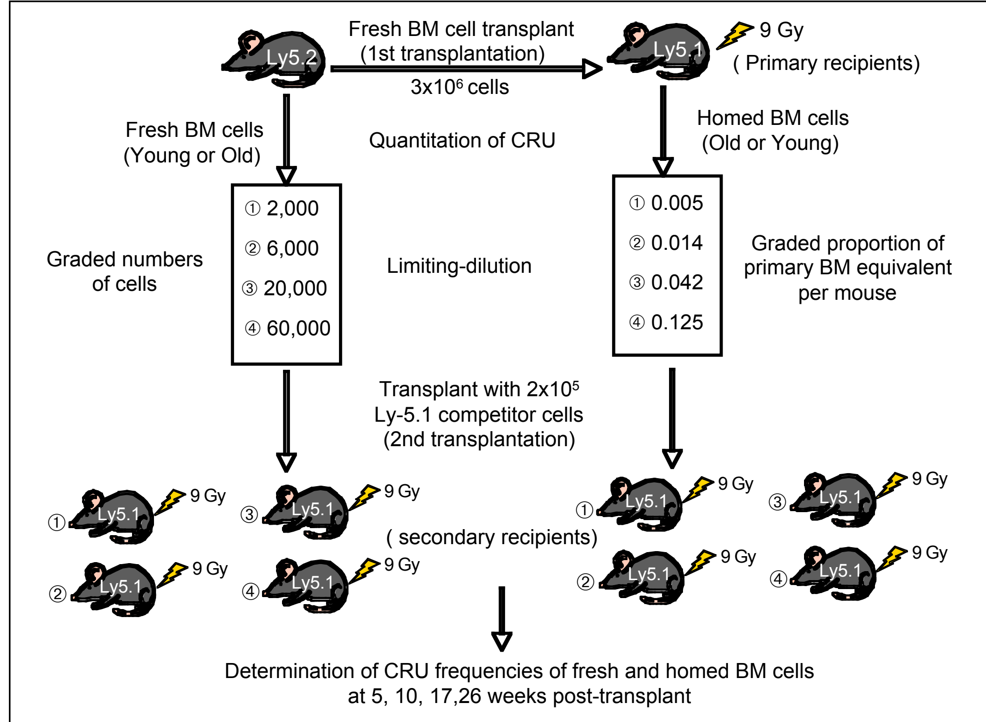

Figure 5.2 Experimental design for competitive repopulation studies.

Lethally irradiated, young or old Ly-5.1 mice were intravenously injected with 3 x 10^6 old or young Ly-5.2 BM cells. Twenty-four hours later, marrow from the primary recipients was harvested to assay the number of Ly-5.2$^+$ stem cells that had homed there following transplant. The frequencies of CRU in the initial BM suspensions and in the BM of primary mice after homing were determined by competitive repopulation of two sets of Ly-5.1 mice that were transplanted with graded numbers of fresh or “homed” BM cells admixed with $2x10^5$ Ly-5.1 competitor BM cells. Donor (Ly-5.2)-derived lymphoid and myeloid cells in PB were then measured at 5, 10, 17 and 26 weeks after transplantation. CRU frequencies were calculated from the proportions of negative mice in each cell dose group as described in the Methods.

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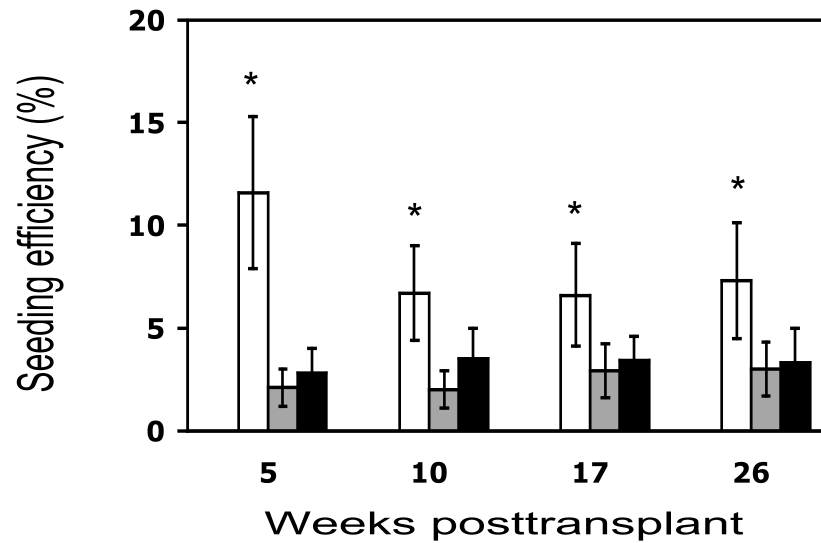

Figure 5.3 Age-related decline in the homing efficiency of murine HSCs. Lethally irradiated, young or old Ly-5.1 mice were intravenously injected with 2-3 x 10^6 old or young Ly-5.2 donor cells as depicted in Figure 2. The number of CRU recovered from the BM 24 hours later was determined by limiting-dilution competitive repopulation assays conducted in young secondary recipients that were assessed for donor engraftment at 5, 10, 17 and 26 weeks after transplantation (see Tables 1 and 2). The homing efficiency of HSCs was calculated by dividing the number of CRU recovered in the primary BM 24 hours after transplantation, by the number of CRU initially injected, and multiplying by 100%. Three different transplantation groups, young cells into young (white bars), young cells into old (gray bars), and old cells into young recipients (black bars), are depicted. Data for young to old, and old to young groups are derived from the experiments described in Tables 1 and 2. Data for the young to young group, although collected contemporaneously with the other transplant groups

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were reported previously by Szilvassy et al., and are shown for comparison. All values are mean ± SEM, and statistically significant differences are designated with an * ($P<0.05$).

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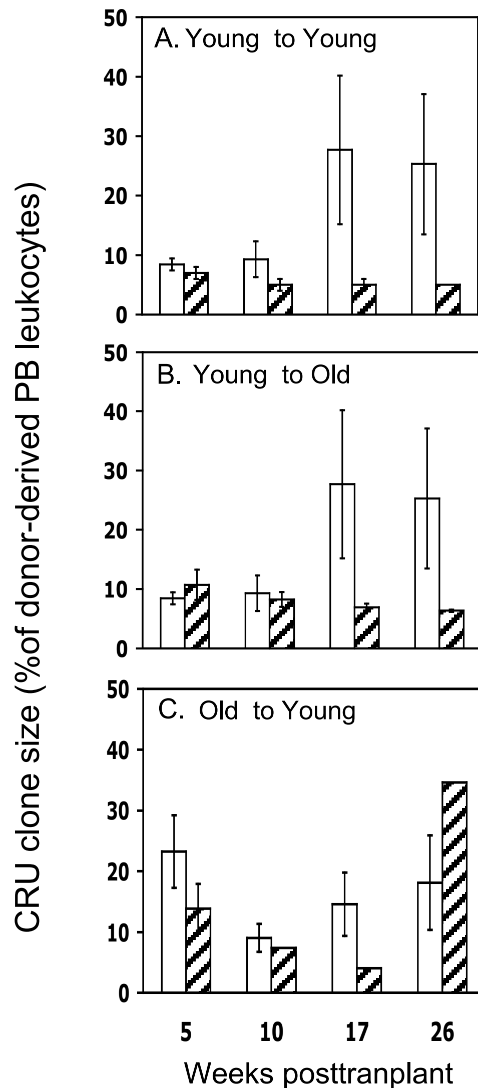

Figure 5.4 Effect of aging and prior transplantation on the proliferation potential of CRU

Following determination of the CRU frequency in fresh and “homed” BM (Tables 1 and 2), it was possible to retrospectively identify mice that had been injected with <0.3 CRU, and in which both the lymphoid and myeloid compartments were subsequently repopulated with donor stem cells. On the basis of Poisson

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statistics, it is 95% probable that such mice were engrafted with a single HSC. Clone sizes generated by single, fresh (open bars) or “homed” (hatched bars) CRU as a function of donor and recipient age are represented as the mean ± SEM percentage of donor (Ly-5.2+)-derived PB leukocytes assessed 5 to 26 weeks after transplantation (4 to 8 mice per group from 2 pooled experiments). Note that data for the young to young group was collected contemporaneously to other groups but in part reported previously in reference.

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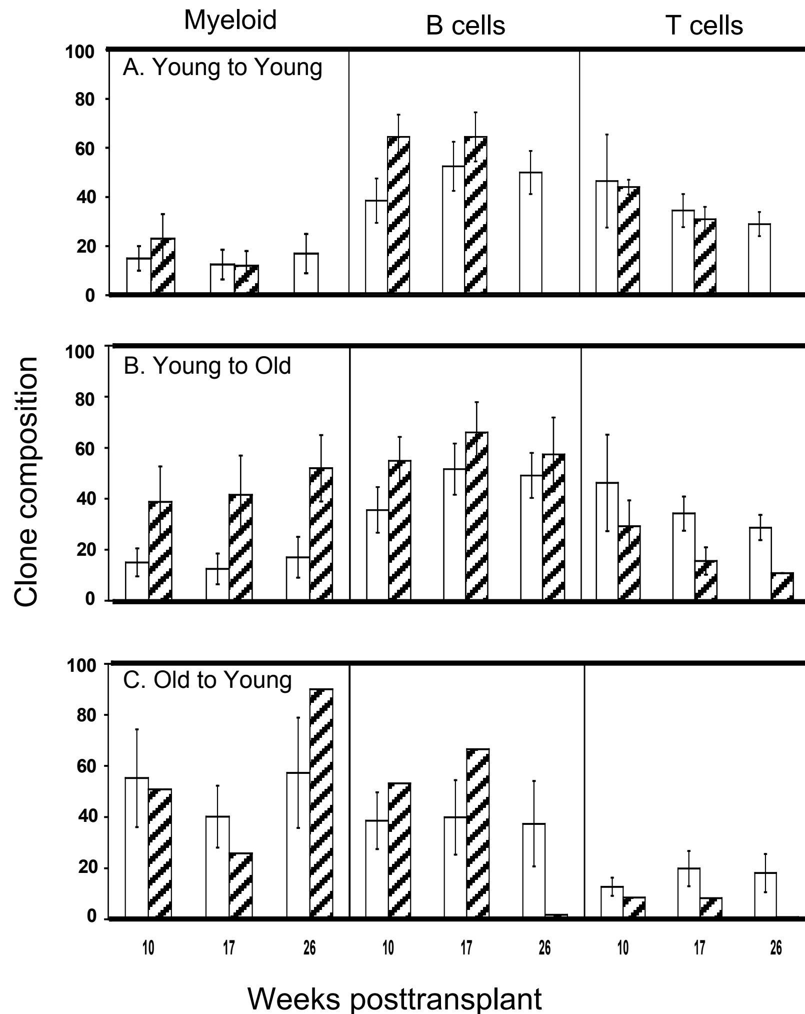

Figure 5.5 Effect of aging and transplantation on the differentiation potential of CRU

Mice that had been repopulated with a single, fresh (open bars) or homed (hatched bars) CRU were identified as described in the Figure 5.4 legend and Methods. The lineage composition of clones generated by single, young or old HSCs in old or young hosts was defined as the proportion of donor (Ly-5.2$^+$)-derived leukocytes expressing markers for B (CD45R/B220$^+$) or T (CD90/Thy-

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1.2$^+$) lymphocytes, or myeloid (Gr-1/Ly6G$^+$ and Mac-1/CD11b$^+$) cells. Values shown for the 3 combinations of variably aged donors and recipients represent the mean ± SEM of 2 pooled experiments with 3 to 6 mice per group. Note that data for the young to young group was collected contemporaneously to other groups but in part reported previously in reference.

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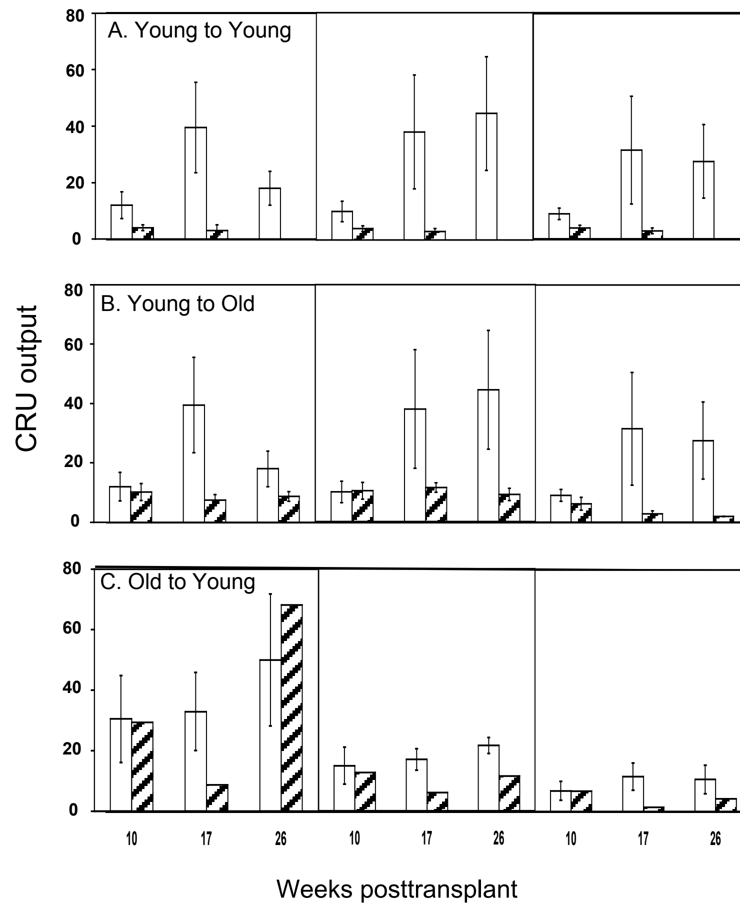

Figure 5.6 Differential contribution of single BM CRU to hematopoietic engraftment

Recipient mice that were repopulated with a single, fresh (open bars) or homed (hatched bars) CRU were identified as described in the Figure 5.4 legend and Methods. The differential contribution of individual CRU to engraftment was defined as the proportion of all circulating B (CD45R/B220$^+$) or T (CD90/Thy-1.2$^+$) lymphocytes, or myeloid (Gr-1/Ly6G$^+$ and Mac-1/CD11b$^+$) cells that were Ly-5.2$^+$. Values shown for the 3 combinations of variably aged donors and recipients represent the mean ± SEM of 2 pooled experiments with 3-6 mice per

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group. Note that data for the young to young group was collected contemporaneously to other groups but in part reported previously in reference.

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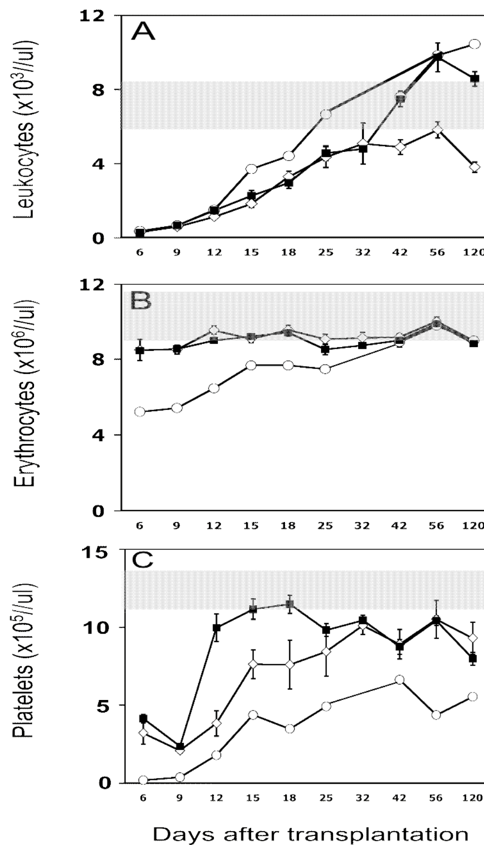

Figure 5.7 Differential engraftment kinetics of young or old BM cells in old or young recipients

2 x 10^6 young (◊) or old (■) Ly-5.2 BM cells were injected into lethally irradiated young mice, or 3 x 10^6 young Ly-5.2 BM cells were injected into lethally irradiated old Ly-5.1 mice (O). Shown are the mean ± SEM number of PB leukocytes (A), erythrocytes (B), and platelets (C) counted on the indicated days after transplantation (pooled data from 2 experiments with 10 mice per group). The absence of error bars for specific data points indicates that fewer than three animals were available for analysis. Note that the time after transplantation is not depicted on a linear scale. Shaded areas indicate normal ranges of blood cell

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counts in age-matched B6.SJL control mice. Data for the young to young group was collected contemporaneously to other groups but in part reported previously in reference.

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Table 5.1 Determination of the number of intravenously transplanted young CRU that home to the BM of old irradiated mice

Cell type injected	No. cells (fresh) or fraction of 1° BM (homed) injected per mouse	Proportion of negative mice#			
		5 weeks	10 weeks	17 weeks	26 weeks
Fresh Young BM	$2X10^3$	15/16	15/16	16/16	16/16
	$6X10^3$	12/16	12/16	12/16	12/16
	$2X10^4$	3/15	4/15	7/15	7/14
	$6X10^4$	0/8	0/8	0/8	0/8
1/CRU frequency* (±SEM)		15,242 (12,124~19,163)	16,814 (13,350~21,178)	23608 (18,571~30,012)	24,578 (19,232~31,409)
No. CRU injected¶ (±SEM)		197 (157~247)	178 (142~225)	127 (100~162)	122 (96~156)
Homed Young BM	0.005	8/8	8/8	7/7	7/7
	0.014	13/14	13/13	13/13	13/13
	0.042	11/14	10/14	11/14	9/11
	0.125	7/14	6/14	8/14	8/14
CRU frequency: £ 1 per 1° BM Fraction (±SEM)		0.182 (0.134~0.247)	0.159 (0.119~0.213)	0.231 (0.166~0.323)	0.247 (0.174~0.352)
1/CRU frequency: ‡ No. CRU Recovered per 1° BM (±SEM)		5.5 (4~7.5)	6.3 (4.7~8.4)	4.3 (3.1~6.0)	4.0 (2.8~5.7)

Lethally irradiated old (20 to 22 months) Ly-5.1 mice were injected with $3X10^6$ young (6 to 8 weeks) Ly-5.2 BM cells and their marrow was harvested 24 hours later. The frequency of CRU in this primary BM, and in the B6 suspension injected initially, were determined by transplanting graded numbers of fresh or

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“homed” BM cells into separate sets of lethally irradiated Ly-5.1 recipients that were analyzed for donor (Ly-5.2^{+})-derived engraftment at the indicated times. Pooled data from 2 independent experiments.

Negative mice are defined as animals in which <5% of the circulating B and/or T lymphocytes and/or myeloid cells were derived from donor (Ly-5.2^{+}) stem cells at the time of analysis.

* Shown are the number of BM cells containing one CRU. The range in CRU frequencies defined by ± 1 SEM is shown in parentheses.

¶ Absolute values were determined by multiplying 1/CRU frequency (*) by 3X10^{6} cells, the number of fresh cells injected into primary recipients.

£ Frequencies of homed CRU are expressed as the proportion of primary (1°) BM cells that contain 1 CRU.

‡ The absolute number of CRU that homed to the total primary BM was determined by inverting the frequencies of homed CRU (£).

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Table 5.2 Determination of the number of intravenously transplanted old CRU that home to the BM of young irradiated mice

Cell type injected	No. cells (fresh) or fraction of 1° BM (homed) injected per mouse	Proportion of negative mice			
		5 weeks	10 weeks	17 weeks	26 weeks
Fresh Old BM	$2X10^3$	12/15	12/15	11/14	11/14
	$6X10^3$	5/14	5/14	8/14	8/14
	$2X10^4$	1/14	1/14	2/14	3/14
	$6X10^4$	0/8	0/8	0/8	0/8
1/CRU frequency (±SEM)		7,006 (5,548~8,848)	7,006 (5,548~8,848)	10,019 (7,923~12,671)	11,304 (8,932~14,306)
No. CRU injected (±SEM)		428 (339~541)	428 (339~541)	299 (237~379)	265 (210~336)
Homed Old BM	0.005	6/6	6/6	6/6	6/6
	0.014	10/12	8/9	7/8	4/5
	0.042	9/14	8/13	8/13	9/13
	0.125	4/10	4/10	4/10	4/10
CRU frequency: 1 per 1° BM Fraction (±SEM)		0.113 (0.085~0.151)	0.116 (0.086~0.157)	0.115 (0.085~0.155)	0.124 (0.091~0.169)
1/CRU frequency: No. CRU Recovered per 1° BM (±SEM)		8.8 (6.6~11.8)	8.6 (6.4~11.6)	8.7 (6.5~11.8)	8.1 (5.9~11)

The experimental design and determinations of the frequencies and absolute numbers of CRU that were transplanted and recovered after homing is the same as described in Table 1, except that donor cells were harvested initially from old (20 to 22 months) mice and young mice (6 to 8 weeks) were used.

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CHAPTER SIX

General Discussion and Conclusions

The primary purpose of the first part of this dissertation study was to evaluate the general hypothesis that specific gene and their allelic variants contribute to strain-specific difference in HSC numbers between C57BL/6 (B6) and DBA/2 (D2) mice. Following a classic forward genetic approach, three specific aims were derived from this underlying hypothesis: first, linkage analyses were performed to determine quantitative trait loci (QTL) linked to strain-specific variation in HSC number and phenotypes were measured in congenic mice to validate the QTL mapping. Second, the biological processes related to HSC number regulation were investigated in congenic mouse models, including cell cycle kinetics, apoptosis and self-renewal. Lastly, by using microarray-based candidate gene method, a candidate gene was identified that might be involved in HSC number regulation in mice. The noteworthy findings in this dissertation project are that 3 QTL on chromosomes 3,5 and 18 were not only mapped but also confirmed phenotypically in congenic mouse strains, in which the D2 allele increased whereas the B6 allele decreased HSC number. Surprisingly, in the context of this dissertation, the chromsome 3 QTL was shown to affect the cell cycle kinetics, apoptosis, and the self-renewal of HSCs. The D2 allele of the QTL increased cycling and self-renewal whereas decreased apoptotic rates of HSCs. Furthermore, a small number of differentially-expressed genes was identified in chromosome 3 congenic HSCs and the differential expression of one candidate,

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latexin, was found to relate to changes in HSC numbers. The B6 allele was associated with up-regulated latexin expression and decreased HSC numbers, whereas the D2 allele was associated with down-regulated latexin expression and increased HSC numbers. Therefore, my current studies not only prove the feasibility of forward genetics leading to gene discovery, but also report the strong evidence of the potential functions of latexin in HSC number regulation. These findings are important for understanding molecular mechanisms of stem cell regulation and helpful for developing effective stem cell expansion strategies that are clinically applied in gene and cell therapy (Morrison et al. 1997; Sorrentino 2004; Weissman 2000).

The tremendous potential for reconstituting the hematopoietic system of HSCs has allowed the development of transplantation of HSCs as a clinical strategy for the treatment of hematological disorders. However, the obstacle for the successful application of this therapy is the limited number of HSCs available from primary cell sources (bone marrow, periperal blood or cord blood) (Karlsson 2004; Sauvageau et al. 2004). Great efforts have been made to design protocols not only to expand HSC populations but also maintain their functions (self-renewal and multi-lineage differentiation) (Bernstein 2004; Brown and Weissman 2004). Recently, some regulatory molecules, such as WNT-protein family (Wnt, ß-catenin), Notch family of receptors (Notch1), cell-cycle genes (cyclin D, some CKIs) and Homeobox genes (especially HoxB4) have been identified that show promise for harnessing the amplification of HSCs in animal models and even in clinical settings (Antonchuk et al. 2002; Duncan et al. 2005; Kozar et al. 2004;

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Reya et al. 2003; Stier et al. 2002; Walkley et al. 2005). Our current findings add an additional potential regulator, latexin, to this list by demonstrating that the reverse relationship between latexin expression and the HSC pool size.

The underlying mechnisms by which latexin controls HSC pool size are not yet clear. Latexin is the only known endogenous carboxypeptidase inhibitor (CPI) in mammals, and recombinant rat latexin has been shown to inhibit pancreatic carboxypeptidase A1, A2 and A3 (CPAs) (Normant et al. 1995). Latexin was also found to be widespread in humans, and it specifically inhibits human CPA4, a member of the A/B subfamilies of mammalian metallocarboxypeptidases (A/B MCPs), but not other types of MCPs (Liu et al. 2000b; Pallares et al. 2005). Comprising over 220 residues, the latexin protein is significantly larger than CPIs from plant and parasites (40-70 residues). Moreover, lack of substrate-interaction site that is located in C-terminus of latexin protein suggests that the mechanisms by which latexin inhibit CPA differ from those of non-mammalian CPIs (Reverter et al. 2000). Combining all available information about latexin and our microarray results, I propose that several potential regulatory pathways (or mechanisms) by which latexin regulates murine HSC numbers may exist (Figure 6.1). First, the effects of latexin on HSCs may reside in its carboxypeptidase A (CPA) inhibitor activity (Normant et al. 1995). CPA is involved in degradation or maturation of protein or peptide by enzymatically cleaving C-terminal amino acids of its substrate protein (Vendrell et al. 2000). Some of these substrates, although yet unknown, may play important roles in regulating murine HSC number. Therefore, latexin may interfere with the

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normal metabolism of these regulators by inhibiting CPA activity, which in turn affect HSC numbers. Secondly, the structural existence of potential cGMP-dependent protein kinase phosphorylation sites in the latexin molecule indicates that it might be one of the members in G protein-coupled receptor signaling pathway and regulate other downstream molecules (Callahan 1999), such as mitogen-activated protein kinase 8 (Mapk8) and/or Ras, guanyl releasing protein 2 (Rasgrp2), which were found to be differentially expressed in my microarry studies (Table 4.1). Thirdly, the physical protein structure of latexin demonstrates two potential $Ca2^{+}$/calmodulin-dependent protein kinase sites, suggesting it may also be involved in the calcium-mediated signaling pathway (Callahan 1999). This hypothesis is strongly supported by the fact that two members of the S100a family, which bind calcium ions, were diffentially expressed (Table 4.1). Finally, the similarity of DNA sequence, amino acid sequences, and pysical protein structure between latexin and the cystatin family of protein and human tumor suppressor tazarotene-induced gene 1 (TGI1) indicates that other mechanisms might also exist by which latexin regulates HSC functions (Aagaard et al. 2005; Pallares et al. 2005). The cystatin family functions as cysteine proteinase and plays important roles in several molecular functions, including extracellular matrix degradation, stimulation of mouse fibroblast proliferation as well as inhibition of osteoclatic bone absorption (Balint et al. 2003; Newman 2002). Moreover, the findings that each of two topologically equivalent subdomains comprised of human latexin protein was strongly similar to that of cystatin indicate the evolutionary conservation between these two proteins. More interestingly, the

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finding that the loss of TGI1 expression contributed to the development of prostate cancer (Jing et al. 2002; Lotan 2002) and that downregulated TGI1 expression was mediated by methylation of the promoter and CpG island of the TGI1 gene (Kwong et al. 2005; Youssef et al. 2004; Zhang et al. 2004) provide a hint that latexin may be involved in hematological malignancy and differential expression of latexin might be due to the different methylation states in its regulatoy region of the DNA sequence. Altogether, these potential mechanisms may synergistically or independently affect HSC number-related biological processes. Our phenotypic characterization of chromosome 3 congenic mouse models suggests that latexin expression level probably relates to the cycling, apoptosis and self-renewal of HSCs. It also probably affects other functions of HSCs, such as migration and cell growth through regulating genes like tubulin, alpha1 (Tuba1) and pituitary tumor-transforming1 (pttg1).

In the second part of the current studies, I focused on another important step that is involved in stem cell transplantation: the homing and engraftment of HSCs. By using functional assays for progenitor and stem cells, we first reported the homing efficiencies of young or old donor cells into young or old recipients. The results indicated that the homing of primitive heamtopoietic cells were not efficient and were significantly decreased with aging of both the donor and the recipients. They also illustrated on the other hand that large numbers of HSCs are needed for transplantation when old donors or recipients involved. In addition, the proliferation and differentiation capabilities of functional HSCs were impaired by the homing process itself as well as the age of donors and

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recipients. More importantly, the hematopoietic reconstitution dynamics following transplantion of bone marrow cells were also affected by the aging process. These results may provide useful information for clinical settings when the number of older individuals serving as donors for elderly patients increases.

In conclusion, we may still be many steps away from clinical application of these findings, but new discoveries concerning regulation of stem cell numbers and their homing and engraftment capabilities may lead us one step further toward this goal. My findings regarding latexin and the effects of aging on homing and engraftment in this dissertation may add new information in this diretion. However, many detailed studies remain to be done in the future in order to reveal the underlying mechanisms for both factors. First of all, temporary knockdown of latexin expression in HSCs througn small RNA interference (SiRNA) technology will help to further validate latexin as the candidate gene. Secondly, generation of a knockout mouse model will be a critical step to investigate the in vivo functions of latexin and the underlying molecular mechanisms. Thirdly, screening of nucleotide polymorphisms in the regulator regions of latexin DNA sequency will be important to understand underlying genetic variations responsible for natural viarions in HSC numbers (Flint and Mott 2001). Fourthly, since we have found HSC numbers change with age, further investigations will be performed to study the effects of aging on latexin expression and its relationship to experimentally induced changes in the population size of HSCs. Most importantly, our final goal is to use these findings to develop effective HSC expansion strategy that will be clinically applicable to the treatment of disease. To this end, we will detect the

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expression of latexin in human HSCs and study its effects in hematopoietic system under the normal, and neoplastic conditions. Finally, we will further investigate the additive effects of donors and recipients' aging on homing and engraftment by setting up transplantion of old bone marrow cells into old recipients. In addtion, we will analyze and compare gene expression profiles between young and old HSCs to uncover the underlying mechanisms.

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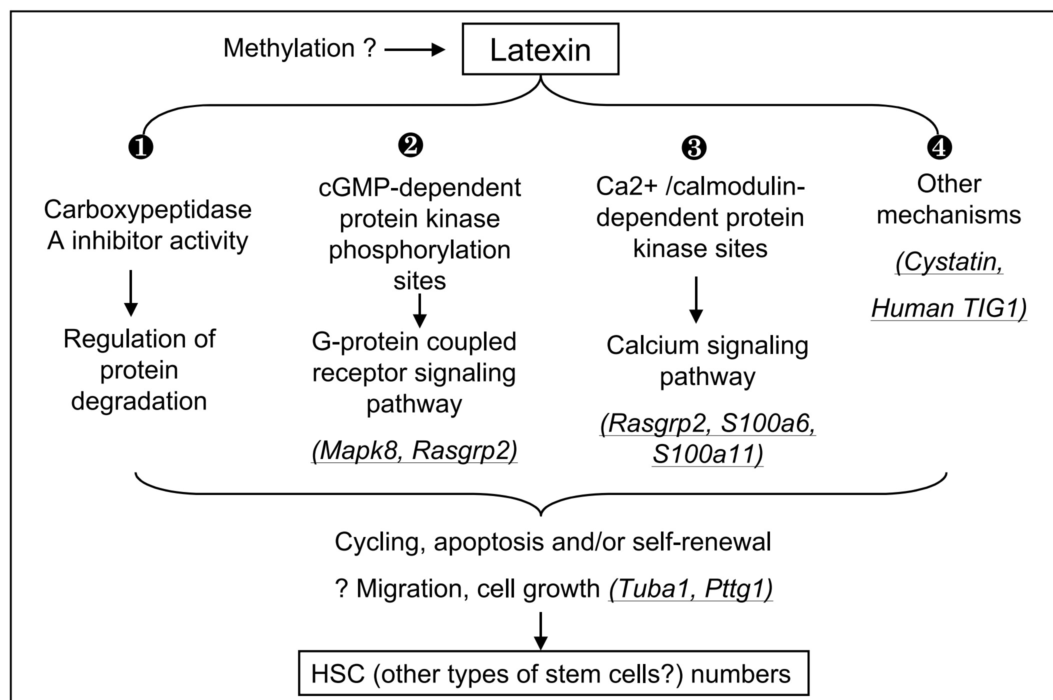

Figure 6.1 Models of potential latexin-involved biological processes. According to current available information about latexin and our microarray results, latexin might be involved in four possible pathways which synergistically or independently regulate the numbers of HSCs and/or other types of stem cells.

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Appendix

March 10, 2005

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Publications

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2. **Ying Liang,** Gary Van Zant. The mechanisms underlying quantitative trait loci on mouse chromosome 3 and 5 that regulate hematopoietic stem cell number. 1st Annual Midwest Blood Club Symposium. April 12 ~ 13, 2003. Cincinnati, OH.

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