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Genetic and Environmental Factors Suggest that Dietary Fatty Acid Content, Lipid Metabolism, and Bone Properties are Key Regulators of Myeloid Progenitor Cell Frequency

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GENETIC AND ENVIRONMENTAL FACTORS SUGGEST THAT DIETARY FATTY ACID CONTENT, LIPID METABOLISM, AND BONE PROPERTIES ARE KEY REGULATORS OF MYELOID PROGENITOR CELL FREQUENCY

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Abstract

Acute myelogenous leukemia (AML) and its precursors are the result of the dysregulation of hematopoiesis. Hematopoiesis proceeds in a stepwise manner, beginning with hematopoietic stem cells, continuing to develop into various stages of progenitor cells, and finally becoming fully functional blood cells. As this process goes awry, immature, functionless cells of the myeloid lineage proliferate out of control. Discerning how myeloid progenitor frequency is regulated allows for a better understanding of how the process may lose control. Hematopoiesis has been shown to depend on genetic and environmental factors. In this work, I have added to this knowledge base by providing novel information about genetic and environmental components for the regulation of blood cell development. By performing colony forming cell (CFC) assays to determine myeloid progenitor cell frequency in 27 strains of mice, and using bioinformatics capabilities in the form on SNP-based *in silico* mapping, I have found patterns among genes located in chromosomal loci of interest for regulating this phenotype. These patterns suggest that lipid metabolism, insulin sensitivity, and bone properties are key regulators of myelopoiesis. Additionally, I have found that polyunsaturated fatty acid (FA) content in the diet is a key environmental regulator. Mice fed diets rich in omega-3 FAs have significantly lower middle and late stage progenitor cell frequency and increased common myeloid progenitor frequency over those fed more similarly to the Western diet. Collectively these data suggest that the content of diet, how it is metabolized by the body, and how this affects the bone, regulates the frequency of myeloid progenitor cells. Further research into these processes may provide novel biomarkers for disease progression and improved therapeutic options for AML.

Dedication

I would like to dedicate this work to my parents, Stewart and Darlene Varney. It is through their constant encouragement, support, and guidance that I have learned that with faith, hard work, humility, and perseverance, making a difference in the world is possible. They taught me to pursue what I am passionate about and not to give up when faced with what seem like immense challenges. They have always been there to see me through when I've needed encouragement and to celebrate with me when I've succeeded. For their love and for all that they have taught me, I am truly grateful.

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Additionally, I would like to thank the NASA West Virginia Space Grant Consortium and the Division of Science and Research for the West Virginia Higher Education Policy Commission. NASA West Virginia Space Grant Consortium has provided me with multiple fellowships to fund this work. As a part of this funding, they have also encouraged me to attend conferences where I was able to present my work and

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Symbols/nomenclature

ALA – α-linolenic acid

AML – Acute myelogenous leukemia

ARA – Arachidonic acid

ATRA – All-*trans* retinoic acid

CFC – Colony forming cell

CFU – Colony forming unit

CLP – Common lymphoid progenitor

CML – Chronic myelogenous leukemia

CMP – Common myeloid progenitor

COX – Cyclooxygenase

DHA – Docosahexanoic acid

DPA – Docosapentanoic acid

E – Erythrocyte

EMP – Erythrocyte megakaryocyte progenitor

EPA – Eicosapentanoic acid

FA – Fatty acid

G – Granulocyte

G-CSF – Granulocyte colony stimulating factor

GEMM – Granulocyte erythrocyte macrophage megakaryocyte

GM – Granulocyte macrophage

GMP – Granulocyte monocyte progenitor

GM-CSF – Granulocyte macrophage colony stimulating factor

GWAS – Genome wide association study

HSC – Hematopoietic stem cell

IL-3 – Interleukin 3

IL-3Rα – Interleukin 3 receptor alpha

IL-6 – Interleukin 6

IMDM – Iscove's modified Dulbecco's medium

IPA – Ingenuity pathways analysis

LNA – Linoleic acid

LT-HSC – Long term hematopoietic stem cell

M – Macrophage

M-CSF – Macrophage colony stimulating factor

MEP – Megakaryocyte erythrocyte progenitor

MP-B – Basophil progenitor

MP-E – Erythrocyte progenitor

MP-Es – Eosinophil progenitor

MP-M – Monocyte progenitor

MP-Meg – Megakaryocyte progenitor

MP-N – Neutrophil progenitor

MPP – Multipotent progenitor

n-3 – Omega 3

n-6 – Omega 6

PGE2 – Prostaglandin E2

PUFA – Polyunsaturated fatty acid

QTL – Quantitative trait loci

REML – Restricted maximum likelihood

SCF – Stem cell factor

SEM – Standard error of the mean

SNP – Single nucleotide polymorphism

ST-HSC – Short term hematopoietic stem cell

WBC – White blood cell

Chapter 1: Introduction

Hematopoiesis

Hematopoiesis refers to the process by which blood cells develop. During hematopoiesis, a continuum of differentiation exists, such that long term hematopoietic stem cells (LT-HSCs) progressively differentiate to produce terminally differentiated cells of the blood (Fig1.1). LT-HSCs, which have infinite self-renewal capabilities, divide asymmetrically producing short term hematopoietic stem cells (ST-HSCs), which have limited self-renewal capacity. ST-HSCs give rise to highly proliferative multipotent progenitor cells (MPPs), which differentiate further, giving rise to the common progenitors of the lymphoid and myeloid lineages [1-3].

During myelopoiesis, the common myeloid progenitor gives rise to middle stage progenitors including granulocyte monocyte progenitors (GMPs) and erythrocyte megakaryocyte progenitors (EMPs). These have more limited differentiation potential. The common myeloid progenitor also gives rise to the late stage or committed progenitors for the formation of basophils and eosinophils. The GMPs can then differentiate further to committed progenitors for granulocytes and monocytes, whereas EMPs can differentiate further to committed progenitors for erythrocytes and megakaryocytes. These progenitors make up the myeloid progenitor cell compartment (Fig.1.1) [4].

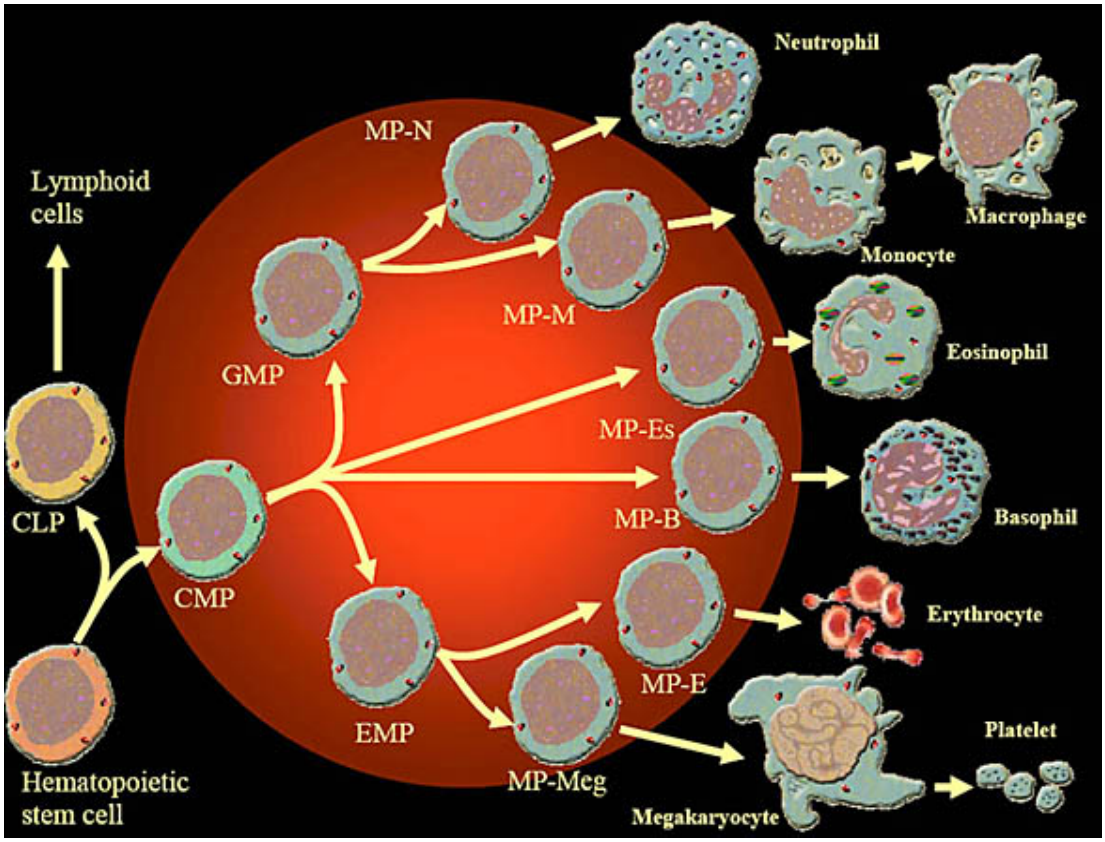


Figure 1.1: Progenitor cells represent a continuum of differentiated cell types derived from the HSC. Successive stages of differentiation of the lymphoid lineage eventually lead to the formation of mature cells, including B and T cells and natural killer cells. Successive stages of differentiation of the myeloid lineage eventually lead to formation of mature neutrophils, basophils, macrophages, megakaryocytes and erythrocytes. The cells within the red background make up the myeloid progenitor cell compartment. Abbreviations: hematopoietic stem cell (HSC), common myeloid progenitor cell (CMP), common lymphoid progenitor cell (CLP), erythroid and megakaryocyte progenitor cell (EMP), and granulocyte and monocyte progenitor cell (GMP). The committed progenitor cells are abbreviated as follows: neutrophil (MP-N), monocyte (MP-M), eosinophil (MP-Es), basophil (MP-B), erythrocyte (MP-E), and megakaryocyte (MP-Meg).

This process of blood cell development occurs first within the yolk sac of embryos. As development continues, it takes place in the liver, spleen, and lymph nodes. As bone marrow develops, it becomes the location of blood development. Maturation and activation of lymphoid cells occur in the thymus, spleen, and lymph nodes [5]. Bone marrow is found within the interior of bones and exists in two forms. Red marrow consists mainly of hematopoietic tissue, and yellow marrow consists mostly of fat cells. As aging occurs, more and more red marrow is converted to yellow marrow, but in cases of severe blood loss, the body can convert yellow marrow to red marrow to increase blood production. The stroma of the bone marrow consists mostly of yellow marrow. Additionally it contains fibroblasts, macrophages, adipocytes, osteoblasts, osteoclasts, endothelial cells, and mesenchymal stem cells. The stroma of bone marrow forms the hematopoietic microenvironment, containing components that can influence the process of hematopoiesis [6.7].

Some hematopoietic cells in the bone marrow exist adjacent to endosteal bone surface. The endosteum is a thin layer of connective tissue that lines the surface of the bony tissue that forms the medullary or bone marrow cavity of long bones. Endosteum is lined primarily by osteoblasts, which are responsible for bone formation and have been shown to produce a variety of cytokines including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-6 (IL-6) [8]. Due to their role in producing these cytokines that are important for hematopoietic cell differentiation, it has been hypothesized that osteoblasts are key regulators of hematopoiesis [9]. Osteoclasts, on the other hand, are responsible for bone resorption. High levels of calcium, magnesium, phosphate and products of collagen are

released as osteoclasts break down mineralized bone. Findings from the Kollet laboratory suggest that, in stress-induced situations, osteoclasts degrade endosteal components and promote the mobilization of hematopoietic progenitor cells [10]. The opposing effects of bone-forming osteoblasts and bone-resorbing osteoclasts control the content of bone inner wall and the endosteum region and, therefore, the environment surrounding the location of hematopoiesis.

Acute myelogenous leukemia and its precursors

Hematopoiesis is known to be influenced by genetic and environmental factors. In situations in which this process becomes dysfunctional, hematopoietic malignancies may develop. Myeloproliferative diseases of the bone marrow are conditions in which excess cells of the myeloid lineage are produced. These diseases may develop into myelodysplastic syndromes or acute myelogenous leukemia (AML). Myelodysplastic syndromes are dysplasias or ineffective production of the myeloid cells in the bone marrow. These syndromes have risk for transformation into AML. In AML, precursor cells of the myeloid lineage undergo impaired differentiation and clonal expansion. The clinical diagnosis for AML occurs when the percentage of these immature and therefore functionless cells rises beyond a particular level in the bone marrow (20-30%) [11]. In AML, the overproduction of these cells prevents the production of normal functioning red and white blood cells. Without the proper amount of functioning red and white blood cells, patients become anemic and slow to fight off infections. Understanding why immature myeloid cells proliferate rapidly and cease to differentiate is essential in understanding why these hematopoietic malignancies can occur. My research is designed

to improve this understanding by identifying genetic and environmental factors that control the frequency of myeloid progenitor cells in the bone marrow.

Genetics of hematopoiesis

A number of genes are involved in controlling the process of hematopoiesis. *Hox, Stat*, and *Gata* genes in addition to *Pu.1, C-myb, Runx1, Slc*, *IL-3Rα,* and others have been shown regulate this process [12-21]. Some of these are also observed in chromosomal translocations in leukemias. Hox genes control differentiation, proliferation, and cell fate during hematopoiesis [12-14]. *Stat-3* and *Stat-5* allow for survival and differentiation of myeloid hematopoietic cells [15]. *Gata-1* and *Pu.1* control hematopoietic cell fate by antagonizing one another through direct physical interaction [16]. *Gata-1* promotes erythromegakaryocytic differentiation while *Pu.1* regulates myelolymphoid differentiation. *Gata-1* directly binds to and suppresses *Pu.1*. Conditional knock-out studies have indicated that *c-Myb* is required for self-renewal of hematopoietic stem cells [17]. *Runx1* is a master regulator of hematopoiesis [18]. *Scl* controls transcriptional programs during embryonic hematopoiesis and is vital to megakaryocyte and erythrocyte development [19-20]. Deletions in *IL-3Rα* are known to cause decreased hematopoietic progenitor cell frequency [21]. The chromosomal region containing this gene served as a control for our studies as it has been shown that several of the mouse strains we included in our studies contain the 5bp deletion in *IL-3Rα* that causes low myeloid progenitor cell frequency.

Chromosomal translocations, deletions, and genetic mutations are common in hematopoietic malignancies. Philadelphia chromosome is a reciprocal translocation

between chromosomes 9 and 22 resulting in an oncogenic fusion protein, BCR-ABL. This translocation occurs in the myeloproliferative disease of chronic myelogenous leukemia (CML) [22]. Other myeloproliferative diseases are often associated with mutations in JAK2 [23-25]. One type of myelodysplastic syndrome is also associated with JAK2 mutations [26]. A particular myelodysplastic syndrome called 5q- syndrome is caused by deletions in the long arm of chromosome 5. These deletions cause anemia and megakaryocytic dysplasia. AML is associated with characteristic chromosomal abnormalities such as translocations between chromosomes 8 and 21 (RUNX1/RUNX1T1), translocations between chromosomes 1 and 17 (RARA/PML), or inversions in chromosome 16 (CBFB/MYH11) among others. These chromosomal aberrations are key indicators of disease prognosis. There are also several gene mutations that have been shown to influence the outcome of the disease. These genes include Flt3, Npm1, and Kit. Flt3 internal tandem duplications are associated with poorer prognosis in AML. Npm1 gene mutations are the most frequent genetic alteration found in AML. Kit is a cytokine receptor expressed on hematopoietic stem cells and involved in promoting stem cell maintenance. Survival rates and relapse risk for AML rely heavily on cytogenetic constitutions and the presence of mutations in the genes mentioned above [27-30].

Table 1.1: Survival and relapse rate associations with chromosomal abnormalities

Risk Category	Abnormality	5-year survival	Relapse rate
Good	t(8;21), t(15;17), inv(16)	70%	33%
Intermediate	Normal, +8, +21, +22, del(7q), del(9q), Abnormal 11q23, all other structural or numerical changes	48%	50%
Poor	-5, -7, del(5q), Abnormal 3q, Complex cytogenetics	15%	78%

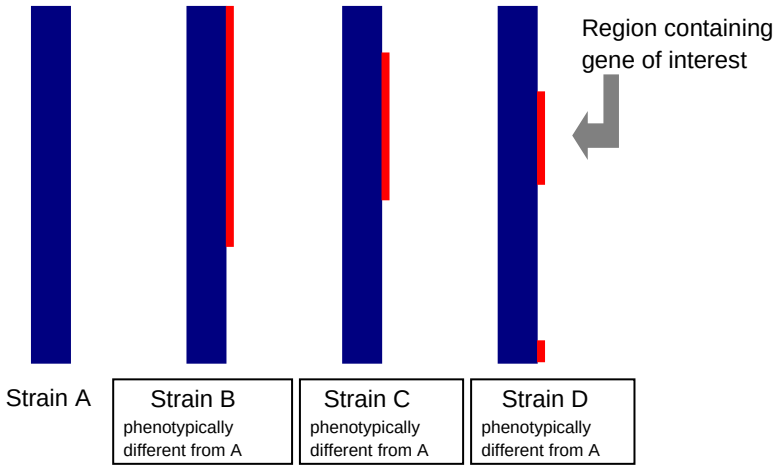
Strategies for the identification of genes contributing to phenotype

As genetics has already been shown to play a vital role in regulating self-renewal, proliferation, differentiation, survival, and cell fate in hematopoiesis, and genetic abnormalities can lead to hematological malignancies like AML, further investigation to discover additional susceptibility genes is warranted. There are multiple ways in which genetic susceptibility studies are performed. Traditional genome wide association studies (GWAS), including traditional quantitative trait loci (QTL) mapping, and *in silico* mapping based on single nucleotide polymorphisms (SNPs) are ways in which this can be accomplished. Their power in doing so, however, differs.

GWAS typically compare two groups, a disease group and a normal group. Genomic DNA is compared for selected markers of variation or SNPs. Genetic variations that are more frequent in people with the disease are considered associated with the disease. As these studies tend to point to broad genomic regions, researchers must use other methods (such as DNA sequencing) to identify the exact genetic change involved in the disease [31]. Traditional QTL mapping studies in mice are GWAS that are based on the intercrossing of 2 strains of inbred mice that differ for the phenotype of interest. F_1 generation mice are genetically identical to one another and are true hybrids of the parental strains. F_1 generation mice are then subject to brother-sister mating to form F_2 generation mice, which are phenotypically and genotypically diverse. Phenotype and genotype comparisons are made to identify which chromosomal loci correlate with the particular phenotype studied. Traditional QTL mapping in mice may use backcrosses as well [32]. Backcrossing involves the mating of F_1 generation mice back to a parental strain.

SNP-based *in silico* mapping studies utilize known SNP information across the genome to associate phenotypic data with available genetic information (genotypes). However unlike traditional QTL studies, *in silico* mapping investigates several (≥25) mouse strains. These comparisons allow the identification of chromosomal regions containing genes responsible for the evaluated phenotype (Fig1.2). The recent availability of denser marker sets (sets of known SNP information) has provided greater resolution for this technique. Typical QTL studies have a 2-5cM resolution, whereas, in SNP based *in silico* mapping studies, the relatively even spacing of the SNPs every 300kb allows for mapping traits within 0.5cM regions [33]. SNP based *in silico* mapping makes comparisons that are based on regions of the genome inherited from common ancestors as opposed to traditional QTL studies that rely only on loci inherited from different progenitors. Comparison across a greater number of strains provides greater statistical power. In addition, SNP based *in silico* mapping takes into account a wider range of phenotypic diversity by utilizing quantitative traits rather than traditional studies that only take into account dichotomous (completely opposing) phenotypes, such as presenting with a disease or without it. This type of haplotype associated mapping has successfully proven to be more powerful in narrowing chromosomal locations containing genes of interest than traditional QTL mapping in multiple studies [34, 35]. Table 1.2 summarizes the differences in the two approaches.

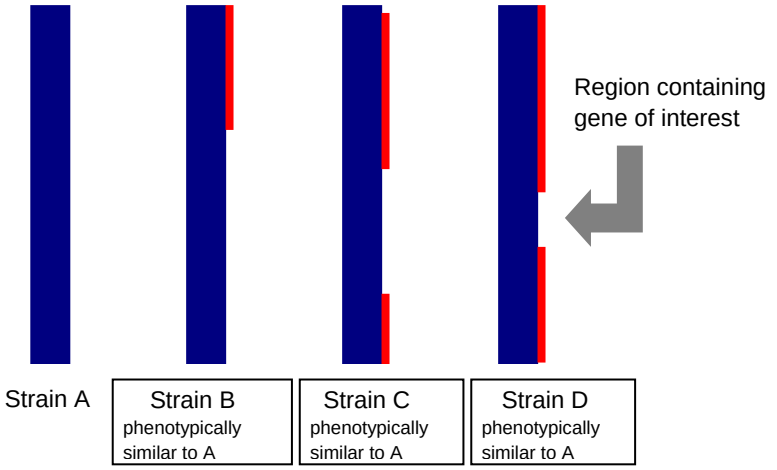
A



Blue bar represents a particular chromosome

Red bar represents regions of the chromosome that are genetically different from Strain A based on SNP data

B



Blue bar represents a particular chromosome

Red bar represents regions of the chromosome that are genetically different from Strain A based on SNP data

Figure 1.2: Hypothetical examples of how *in silico* comparisons are made.

Phenotypic similarities and differences are compared to SNP similarities and differences among mouse strains to identify chromosomal regions of interest that control phenotype. A. These comparisons take advantage of differences at SNP sites throughout the genome. B. These comparisons take advantage of similarities at SNP sites throughout the genome.

Table 1.2: Comparison of genome wide mapping techniques in mice

	Traditional QTL	**SNP Based *In Silico***
Genetic diversity	Utilizes 2 background strains	Utilizes ≥25 strains
Comparisons	Differences of 2 progenitor strains	Differences and similarities of ≥25 strains
Resolution	2-5cM	0.5cM

Some of these strategies have been useful in identifying chromosomal loci important to hematopoiesis. QTLs found for hematopoietic stem cell frequency have been very large, in the range of 10 or more cMs, when using traditional QTL studies, making it difficult to determine genes of importance [36-38]. One group identified 2 QTLs in regulating hematopoietic parameters in B6AKRF2 mice, which are derived from intercrossing AKR and C57BL/6 mice. They found that a QTL in chromosome 12 regulated neutrophils in the blood and a QTL at chromosome 2 regulated stem cell numbers in the bone marrow [39]. Interestingly, the gene *Alpq1* was located in this region. As you will find in chapter 2, our studies pointed to a region containing *Alpq3*. These genes were both identified in GWAS for alcohol preference [40]. Chronic alcohol use induces bone loss through the suppression of Wnt signaling [41]. Another group found a region in chromosome 2 important for response of progenitors to early cytokines [38]. These studies were limited in that they only investigated strains that were progeny of 2 background strains. This same group

investigated quantitative genetic variation in the hematopoietic stem cell and progenitor cell compartment properties and found them to be associated with life span QTLs [42]. Resolution in these studies was much lower than attainable by *in silico* mapping. We show in chapter 2 that *in silico* mapping offers much higher resolution for identifying these regions. Many regions in our study were much smaller regions within the previously mentioned QTLs for hematopoietic stem and early progenitor cell frequency.

Environment and hematopoiesis

Environmental influences have also been shown to influence hematopoiesis and risk for hematological malignancies. Radiation, benzene, and cold exposure have been shown to affect the process of hematopoiesis in a profound way [43-47]. Atomic bomb survivors have a high incidence of myelodysplastic syndromes as well as AML [48]. Treatment of other diseases, such as other cancers and ankylosing spondylitis, with radiation is also associated with these hematopoietic malignancies [49, 50]. Additionally, prior to modern radiation safety precautions, radiologists had increased incidence of AML and its precursors [51]. Benzene exposure also contributes to leukemia risk. It has toxic effects on bone marrow, such that progenitor cell colony formation is significantly declined with increasing benzene exposure [52]. Cold exposure has been shown to down-regulate the expression of many genes encoding hematopoietic transcription factors *(Runx1*, *Scl*, *C-myb*, and *Gata-2*) and particularly erythropoiesis related factors (*Klfd*, *Hbaa1*, *Ba1*, *Gata-1*, *Epo*, *and Epor*). Some myelopoiesis related factors, however, were

up-regulated in these low temperature conditions [47]. In chapters 3 and 4, we demonstrate that the content of FAs in the diet control myeloid progenitor cell frequency.

Lipids and hematopoiesis

Studies for cancer prevention and treatment have in recent years turned to the diet as it involves opportunities for natural ways to achieve these goals. Focus has centered on FA consumption in the diet and how it affects cancer risk. Particular interest for preventative measures as well as treatment options have focused on polyunsaturated fatty acids (PUFAs).

FAs are carboxylic acids with hydrocarbon chains ranging from 4 to 28 carbons in length and varying in their degree of saturation with hydrogen molecules. PUFAs are FAs that contain more than one double bond. They are structural components of membrane phospholipids and are the precursors to many signaling molecules. Omega-3 (n-3) and omega-6 (n-6) are PUFAs in which the n-3 and n-6 refer to the placement of the first double bond from the omega end of the FA chain (Fig1.3). n-3 FAs consist primarily of α-linolenic acid (ALA), eicosapentanoic acid (EPA), and docosahexanoic acid (DHA). In several research studies, n-3 FAs have been found to have suppressive effects on cancer [53-56]. n-6 FAs consist of linoleic acid (LNA) and arachidonic acid (ARA), among others. In many studies, n-6 FAs appear to promote cancer development [53, 55, 57]. n-3 and n-6 FAs have antagonizing effects in the body. n-6 FA prostaglandins tend to be pro-inflammatory and pro-proliferative, while n-3 FA prostaglandins are associated with the opposite qualities [58]. Suppression of n-6 FA-derived eicosanoids has been proposed as a strategy for chemoprevention [59]. As n-3 FAs compete for the same metabolic

enzymes as n-6 FAs, the ratio of n-3 to n-6 FAs in the diet is increasingly studied in addition to the content of these FAs individually.

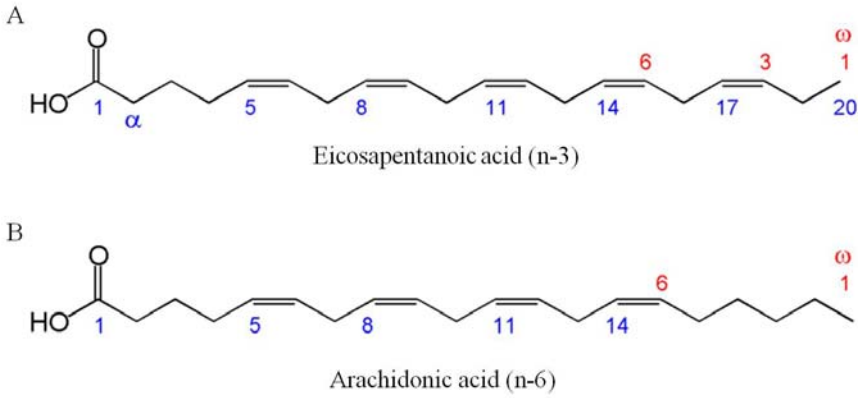


Figure 1.3: Examples of n-3 and n-6 fatty acids. A. Eicosapentanoic acid with first double bond in n-3 position. B. Arachidonic acid with first double bond in n-6 position.

The broad category of lipids contains FAs among others. Lipids have function in cell membranes, signaling, and energy storage. They have been shown to have several effects on hematopoiesis. Prostaglandin E2 (PGE2), derived from ARA, preferentially expands ST-HSCs/MPPs [60]. It also decreases macrophage colony stimulating factor (M-CSF) production by bone marrow stromal cells [61]. Many studies have shown that various lipoxygenases have key roles in regulating hematopoiesis as well [62-64]. Lipoxygenases are enzymes that catalyze the oxidation of PUFAs into lipidhydroperoxides. Many lipoxygenase isozymes are involved in the metabolism of eicosanoids. Recent studies have also investigated the functions of lysosomal acid lipase in hematopoiesis. It has been shown to play a critical role in myelopoiesis during hematopoietic development, differentiation, and homeostasis. Lysosomal acid lipase is a key enzyme involved in cleaving cholesteryl esters and triglycerides. This cleavage

generates free FAs and cholesterol in lysosomes. In lysosomal acid lipase knock out mice, there is increased proliferation of HSCs and myeloid progenitor cells. The microenvironment in these mice does not support normal hematopoiesis [65]. Valproic acid, a clear liquid FA at room temperature, is also involved in control of hematopoiesis. This histone deacetylase inhibitor is known to induce differentiation or apoptosis in leukemic blast cells. It is also involved in HSC proliferation and enhances the potential of interleukin 3 (IL-3) to stimulate megakaryopoiesis and erythropoiesis [66, 68].

A role for dietary lipids has been established in hematopoiesis. Ratios of n-3 to n-6 FAs in the diet have been shown to have effects in prevention or progression (depending on the ratio) of cancer [68, 69]. As progenitor cell frequency is important to understanding leukemia progression, we decided to investigate how ratios of dietary n-3 to n-6 FAs affect stem and progenitor cell biology in myelopoiesis. In chapters 3 and 4 we show that these ratios indeed play a significant role in regulating myeloid progenitor cell frequency.

Overall, this work proposes that genetic and environmental factors have related roles in controlling myeloid progenitor cell frequency. In chapter 2, we propose that genetic factors control hematopoiesis and that they can be identified by investigating polymorphisms associated with the control of myeloid progenitor cell frequency among inbred mouse strains. Our results suggest that genes that control metabolism and bone properties regulate this process. In chapters 3 and 4, we show that the environmental factor of the diet plays a significant role in controlling myeloid progenitor cell frequency.

Collectively, this work supports the roles of dietary FA content, lipid metabolism, and bone properties in regulating hematopoiesis.

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Chapter 2: *In silico* mapping suggests that bone properties and metabolism are key regulators of hematopoietic progenitor cell frequency in mice.

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Abstract

Upon discovering that various strains of inbred mice have significantly different myeloid progenitor cell pools, we hypothesized that polymorphisms between inbred strains could be used to identify genes that regulate myeloid progenitor cell frequency in the bone marrow. This information will be useful in understanding how hematopoietic malignancies develop. We used hematopoietic progenitor cell assays, determining progenitor cell frequency in 27 strains of inbred mice, and single nucleotide polymorphism (SNP) based *in silico* mapping techniques to investigate our hypothesis. These techniques returned several chromosomal loci containing cancer and hematological disease related genes. Additionally, a control region containing interleukin 3 receptor alpha (*IL-3Rα*), which is known to be involved in controlling myeloid progenitor cell frequency, had a strong association with the regulation of this phenotype in these *in silico* mapping studies. Upon meta-analysis of our data, we found that many chromosomal regions that presented with strong association or as regions of interest contain genes involved in bone formation, bone quality, and bone mechanics as well as lipid metabolism and insulin metabolism. These genes of interest suggest a complex combination of involvement in the properties of the bone specifically providing a support mechanism for the regulation of myeloid progenitor cell frequency. Further investigation of these genes may yield novel information as to how hematopoiesis is controlled, and therefore why it may go awry in hematopoietic malignancies, such as acute myelogenous leukemia.

Introduction

The process of hematopoiesis has been shown to depend on genetic and environmental factors. Fatty acid (FA) composition of the diet, radiation exposure, benzene exposure, and cold exposure are all environmental factors that affect this process of blood cell development [1-6]. Several genes have also been shown to be involved in controlling hematopoiesis. *Stat*, *Gata*, and *Hox* genes as well as *Pu.1*, *Runx1*, *Scl*, *IL-3Rα*, and many more genes have been shown to regulate this process [7-13]. *Stat-3* and *Stat-5* allow for survival and differentiation of myeloid hematopoietic cells [7]. *Gata-1* and Pu.1 control hematopoietic cell fate by antagonizing one another [8]. *Hox* genes control differentiation, proliferation, and cell fate during hematopoiesis [9-11]. *Runx1* is a master regulator of hematopoiesis [12]. *Scl* controls transcriptional programs during embryonic hematopoiesis and is vital to megakaryocyte and erythrocyte development [13-14]. Deletions in *IL-3Rα* are known to cause decreased hematopoietic progenitor cell frequency [15]. In our studies, we are continuing the search for genes involved in controlling hematopoiesis.

Hematopoiesis is a process that occurs in a stepwise manner, beginning with hematopoietic stem cells, continuing to develop into various stages of progenitor cells, and finally becoming fully functional blood cells. In hematopoietic malignancies, such as acute myelogenous leukemia (AML), this process is dysfunctional, allowing for the expansion of the myeloid progenitor cell compartment. This compartment contains early progenitors called granulocyte erythrocyte megakaryocyte macrophage (GEMM) progenitors that have the potential to develop into several blood cell types. It contains middle stage progenitors, such as granulocyte macrophage (GM) progenitors, that

develop from GEMM progenitors. These middle stage progenitors have less potential than their precursors. This compartment also contains late stage or committed progenitors, such as erythrocyte (E), granulocyte (G), and macrophage (M) progenitors. E progenitors also have GEMM progenitors as a precursor as well as a middle stage precursor that cannot be assayed with our colony forming cell (CFC) technique. These late stage progenitors, however, can only develop into erythrocytes, which have the primary function of delivering oxygen to tissues throughout the body. G and M progenitors develop from GM progenitors and only have the potential to develop into granulocytes or macrophages, respectively. Granulocytes and macrophages are cells of the immune system that defend against infection and foreign materials. Understanding more completely how the processes of development and differentiation of these progenitor cells are genetically controlled may provide valuable information as to how they may malfunction in cancers of the blood and lymph.

Myeloproliferative diseases of the bone marrow are conditions that involve an excess of progenitor cells of the myeloid lineage being produced. This precursor disease may evolve into myelodysplastic syndrome or AML. Myelodysplastic syndromes are bone marrow cell disorders in which the ineffective production of myeloid blood cells increases risk for transformation to AML. In AML, normal bone marrow is overtaken by immature functionless cells of the myeloid lineage, which causes a drop in normal blood cells and platelets. Learning how hematopoietic cell frequency is controlled at the progenitor cell level is vital to understanding why it becomes altered in these hematological disorders.

In order to determine how this process is genetically controlled, our lab used hematopoietic progenitor cell assays and *in silico* mapping techniques to identify chromosomal regions containing genes that control myeloid progenitor cell frequency [16]. We have since increased the number of inbred strains studied from 10 to 27. We also enhanced our assay to investigate the frequency of individual progenitor types. *In silico* mapping databases have been improved to include increased sets of known SNP information throughout the genome (denser marker sets) as well. This has increased statistical power in our studies.

Materials and methods

Animals

All mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Strains include 129S1/SvImJ, A/J, AKR/J, BALB/cJ, BALB/cByJ, BTBRT+tf/J, C3H/HeJ, C57BL/6J, C57BL/10J, C57BLKS/J, C57BR/cdJ, C57L/J, C58/J, CBA/J, DBA/1J, DBA/2J, FVB/NJ, I/lnJ, LP/J, MA/MyJ, NZB/BINJ, NZW/lacJ, PERA/EiJ, PL/J, SEA/GnJ, SM/J, and WSB/EiJ. Strains were chosen based on previously published results [17]. Only male mice were used to avoid cyclic changes in hormones. Mice were housed in the AAALAC accredited animal facilities of the Marshall University School of Medicine. All animal use and care was approved by the Marshall University Institutional Animal Care and Use Committee. Bone marrow was harvested at 10-14 weeks of age.

Colony forming cell assays

CFC assays were performed upon bone marrow isolated from mice as previously described [16, 18]. Briefly, bone marrow was harvested by flushing from the femurs with Iscove's Modified Dulbecco's Medium (IMDM), and cells were counted and seeded in 4 well plates at a density determined empirically by a pilot study conducted with a broad and consistent range of seeding densities. After linear response of colony production to seeding density was ensured by the pilot study, seeding densities were chosen to produce 10-30 colonies for each well (4-well plates). Bone marrow was cultured in 1% semi-solid Methocult M3434 (StemCell Technologies), supplemented with 0.4% autochthonous sera. Cells were incubated for 6-7 days to allow colony formation. Colonies with a minimum cell number of 20 were scored as positive using an inverted microscope at 40X magnification. Colonies were counted based on morphological features that are associated with each progenitor type.

***In silico* mapping**

The SNPs used for genome-wide association tests are found in the Mouse Diversity Array [19]. A total of 194,363 informative SNPs were used for SNPster and efficient mixed model association (EMMA) analysis. Our data set was analyzed using SNPster genome-wide association software (http://snpster.gnf.org), which gives a phenotype/genotype correlation score in the form of -logp [17, 20]. These -logp scores are used to identify regions of the genome that have a high probability of non-random correlation. Heritability was measured based on standard deviation within the strains as compared with the variability between strains. This measure of heritability is

incorporated in the algorithms used in the analyses. Heritability is rated on a percentage scale in which a higher percentage reflects a higher heritability. Figures and tables show -logp values based on SNPster analysis. Based on the -logp value of the *IL-3Rα* control region (3.02, p-value of 0.00095) and the need to minimize spurious associations, regions with -logp values above 2.5 (p-value of 0.0032), were considered of interest, and regions with a -logp value above 2.8 (p-value of 0.0016) were considered of strong association. SNPster -logp values are not corrected for multiple comparisons.

We also employed the EMMA R package for genome-wide association scans as an alternative to SNPster analysis. In this model, restricted maximum likelihood (REML) was used to estimate the variance component, which is followed by a t-test. This method accounts for strain relatedness to reduce the number of false positives [21].

Evaluation of *Interleukin-3 Receptor Alpha*

Genomic DNA was obtained from mouse liver. To amplify intron 7 of the murine IL-3Rα gene, 100ng of genomic DNA from each strain and two primers (STP-44 and STP-23) were used. The primers used were 5'-AACAGATTCCACCATGGCCTCC-3' (STP-44) and 5'-TCTGACCTCGACTTGACCCGG-3' (STP-23). PCR was performed by 31 cycles of 94°C for 1 minute, 53°C for 2 minutes, and 72°C for 3 minutes using AmpliTaq Gold Faststart Polymerase (Applied Biosystems). PCR products were digested with DdeI endonuclease (Promega) and analyzed on a 2% agarose gel using ethidium bromide for detection of bands. A Mann Whitney test (using SigmaPlot) was performed on genotypes A and B.

Meta-analysis

For pathway analysis, we selected the top 50-55 genes within the chromosomal regions with the highest -logp values from the *in silico* mapping analyses. We defined the boundaries of the regions of interest as 90% of the highest -logp value in each peak. Chromosomal regions were then entered into the Mouse Genome Information database to determine the known genes that exist in the regions. The top 50 genes for each phenotype were subjected to literature searches as well as Ingenuity Pathway Analysis v8.7 (IPA, Ingenuity Systems Inc.) analyses to determine gene properties and network interactions. IPA analyzes the combination of genes in signaling networks and statistically evaluates the probability of the network against the probability that a network would form at random. Networks are subject to a Fisher's exact test, and those with p-values less than 0.001 were considered to be significant. Top 50-55 genes lists for each phenotype can be found in the addendum.

Results

Our previous experiments in investigating the myeloid progenitor compartment in multiple strains of inbred mice revealed that myeloid progenitor cell frequency varies among strains [16]. Use of *in silico* mapping to compare these phenotypic similarities and differences to the genetic similarities and differences among them was previously limited due to a relatively low number of strains studied and less informative databases. To increase the power and specificity of this technique, we have increased the amount of strains being studied and refined our assay to study each progenitor type individually as well as total progenitor cell frequency.

In silico mapping utilizes SNP information across the genome to compare phenotypic information to available genetic information about mouse strains. These comparisons allow the identification of chromosomal regions containing genes responsible for the evaluated phenotype. The availability of denser marker sets and an increased number of strains being studied provided a greater statistical power for this technique [19].

Twenty-seven mouse strains were evaluated for myeloid progenitor cell frequency. These strains have varied ancestry, thus allowing *in silico* mapping techniques to take advantage of closely related strains as well as strains that are more genotypically diverse. The majority of mice were of Castle (129S1/SvImJ, A/J, AKR/J, BALB/cJ, BALB/cByJ, C3H/HeJ, CBA/J, DBA/1J, DBA/2J, I/LnJ, LP/J, NZB/BINJ, NZW/lacJ, SEA/GnJ, and SM/J) or C-57 related ancestry (C57BL/6J, C57BL/10J, C57BLKS/J, C57BR/cdJ, C57L/J , C58/J, and MA/MyJ). Additional strains were of Swiss (FVB/NJ), wild(PERA/EiJ and WSB/EiJ), other (PL/J), or unknown (BTBRT+tf/J) ancestry. There was no correlation between ancestry and phenotypic data. GEMM, GM, G, M, and E progenitor cell frequencies were evaluated as well as total myeloid progenitor cell frequency. Phenotypic data was then compared to genotypic information using *in silico* mapping.

Early progenitor frequency and *in silico* mapping

The earliest progenitor type evaluated by CFC assays was the GEMM progenitor. This phenotype varied among inbred mouse strains, with a range of 0.005-0.449 colonies/1000 cells seeded, and an 89.8-fold difference between the strain with the

highest progenitor cell frequency and the strain with the lowest progenitor frequency. Phenotypic data for GEMM progenitor cell frequency is shown in Figure 2.1A as colonies counted per 1000 cells seeded. *In silico* mapping revealed several regions with -logp values above 2.5, and several regions with a -logp value above 2.8. *In silico* mapping results using SNPster software are shown in Figure 2.1B. EMMA analysis was also performed for this data. It showed similar results, with the region in chromosome 14 having significant genome wide association after tests for multiple comparisons. Table 2.1 shows chromosomal regions with -logp values above 2.5, and the genes that are known to be located in those particular regions. Genes that are associated with leukemia or known to be involved in hematopoiesis are shown in bold.

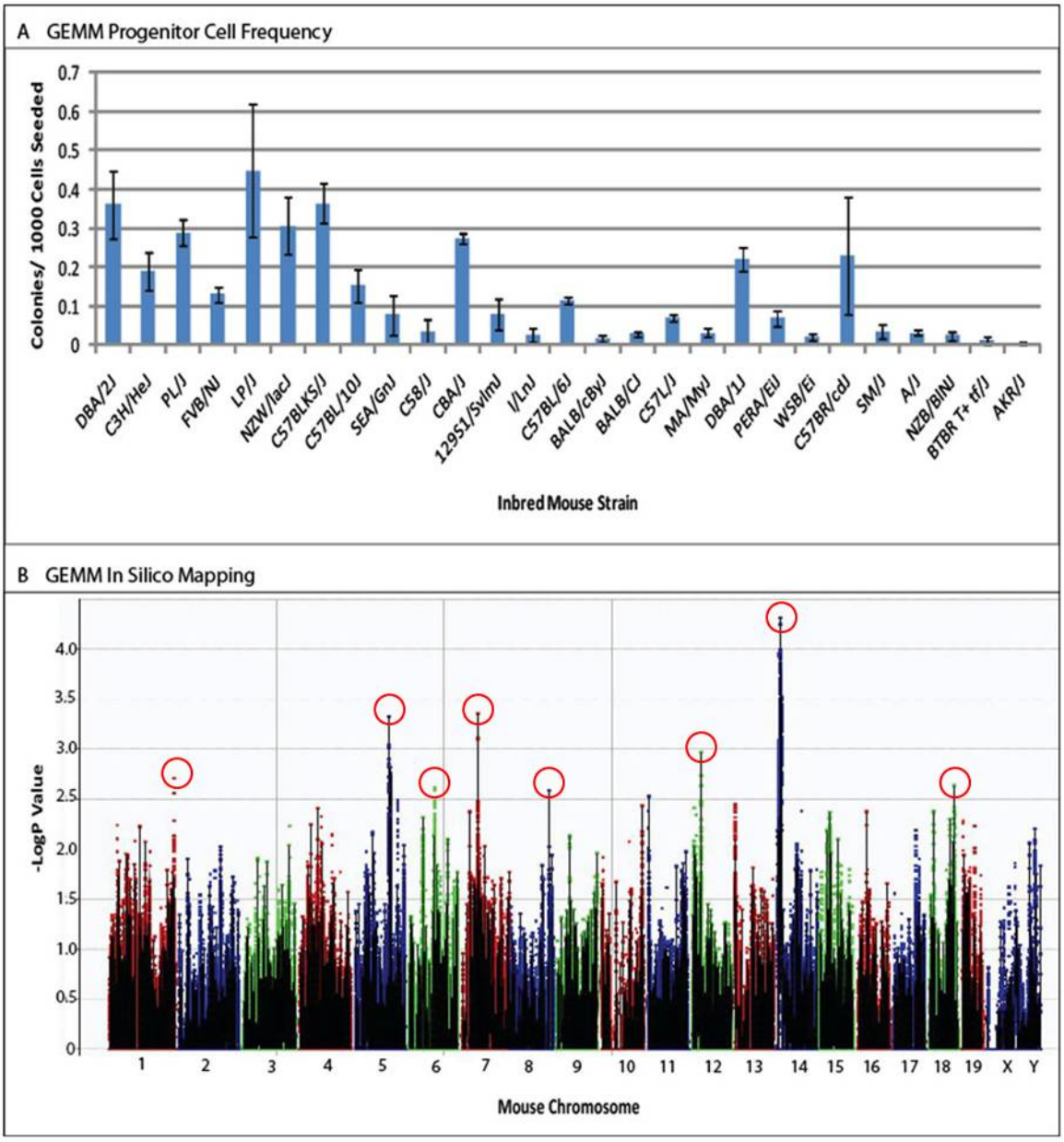

Figure 2.1: Frequency of GEMM progenitor cells and *in silico* mapping. A: GEMM progenitor cell frequency as measured by CFC assays. Results are shown as colonies/1000 cells seeded. Error bars represent SEMs (n = 3-5). B: *In silico* mapping results when phenotypic information from part A was compared with genotypic information based on SNP information for the various inbred mouse strains. Results are shown as a -logp value. Circles point out peaks with a -logp value above 2.5.

Table 2.1: Genes of interest in regulating GEMM frequency

Genes located in chromosomal region with –logp values above 2.8		
Gene	**Region**	**-logp value**
Ptprg, protein tyrosine phosphatase, receptor type, G	**14: 12608073-12623845**	**4.309804**
Cadps, Ca2+-dependent secretion activator	14: 13451373-13528685	4
Fhit, fragile histidine triad gene	**14: 10693745-10736604**	**3.946922**
Rthyd3, resistance to thymic deletion 3	14: 13387864-13423496	3.928118
n-R5s45, nuclear encoded rRNA 5S 45	14: 13196426-13337728	3.850781
Synpr, synaptoporin	14: 14291097-14489374	3.793175
Atxn7, ataxin 7	14: 14725638-15083409	3.777284
Olfr720, olfactory receptor 720	14: 14725638-15083409	3.777284
Psmd6, proteasome (prosome, macropain) 26S subunit, non-ATPase, 6	14: 14725638-15083409	3.777284
Thoc7, THO complex 7 homolog (Drosophila)	14: 14725638-15083409	3.777284
Rpl19-ps4, ribosomal protein L19, pseudogene 4	14: 14552540-14662129	3.725843
Sntn, sentan, cilia apical structure protein	14: 14290630-15106086	3.671621
Fezf2, Fez family zinc finger 2	14: 13174979-13450378	3.659556
Lrrc3b, leucine rich repeat containing 3B	14: 16103926-16294228	3.518558
Ngly1, N-glycanase 1	14: 17064860-17090557	3.497573
Oxsm, 3-oxoacyl-ACP synthase, mitochondrial	14: 17064860-17090557	3.497573
Rpl21-ps4, ribosomal protein L21, pseudogene 4	14: 12042049-12260227	3.469801
Iglon5, IgLON family member 5	7: 50735869-50778325	3.354578
Arhgap24, Rho GTPase activating protein 24	**5: 102870089-102911460**	**3.321482**
Tmc1m4, Tmc1 modifier 4	5: 102870089-102911460	3.321482
Appd2, APP associated premature death 2	14: 17434991-17778674	3.254926
Rpl31-ps3, ribosomal protein L31, pseudogene 3	14: 11970057-12401283	3.219683
Olfr721-ps1, olfactory receptor 721, pseudogene 1	14: 15224397-15419224	3.192465
Olfr31, olfactory receptor 31	14: 14162704-15177633	3.162412
Rarb, retinoic acid receptor, beta	**14: 17260939-17434727**	**3.109579**
Top2b, topoisomerase (DNA) II beta	**14: 17260939-17434727**	**3.109579**
Wbcq6, white blood cell quantitative locus 6	**14: 17260939-17434727**	**3.109579**
Nek10, NIMA (never in mitosis gene a)- related kinase 10	14: 15683725-15737034	3.084601
n-R5s44, nuclear encoded rRNA 5S 44	14: 9779720-9981564	3.059484
Il3ra, interleukin 3 receptor, alpha chain	**14: 13985651-15492174**	**3.024569**
Slc4a7, solute carrier family 4, sodium bicarbonate cotransporter, member 7	14: 11799713-15618059	2.995591
Evi5, ecotropic viral integration site 5	**5: 108163334-108178591**	**2.818136**
Genes located in chromosomal region with –logp values above 2.5		
Gene	**Region**	**-logp value**
Ep400, E1A binding protein p400	**5: 111108675-111133204**	**2.759341**
Ptpn13, protein tyrosine phosphatase, non-receptor type 13	**5: 103855361-103866161**	**2.71364**
Idd21.1, insulin dependent diabetes susceptibility 21.1	18: 78923250-78937776	2.630284
Bmd8, bone mineral density 8	6: 80953210-80967127	2.614961
Efw, epididymal fat weight	6: 80953210-80967127	2.614961
Klhl8, kelch-like 8 (Drosophila)	5: 104324701-104326563	2.600348
Bmd39, bone mineral density 39	8: 118356974-118360978	2.582694
Tpi-rs9, triosephosphate isomerase related sequence 9	14: 10871174-18415403	2.569667
Ascc2, activating signal cointegrator 1 complex subunit 2	14: 9997616-18431455	2.548268

The top region (-logp value of 4.3), located in chromosome 14, has a size of 15.8kbp. It contains protein tyrosine phosphatase, receptor type g (*Ptprg*), a known tumor suppressor gene in mice and humans [22]. It is regulated by estrogen signaling and is associated with control of peak bone mineral density in mice [23, 24]. The second region (-logp value of 4.0), also located in chromosome 14, is 77.31kbp, and contains the gene calcium2^{+}-dependent secretion activator (*Cadps*). This gene's expression is associated with neuroendocrine tumors called pheochromocytomas, which may be malignant [25]. Cadps also has known function in controlling stability and recruitment of insulin granules [26]. A third peak (-logp value of 3.9) also located in chromosome 14 spans 42.86kbp and contains the fragile histidine triad gene (*Fhit*). This gene is a known tumor suppressor with an aphidicolin-inducible fragile site (FRA3B) known for deletions that are frequently observed in de novo acute leukemias [27]. Fhit is associated with type 2 diabetes and has been shown to inhibit prostaglandin E2 mediated cancer progression [28, 29].

IL-3Rα genotyping as validation of technique

To validate the accuracy of our *in silico* mapping techniques, the region containing interleukin 3 receptor alpha (*IL-3Rα)* presented as a significant region with a -logp value of 3.02. This region was to serve as a positive control as it presented as a region of interest in previous experiments in our laboratory. It is known to have a deletion that eliminates the consensus sequence of branch points for RNA splicing [15].This deletion causes aberrant splicing of *IL-3Rα*, which results in the production of a shorter form of *IL-3Rα.* This form is not transported to the cell surface and therefore does not

mediate actions of IL-3. In multiple strains of mice, this form of *IL-3Ra*, which contains the 5bp deletion, reduces progenitor cell frequency [15]. We evaluated *IL-3Rα* in the 27 mouse strains for genotype based on a previously published method (Fig.2.2) [15]. *IL-3Rα* can have two forms in mice. The A-type mice have a 5bp deletion, whereas the B-type mice do not. These genotypes were determined by PCR amplification of the region of *IL-3Rα* including intron 7, which is the location of the branch-point deletion, followed by DdeI endonuclease digestion to analyze A and B-type genes. DdeI cuts twice in the wild-type intron 7 mice (type B) and only once in mutant intron 7 with the 5bp deletion (type A). We compared this genotypic information to the phenotypic order (highest progenitor cell frequency to lowest progenitor cell frequency) for the GEMM phenotype (Table 2.2). The genotypic form A is found only in strains with low progenitor cell frequencies. This finding is consistent with the idea that the A type of *IL-3Rα* is involved in contributing to low progenitor cell frequency, and therefore served as a proper control for our study. There are some strains with low progenitor cell frequency that are B-type mice as well. This was anticipated as myeloid progenitor cell frequency is a complex trait. A Mann Whitney test (using SigmaPlot) was performed on genotypes A and B. A and B types of *IL-3Rα* were significantly different with a p-value of 0.0028.

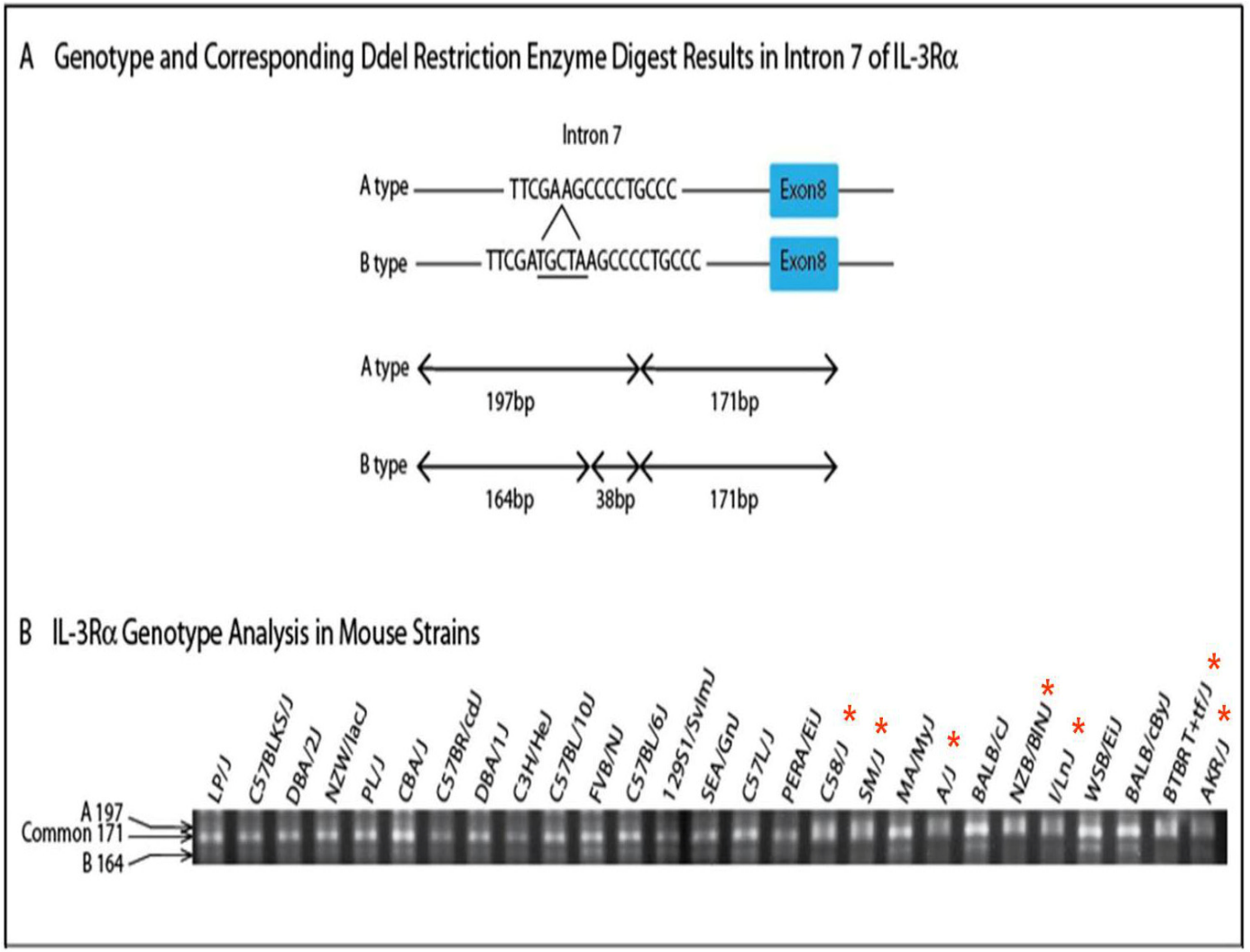
A Genotype and Corresponding DdeI Restriction Enzyme Digest Results in Intron 7 of IL-3Rα
Intron 7
A type
TTCGAAGCCCCTGCCC
Exon8
B type
TTCGATGCTAAGCCCCTGCCC
Exon8
A type
197bp
171bp
B type
164bp
38bp
171bp
B IL-3Rα Genotype Analysis in Mouse Strains
A 197
Common 171
B 164
LP/J
C57BLKS/J
DBA/2J
NZW/lacJ
PL/J
CBA/J
C57BR/cdJ
DBA/1J
C3H/HeJ
C57BL/10J
FVB/NJ
C57BL/6J
129S1/SvImJ
SEA/GnJ
C57L/J
PERA/EiJ
C58/J
SM/J
MA/MyJ
A/J
BALB/cJ
NZB/BINJ
I/LnJ
WSB/EiJ
BALB/cByJ
BTBR T+tf/J
AKR/J

Figure 2.2: Genotype of *IL-3Rα* in mice. A. Type A has a 5bp deletion previously shown to be associated with low progenitor cell frequency. DdeI restriction enzyme digestion results in 2 bands for Type A and 3 bands for Type B IL-3Rα. B. Mouse liver DNA was amplified using primers specific to IL-3Ra. PCR products were then digested with DdeI and run on a 2% agarose gel to determine genotype. Asterisks denote type A strains. A Mann Whitney test (using SigmaPlot) was performed on genotypes A and B. A and B types of *IL-3Rα* were significantly different with a p-value of 0.0028.

Table 2.2: Comparison of GEMM phenotype and IL-3Ra genotype

Comparison of GEMM Phenotype and IL-3Ra Genotype		
Strain	**GEMM Progenitor Cell Frequency (Colonies/1000 Cells Seeded)**	**Genotype**
LP/J	0.448718	B
C57BLKS/J	0.363446	B
DBA/2J	0.360417	B
NZW/lacJ	0.306467	B
PL/J	0.288063	B
CBA/J	0.273611	B
C57BR/cdJ	0.230556	B
DBA/1J	0.220212	B
C3H/HeJ	0.188523	B
C57BL/10J	0.152206	B
FVB/NJ	0.130556	B
C57BL/6J	0.114978	B
129S1/SvImJ	0.079167	B
SEA/GnJ	0.077083	B
C57L/J	0.069031	B
PERA/EiJ	0.068524	B
C58/J	0.034921	A
SM/J	0.034722	A
MA/MyJ	0.032044	B
A/J	0.03197	A
BALB/cJ	0.029405	B
NZB/BINJ	0.024889	A
I/LnJ	0.02375	A
WSB/EiJ	0.022991	B
BALB/cByJ	0.016964	B
BTBRT+tf/J	0.011111	A
AKR/J	0.005	A

Middle-stage progenitor frequency and *in silico* mapping

A middle-stage progenitor type evaluated was the GM progenitor. This phenotype varied among inbred mouse strains, with a range of 0-0.378 colonies/1000 cells seeded and greater than a 147-fold difference in the strain with the highest progenitor cell frequency and the strain with the lowest progenitor frequency (Fig.2.3A). *In silico* mapping revealed one region with a -logp value above 2.8 and multiple regions with a -logp value above 2.5. *In silico* mapping results using SNPster software are shown in Figure 2.3B. Regions in chromosomes 14 and 15 were of genome wide significance after correcting for multiple comparisons using EMMA analysis. Table 2.3 shows chromosomal regions with -logp values above 2.5, and the genes that are known to be located in those particular regions. Genes that are associated with leukemia or known to be involved in hematopoiesis are shown in bold.

The top region for this phenotype spans 27.42kbp in chromosome 14 and contains 4 known genes: atherosclerosis 13 (*Ath13*), cocaine seizure 2 (*Cosz2*), plasma plant sterol 1 (plast1), and plasma plant sterol 1b (*Plast1b*). *Ath13* is a gene found in an atherosclerosis predisposition study using *in silico* QTL mapping [30]. Plant sterols occur naturally in small quantities in vegetable oils and are also available as food additives as they have cholesterol lowering properties [31]. In a genome wide association study, *Cosz2* was found to be associated with cocaine induced seizure thresholds [32].

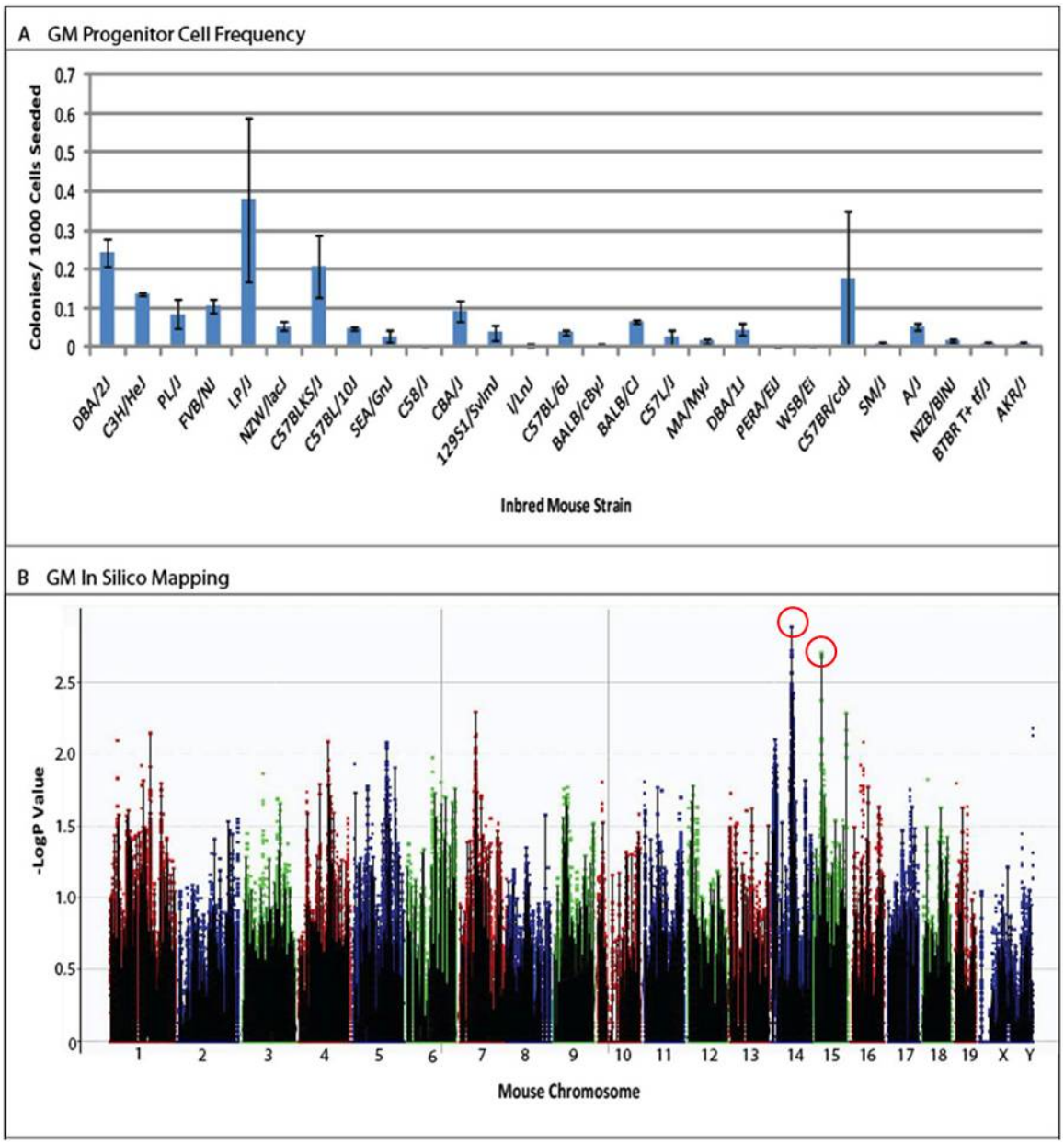

Figure 2.3: Frequency of GM progenitor cells and *in silico* mapping. A: GM progenitor cell frequency as measured by CFC assays. Results are shown as colonies/1000 cells seeded. Error bars represent SEMs (n = 3-5). B: *In silico* mapping results when phenotypic information from part A was compared with genotypic information based on SNP information for the various inbred mouse strains. Results are shown as a -logp value. Circles point out peaks with a -logp value above 2.5.

Table 2.3: Genes of interest in regulating GM frequency

Genes located in chromosomal region with –logp values above 2.8		
Gene	**Region**	**-logp value**
Ath13, atherosclerosis 13	14: 63145626-63173054	2.889386
Cosz2, cocaine seizure 2	14: 63145626-63173054	2.889386
Plast1, plasma plant sterol 1	14: 63145626-63173054	2.889386
Plast1b, plasma plant sterol 1b	14: 63145626-63173054	2.889386
Genes located in chromosomal region with –logp values above 2.5		
Gene	**Region**	**-logp value**
Fbxl7, F-box and leucine-rich repeat protein 7	15: 26533596-26539153	2.705725
Defb43, defensin beta 43	14: 63614409-63639344	2.678805
Defb47, defensin beta 47	14: 63614409-63639344	2.678805

Late-stage progenitor frequency and *in silico* mapping

The late stage progenitor phenotypes (E, G, and M) varied among inbred mouse strains, with ranges of 0.157-0.831, 0.190-0.653, and 0.239-1.055 cells/1000 cells seeded, respectively (Fig. 2.4A, 2.5A, and 2.6A). Fold differences in the strain with the highest progenitor cell frequency and the strain with the lowest progenitor frequency were 5.3-fold in E, 3.4-fold in G, and 4.4-fold in M phenotypes. *In silico* mapping revealed multiple regions in each with -logp values above 2.5. *In silico* mapping results using SNPster software are shown in Figure 2.4B, 2.5B, and 2.6B. Tables 2.4, 2.5, and 2.6 show chromosomal regions with -logp values above 2.5, and the genes that are known to be located in those particular regions. Genes that are associated with leukemia or known to be involved in hematopoiesis are shown in bold.

The top region for E progenitor cell frequency is a 46.23kbp gene in chromosome 5 containing serine/threonine kinase 32b (Stk32b). This gene is associated with the skeletal dysplasias of Ellis-van Creveld syndrome and Weyers acrodental dysostosis [33]. The top region for G progenitors is a 26.31kbp region in chromosome 11 containing the genes, calcium/calmodulin-dependent protein kinase II beta (Camk2b) and YTK6 homolog (Ykt6). Group VIA phospholipase A2 forms a signaling complex with Camk2b

expressed in pancreatic islet beta-cells. This complex participates in insulin secretion. Their expression is co-induced upon differentiation of pancreatic progenitor to endocrine progenitor cells [34]. Ytk6 has been associated with tumor invasion and metastasis [35]. It is also known to mediate protein palmitoylation, the covalent attachment of FAs to cysteine residues of membrane proteins [36]. Another region of significance for this phenotype is a 6kbp region located in Chromosome 8. It contains the genes bone mineral density 39 (Bmd39) and tetratricopeptide repeat domain 29 (Ttc29). Bmd39 contributes to the degree to which bone tissues respond to mechanical loading [37]. Little is known about Ttc29. The top region for M progenitors contains the gene Fhit, discussed above.

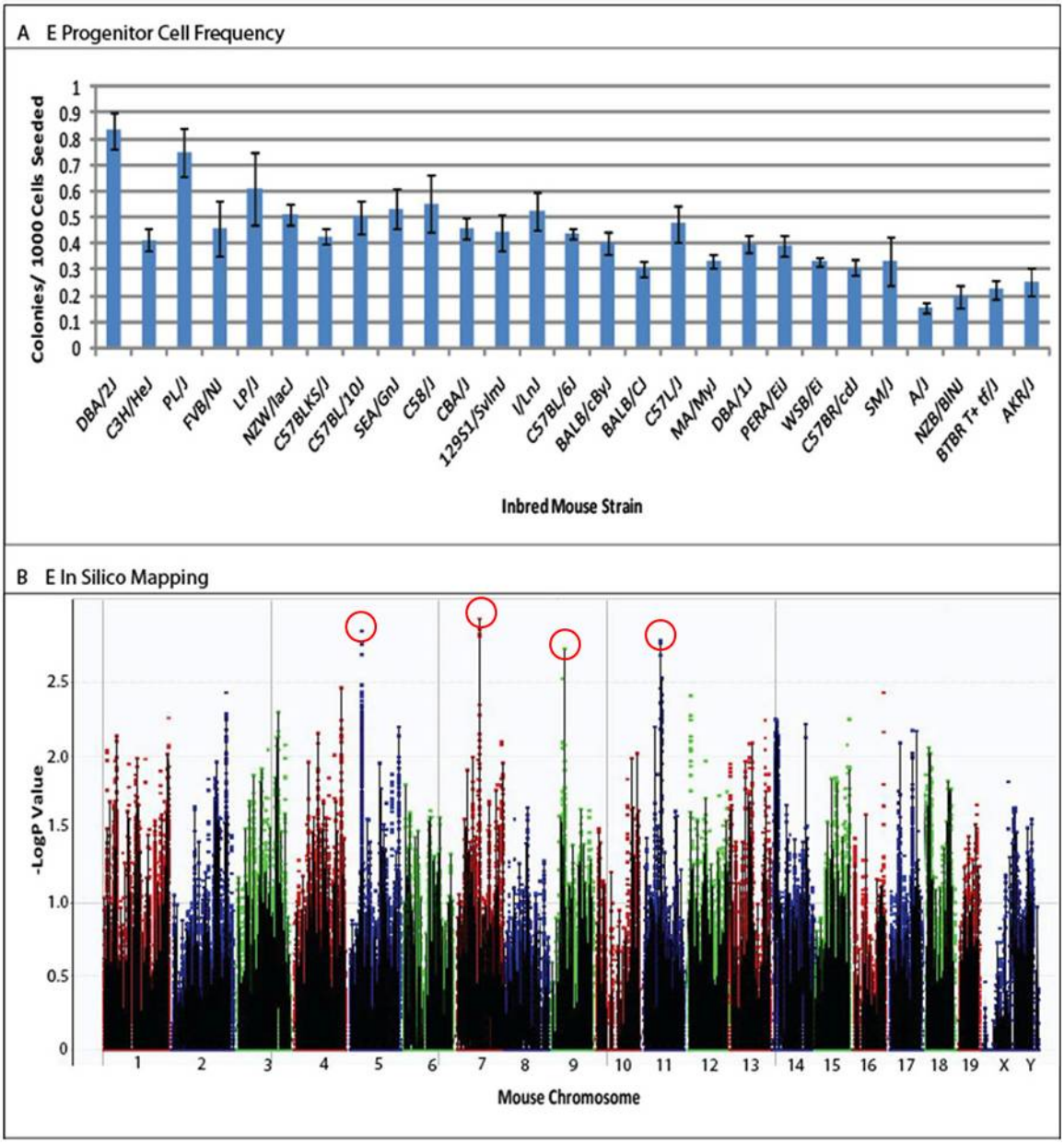

Figure 2.4: Frequency of E progenitor cells and *in silico* mapping. A: E progenitor cell frequency as measured by CFC assays. Results are shown as colonies/1000 cells seeded. Error bars represent SEMs (n = 3-5). B: *In silico* mapping results when phenotypic information from part A was compared with genotypic information based on SNP information for the various inbred mouse strains. Results are shown as a -logp value. Circles point out peaks with a -logp value above 2.5.

Table 2.4: Genes of interest in regulating E frequency

Genes located in chromosomal region with –logp values above 2.8		
Gene	**Region**	**-logp value**
Stk32b, serine/threonine kinase 32B	5: 38038420-38084656	2.849933
Genes located in chromosomal region with –logp values above 2.5		
Gene	**Region**	**-logp value**
Ity2a, immunity to S. typhimurium 2a	11: 52093205-52150216	2.79202
Tcf7, transcription factor 7, T-cell specific	**11: 52093205-52150216**	**2.79202**
W6q3, weight 6 weeks QTL 3	11: 52093205-52150216	2.79202
Alpq3, alcohol preference QTL 3	9: 42048114-42065818	2.728445
Sc5d, sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (S. cerevisiae)	9: 42048114-42065818	2.728445
Crmp1, collapsin response mediator protein 1	**5: 37681204-37711624**	**2.690689**
Evc, Ellis van Creveld gene homolog (human)	5: 37681204-37711624	2.690689
Blmpf2, bleomycin-induced pulmonary fibrosis 2	11: 54038026-54056774	2.532897
Cia40, collagen induced arthritis 40	11: 54038026-54056774	2.532897
Ckdbp2, chronic kidney disease blood pressure locus 2	11: 54038026-54056774	2.532897
Pregq1, pregnancy QTL 1	11: 54038026-54056774	2.532897
Elnv, epilepsy naïve	9: 34385442-34401249	2.527766
Igan3, IgA nephropathy 3	9: 34385442-34401249	2.527766

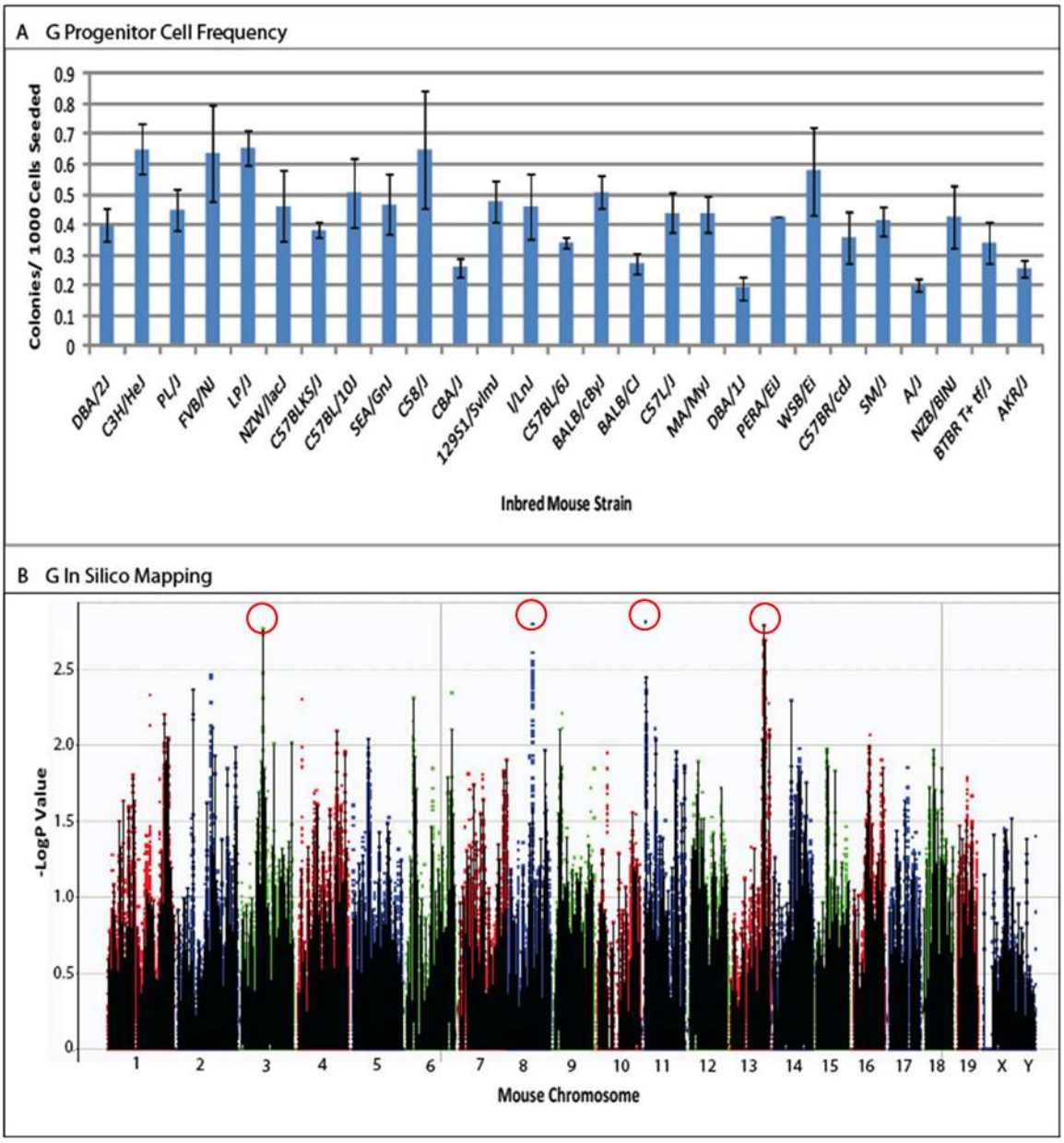

Figure 2.5: Frequency of G progenitor cells and *in silico* mapping. A: G progenitor cell frequency as measured by CFC assays. Results are shown as colonies/1000 cells seeded. Error bars represent SEMs (n = 3-5). B: *In silico* mapping results when phenotypic information from part A was compared with genotypic information based on SNP information for the various inbred mouse strains. Results are shown as a -logp value. Circles point out peaks with a -logp value above 2.5.

Table 2.5: Genes of interest in regulating G frequency

Genes located in chromosomal region with -logp values above 2.8		
Gene	**Region**	**-logp value**
Camk2b, calcium/calmodulin-dependent protein kinase II, β	**11: 5864894-5891207**	**2.815092**
Ykt6, YKT6 homolog (S. Cerevisiae)	11: 5864894-5891207	2.815092
Bmd39, bone mineral density 39	8: 80875682-80881683	2.801206
Ttc29, tetratricopeptide repeat domain 29	8: 80875682-80881683	2.801206
Genes located in chromosomal region with -logp values above 2.5		
Gene	**Region**	**-logp value**
Erbb2ip, Erbb2 interacting protein	**13: 104665228-104684576**	**2.794302**
Tbqt5, tibia bone quality traits 5	13: 104665228-104684576	2.794302
Nln, neurolysin (metallopeptidase M3 family)	13: 104701994-104824890	2.694254
Srsf12, serine/arginine-rich splicing factor 12	13: 104528844-104542789	2.686086
Pou4f2, POU domain, class 4, transcription factor 2	8: 80947042-81014484	2.613413

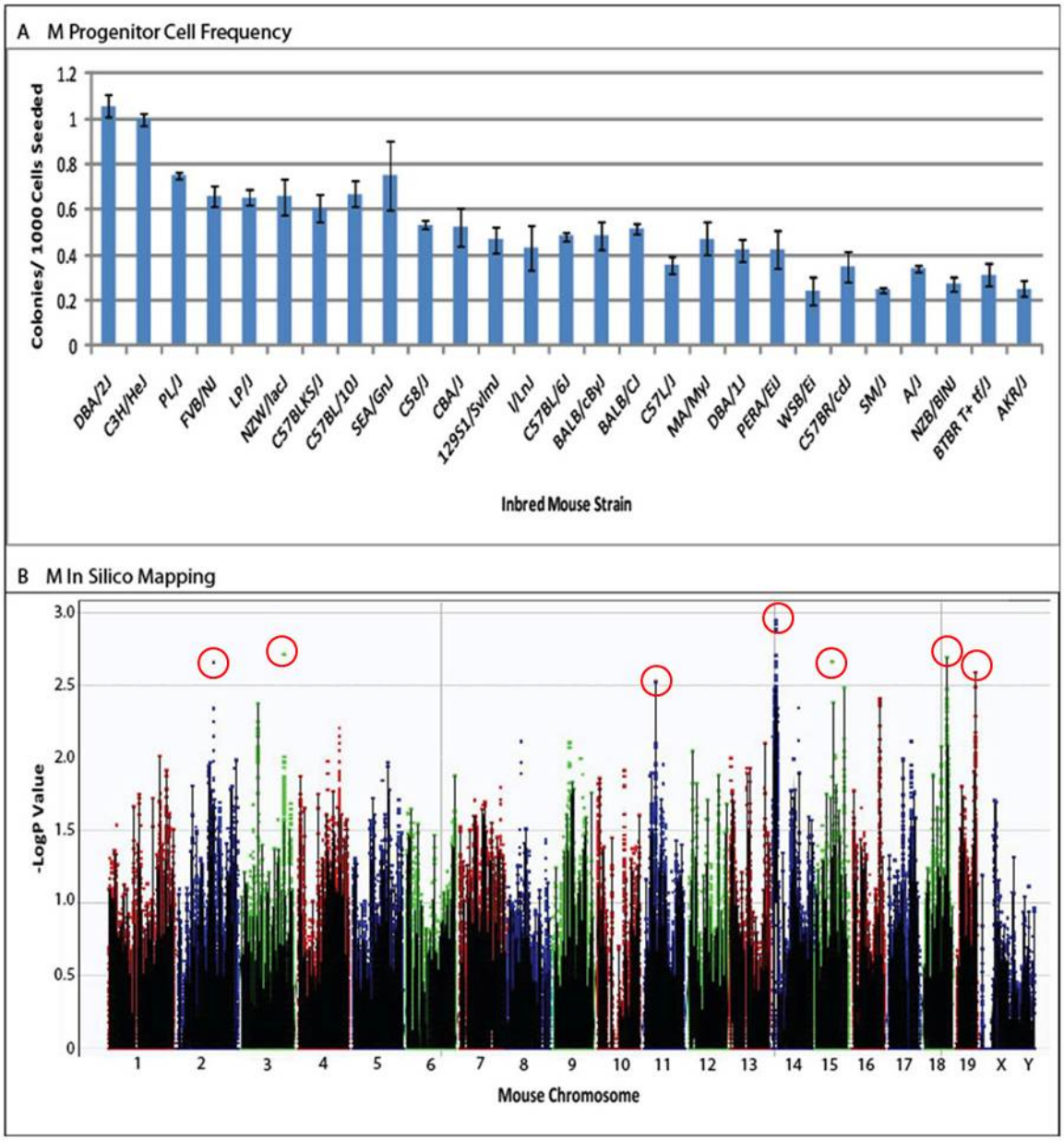

Figure 2.6: Frequency of M progenitor cells and *in silico* mapping. A: M progenitor cell frequency as measured by CFC assays. Results are shown as colonies/1000 cells seeded. Error bars represent SEMs (n = 3-5). B: *In silico* mapping results when phenotypic information from part A was compared with genotypic information based on SNP information for the various inbred mouse strains. Results are shown as a -logp value. Circles point out peaks with a -logp value above 2.5.

Table 2.6: Genes of interest in regulating M frequency

Genes located in chromosomal region with –logp values above 2.8		
Gene	**Region**	**-logp value**
Fhit, fragile histidine triad gene	**14: 11584520-11656529**	**2.94897**
Genes located in chromosomal region with –logp values above 2.5		
Gene	**Region**	**-logp value**
Ctrq2, C. trachomatis resistance QTL 2	3: 131789521-131823292	2.714458
Dkk2, dickkopf homolog 2 (Xenopus laevis)	3: 131789521-131823292	2.714458
Dym, dymeclin	18: 75266222-75267209	2.693152
Idd21.1, insulin dependent diabetes susceptibility 21.1	18: 75266222-75267209	2.693152
Pgis1, proteoglycan induced spondylitis 1	18: 75266222-75267209	2.693152
Bmd4, bone mineral density 4	15: 56618365-56626586	2.660179
Mob5, multigenic obesity 5	2: 110016970-110020628	2.655399
Plast2b, plasma plant sterol 2b	2: 110016970-110020628	2.655399
T2dm2sa, type 2 diabetes mellitus 2 in SMXA RI mice	2: 110016970-110020628	2.655399
Tmc1m1, Tmc1 modifier 1	2: 110016970-110020628	2.655399
Dclre1a, DNA cross-link repair 1A, PSO2 homolog (S. cerevisiae)	19: 56596851-56621288	2.587244
Rpl21-ps4, ribosomal protein L21, pseudogene 4	14: 12042049-12162496	2.564197
Slit3, slit homolog 3 (Drosophila)	11: 35100995-35120899	2.520235
W6q3, weight 6 weeks QTL 3	11: 35100995-35120899	2.520235

Total progenitor frequency and *in silico* mapping

Total progenitor cell frequency varied among strains with a range of 0.855-2.933 colonies/1000 cells seeded and a 3.4 fold difference in the strain with the highest progenitor cell frequency and the strain with the lowest progenitor frequency (Fig.2.7A). *In silico* mapping results using SNPster software are shown in Figure 2.7B. Table 2.7 shows genes within regions that have -logp values above 2.5. Genes that are associated with leukemia or known to be involved in hematopoiesis are shown in bold. Fhit and Ptprg (genes found in regions of significance in other phenotypes) were located in the top chromosomal regions for this phenotype.

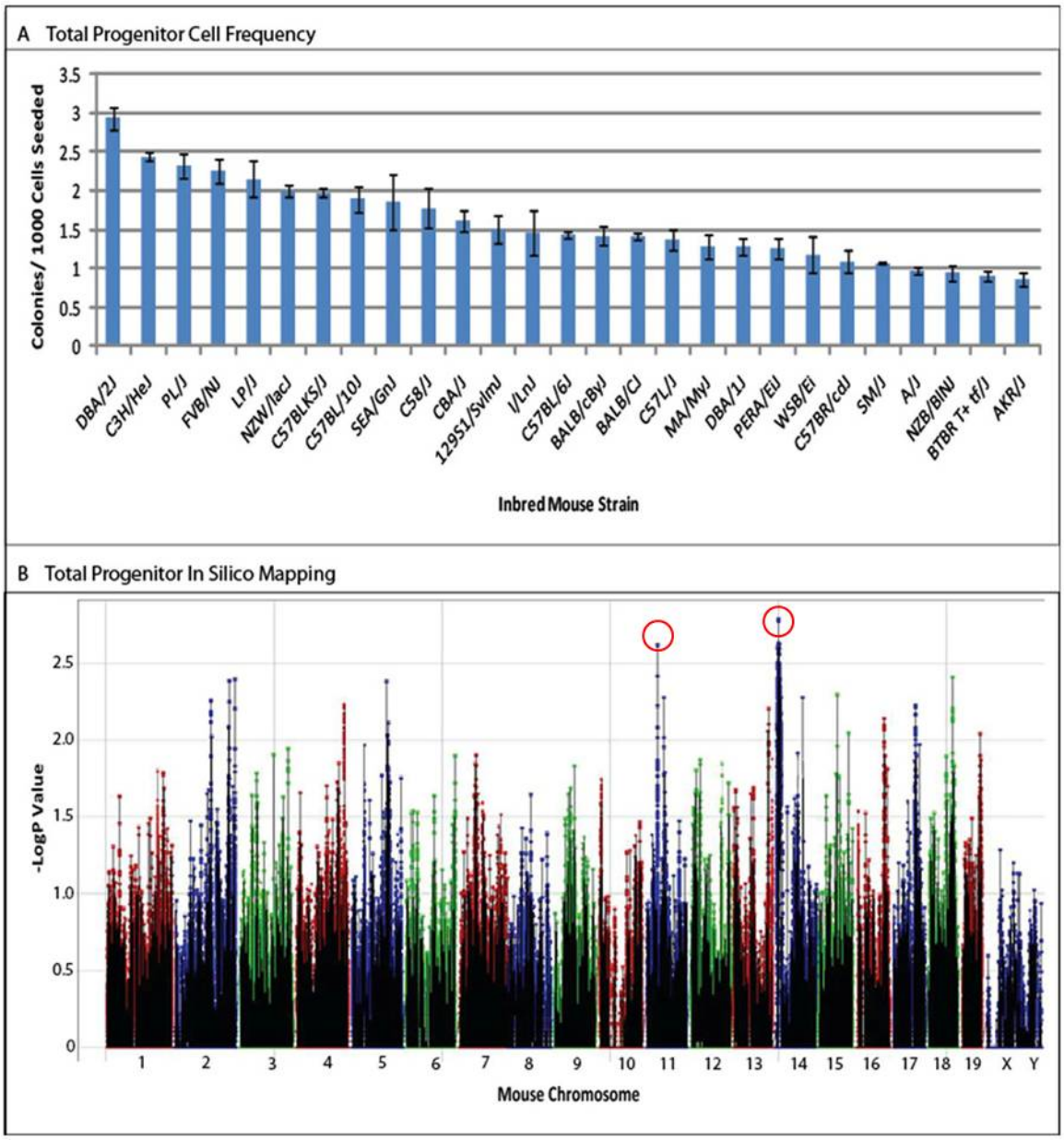

Figure 2.7: Frequency of total myeloid progenitor cells and *in silico* mapping. A: Total progenitor cell frequency as measured by CFC assays. Results are shown as colonies/1000 cells seeded. Error bars represent SEMs (n = 3-5). B: *In silico* mapping results when phenotypic information from part A was compared with genotypic information based on SNP information for the various inbred mouse strains. Results are shown as a -logp value. Circles point out peaks with a -logp value above 2.5.

Table 2.7: Genes of interest in regulating total progenitor cell frequency

Genes located in chromosomal region with –logp values above 2.5		
Gene	**Region**	**-logp value**
Fhit, fragile histidine triad gene	**14: 11584520-11656342**	**2.790107**
Ptprg, protein tyrosine phosphatase, receptor type, G	**14: 12838623-12859093**	**2.629132**
Slit3, slit homolog 3 (Drosophila)	11: 35100995-35120899	2.622682
W6q3, weight 6 weeks QTL 3	11: 35100995-35120899	2.622682
n-R5s44, nuclear encoded rRNA 5S 44	14: 9797430-9881986	2.509633

IPA and literature searches reveal patterns among genes

In addition to confirming our results with the IL-3 Rα control, we performed a meta-analysis of our data by entering the top 50-55 genes (as determined by chromosomal region with the highest -logp values) for each phenotype into Ingenuity Pathways Analysis (IPA) software. We used IPA to investigate the involvement of signaling networks in the regulation of hematopoiesis to determine if there were patterns of involvement in biological processes among these genes. Results indicate overlap of signaling pathways throughout hematopoiesis, but also show that particular progenitor types have genes working in pathways that are specific to their development. A summary of this analysis can be found in Table 2.8. Top signaling pathways include cancer, hematological disease, metabolism, development, cell cycle, cell growth and proliferation, and cell death among others. As top signaling pathways include cancer, hematological disease, genetic disorder, and many cancer related processes, this finding further supports that our method can be used to identify potential tumor suppressors and oncogenes in the regulation of hematopoiesis.

Table 2.8: Top IPA signaling pathway for each phenotype.

IPA Top Signaling Networks					
Phenotype	**Signaling Network 1**	**Signaling Network 2**	**Signaling Network 3**	**Signaling Network 4**	**Signaling Network 5**
Total	Cell Cycle, Cardiovascular System Development and Function, Cellular Development	Behavior, Cardiovascular Disease, Cardiovascular System Development and Function	Cellular Development, Cellular Growth and Proliferation		
GEMM	Gene Expression, Cancer, Hematological Disease	Cell Death, Cell-To-Cell Signaling and Interaction, Cellular Growth and Proliferation	RNA Post-transcriptional Modification, Nervous System Development and Function, Skeletal and Muscular System Development and Function	Lipid Metabolism, Small Molecule Biochemistry, Nucleic Acid Metabolism	Cellular Assembly and Organization, Cellular Compromise, DNA Replication Recombination and Repair
GM	Cancer, Reproductive System Disease, Tumor Morphology	Lipid Metabolism, Carbohydrate Metabolism, Small Molecule Biochemistry	Amino Acid Metabolism, Small Molecule Biochemistry, Cell Death	Cancer, Cardiovascular System Development and Function, Cell Cycle	
E	Endocrine System Disorders, Genetic Disorder, Metabolic Disease	Cellular Development, Hematological Disease, Immunological Disease	Developmental Disorder, Genetic Disorder, Embryonic Development	Cancer, Cardiovascular Disease, Cardiovascular System Development and Function	Genetic Disorder , Metabolic Disease, Connective Tissue Development and Function
G	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	Carbohydrate Metabolism, Small Molecule Biochemistry, Drug Metabolism	Cancer, Reproductive System, Genetic Disorder	Cardiovascular System Development and Function, Skeletal and Muscular System Development and Function, Cell Morphology	
M	Cell Morphology, Neurological Disease, Cell Cycle	Cellular Compromise, Carbohydrate Metabolism, Molecular Transport	Organismal Injury and Abnormalities, Genetic Disorder, Hepatic System Disease		

As IPA was limited in recognizing genes that relatively little is known about in signaling pathways, we also conducted literature searches using PubMed to understand more about the patterns among these top genes. For example, bone mineral density genes, bone quality genes, and many genes related to other bone properties are not recognized in signaling pathways in IPA, yet they make up 18% of the genes in our meta-analysis. Results for these literature searches can be found in Table 2.9. In the literature searches conducted, we found that 11% of genes in our listing for the top 50 genes in each phenotype are known to be associated with the regulation of hematopoiesis. Thirty percent of genes are known to be associated with cancer. Of these, there seems to be a mix of genes related to many cancer processes and types. As progenitor cell frequency is related to AML and its precursors, it is not surprising that 15% of genes returned on the top genes lists are previously known to be involved in leukemias specifically. Taken together, these data also support our use of these techniques to identify tumor suppressors and oncogenes in hematopoietic regulation. In addition, 18% of the top genes are involved in bone properties, 20% are related to FA, fat, or atherosclerosis, and 19% are related to insulin or diabetes. Of the genes in these top genes lists, several have been shown to be involved in regulating hematopoiesis or contributing to leukemia (shown in bold), whereas most are novel candidate genes for these processes.

Table 2.9: Genes located in regions with highest -logp values associated with relevant processes/qualities.

Genes located in regions with highest -logp values associated with relevant processes/qualities	
Cancer (30%)	Cadps, Top2b, Rarb, Ep400, Ptpn13, Net1, Ikbkap, Skts-fp1, Fbxl7, Ints6, Ctsb, Scara5, Msra, Gata4, Pou6f1, Blk, Dleu2, Kcnrg, Mir15a, Mir16-1, Trim13, Elp3, Neil2, Tdh, Tcf7, Crmp1, Ptprt, Cbx5, Smug1, Slc22a4, Cinda3, Stx18, Flt1, Slit3, Ykt6, Erbb2ip, Nln, Mpped2, Opcml, Rgs4, Adamts6, Slc13a1, Hcn1, Dkk2, Dym, Afap1l2, ,Vwa2, Lrrk2, Slc2a13, Myo5b, Dcc, Casp7, Fat1, Mtnr1a, Lama1, Ptprg, Evi5, Nek10, Slc4a7, Sntn, Wasl, Fhit, Lrrc3b, Psmd6
Leukemia (15%)	Il3ra, Ptpn13, Gata4, Blk, Dleu2, Mir15a, Mir16-1, Trim13, Tcf7, Cbx5, Slc22a4, Flt1, Rgs4, Dcc, Casp7, Evi5, Wasl, Fhit, Rarb, Top2b, Net1, Ctsb, Kcnrg, Crmp1, Ptprt, Erbb2ip, Slc13a1, Lrrk2, Lama1, Lrrc3b, Arhgap24, Camk2b, F11
Hematopoiesis (11%)	Il3ra, Ep400, Ptpn13, Gata4, Blk, Dleu2, Mir15a, Mir16-1, Trim13, Tcf7, Cbx5, Slc22a4, Flt1, Rgs4, Dcc, Casp7, Evi5, Wasl, Fhit, Jakmip1, Ptprg, Rarb, Wbcq6
Bone Properties (18%)	Slc22a4, Rgs4, Dcc, Casp7, Wasl, Fhit, Slc13a1, Lama1, Dkk2, Dym, Bmd8, Bmd39, Bmd3, Bmch2, Bmd4, Bmd5, Bmd7, Evc2, Tbqt5, Bmd1, Evc, Pgis1, Ptprg, Mir199a-1, Tcf7, Blk, Rarb, Stk32b, Il3ra, Net1, Ctsb, Agnm2, Bdlnq7, Flt1, Fezf2, Pou4f2, Gata4, Dcdc5, Ptpn13
Fatty acid, Fat, or Atherosclerosis (20%)	Slc22a4, Rgs4, Dcc, Casp7, Wasl, Fhit, Ptprg, Blk, Rarb, Il3ra, Ctsb, Flt1, Gata4, Erbb2ip, Camk2b, F11, Ikbkap, Msra, Tdh, Ykt6, Hcn1, Fat1, Oxsm, Plast1, Plast1b, Lmbrd1, Sc5d, Plast2b, Tcf7, Dleu2, Ptprt, Myo5b, Mtnr1a, Efw, Mob5, T2dm2sa, Dkk2, Bmd8, Scara5, Ath8, Ath13, Mtmr9, Xkr6, Dnm2, Ascc2
Insulin or Diabetes (19%)	Slc22a4, Rgs4, Dcc, Casp7, Wasl, Blk, Ctsb, Flt1, Gata4, F11, Ikbkap, Msra, Plast1, Plast1b, Tcf7, Dleu2, Myo5b, Mtnr1a, Efw, Mob5, T2dm2sa, Fhit, Camk2b, Tdh, Hcn1, Lama1, Crmp1, Pou4f2, Ptpn13, Cadps, Ints6, Pou6f1, Vwa2, Idd21.1, Fam167a, Ap2a1, Idd26, Athsq1, Bglu1, Aod2, Idd21.2

Discussion

The strength of *in silico* mapping method over more traditional methods is evident as it provides denser marker sets and makes comparisons that are based on regions of the genome inherited from common ancestors, as opposed to traditional quantitative trait loci (QTL) studies that rely only on loci inherited from different progenitors. Comparison across a greater number of strains also provides greater statistical power. This type of haplotype associated mapping has successfully proven to be more powerful in narrowing chromosomal regions of interest than traditional QTL mapping in multiple studies [38,

39]. This method has been confirmed to identify chromosomal regions containing genes that are known to be associated with the particular phenotype [19]. This was evident in our study as well, as the region of chromosome 14 containing IL-3Rα presented with a high -logp value. The region containing IL-3Rα was a control for our study. IL-3Rα is mutated in multiple inbred mouse strains causing their progenitor cell frequencies to be reduced.

Our results pointed to regions identified in previous QTL studies, while providing greater resolution. Henckaerts et al. [40] found that the following QTLs are of importance to the hematopoietic progenitor cell compartment: chromosome 4(37.6-48.5 and 66-78.5cM), chromosome 2(72.1-77.2cM), chromosome 9(23-27cM), and chromosome 14 (broad range). We found that chromosome 4(42.13-45.76cM) contains genes that overlap with the previous QTL study. These genes are autoimmune glomerulonephritis in MRL 2 (*Agnm2*) and bone mineral density (*Bmd7*). They are contained within a region important to granulocyte progenitor cell frequency. Chromosome 4(74.75-75.77cM) contains the overlapping genes of atherosclerosis susceptibility QTL 1(*Athsq1*), cytokine induced activation 3(*Cinda3*), and forkhead-associated phosphopeptide binding domain 1 (*Fhad1*), in a region that is important to progenitor cell frequency in erythrocyte, and total frequency phenotypes. Chromosome 2(80.93-85.27) contains one overlapping gene, spontaneous crescentic glomerulonephritis QTL 3(*Scgq3*). Chromosome 9(23.57cM) contains the overlapping gene, sterol-C5-desaturase (*Sc5d*), in a region that is important to erythrocyte development. We also found several very significant regions within chromosome 14. Henckaerts et al. [41] also found that a region of chromosome 2 is important to stem and progenitor cell populations. They found that the region at 73.2-

77.2cM in chromosome 2 is a QTL for early hematopoietic populations. We found a region of chromosome 2 at 80.92-80.96cM that contains genes that overlap the previously discovered QTL. Another group found that a QTL at chromosome 2 regulates stem cell numbers in the bone marrow [42]. Interestingly, the gene alcohol preference QTL 1 (*Alpq1*) was located in this region. Our studies resulted in a region of chromosome 9(20.76cM) containing alcohol preference QTL 3 (*Alpq3*). These were both identified in genome wide association studies for alcohol preference [43]. Chronic alcohol use induces bone loss through the suppression of Wnt signaling [44].

Additionally, a portion of mouse chromosome 11 that presented in the E progenitor phenotype from our study corresponds to human chromosome 5, specifically region 5q31.1. This particular region of chromosome 5 undergoes deletion in 5q- syndrome, which is a disorder that affects bone marrow cells and causes severe macrocytic anemia and myelodysplastic syndromes that may lead to AML. It is characterized by a defect in erythroid differentiation and is often accompanied by erythroid hypoplasia in the bone marrow [45]. It is fitting that this region presented in the E progenitor phenotype. This result further validates our methods. Genes contained in the region are transcription factor 7 (*Tcf7*), CDC42 small effector 2 (*Cdc42se2*), follistatin-like 4 (*Fstl4*), Rap guanine nucleotide exchange factor 6 (*Rapgef6*), and solute carrier family 22 member 4 (*Slc22a4*).

Our meta-analysis of the top 50-55 genes within the chromosomal regions with the highest -logp values for each phenotype revealed interesting patterns. Overall, there were 39 genes associated with bone mineral density, bone quality, bone mechanics, and bone disease. These genes are located in several different chromosomal regions. Seven of

these were bone mineral density genes: *Bmd1*, *Bmd3*, *Bmd4*, *Bmd5*, *Bmd7*, *Bmd8*, *and Bmd39*. Bone mechanical trait 2 (*Bmch2*) also showed up in our meta-analysis as well as tibia bone quality trait 5 (*Tbqt5*). Several additional genes were involved in osteogenesis, skeletal formation, bone morphogenic signaling, osteoclast differentiation, osteoblast differentiation, skeletal size, bone growth plate regulation, and bone disease [46-55]. This finding suggests a role of bone properties in determining how myeloid cells develop. Additional support for the idea of bone properties being key regulators of myeloid progenitor cell frequency lies in the high number of genes related to calcium and estradiol signaling that were located in chromosomal regions of high -logp values as well. The roles of calcium and estradiol in the quality of bone have been well documented. [56-62]

Another interesting pattern involved metabolism. Twenty percent of the genes returned in our analysis were related to FA uptake and oxidation, lipid metabolism, and atherosclerosis susceptibility [63-70]. This finding supports our previous work that shows that the ratio of omega 3 to omega 6 FAs in the diet has a significant effect on progenitor cell frequency in the bone marrow of mice [11]. Furthermore, these qualities have been shown to regulate bone mineral density in humans and mice. High fat diets lower bone mineral density and bone volume [69, 61]. One study also correlates QTLs for body size (body weight, length, and adipose mass) with those identified for skeletal traits, suggesting that many QTLs may regulate bone properties via their effect on body size [72].

Genes associated with glucose metabolism, insulin, and diabetes susceptibility were also present in high numbers in our meta-analysis. These genes have been show to have a major role in bone remodeling and metabolism. Osteoblasts express insulin

receptors and favor glucose metabolism through the hormone osteocalcin. Insulin signaling in osteoblasts is necessary for whole-body glucose homeostasis because it increases osteocalcin activity. Osteoclasts can determine the status and function of osteocalcin and, accordingly, increase or decrease insulin signaling in osteoblasts. This determination then promotes or hampers glucose metabolism in a bone resorption-dependent manner in mice and humans [73]. As insulin stops the use of fat as an energy source, lack of control of insulin levels can have a profound effect on fat stores in the body. Insulin's direct effect on FA synthesis in the body could play a role in the bone properties associated with fat as well. Furthermore, insulin receptor interacting proteins have been shown to regulate hematopoietic stem and progenitor cell pools [74].

Future directions for our lab include determining if the genes located in these chromosomal regions with high -logp values are in fact associated with changes in myeloid progenitor cell frequency. In order to complete this work, we will investigate these novel candidate genes one by one, beginning with those in which nucleotides correlate strongly with phenotypic data and are involved in the processes that our data suggest are regulators of hematopoiesis. We will test these genes for function in hematopoiesis by altering their expression. We will also determine if the particular pathways that emerged the most (bone properties and metabolic processes) are biomarkers for AML progression risk. Additionally, we will validate the role of these genes and pathways in human hematopoiesis and AML progression.

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Addendum for chapter 2

GEMM top genes list

Gene	Region	-logp value
Ptprg, protein tyrosine phosphatase, receptor type, G	**14: 12608073-12623845**	**4.309804**
Cadps, Ca2+-dependent secretion activator	14: 13451373-13528685	4
Fhit, fragile histidine triad gene	**14: 10693745-10736604**	**3.946922**
Rthyd3, resistance to thymic deletion 3	14: 13387864-13423496	3.928118
n-R5s45, nuclear encoded rRNA 5S 45	14: 13196426-13337728	3.850781
Synpr, synaptoporin	14: 14291097-14489374	3.793175
Atxn7, ataxin 7	14: 14725638-15083409	3.777284
Olfr720, olfactory receptor 720	14: 14725638-15083409	3.777284
Psmd6, proteasome (prosome, macropain) 26S subunit, non-ATPase, 6	14: 14725638-15083409	3.777284
Thoc7, THO complex 7 homolog (Drosophila)	14: 14725638-15083409	3.777284
Rpl19-ps4, ribosomal protein L19, pseudogene 4	14: 14552540-14662129	3.725843
Sntn, sentan, cilia apical structure protein	14: 14290630-15106086	3.671621
Fezf2, Fez family zinc finger 2	14: 13174979-13450378	3.659556
Lrrc3b, leucine rich repeat containing 3B	**14: 16103926-16294228**	**3.518558**
Ngly1, N-glycanase 1	14: 17064860-17090557	3.497573
Oxsm, 3-oxoacyl-ACP synthase, mitochondrial	14: 17064860-17090557	3.497573
Rpl21-ps4, ribosomal protein L21, pseudogene 4	14: 12042049-12260227	3.469801
Iglon5, IgLON family member 5	7: 50735869-50778325	3.354578
Arhgap24, Rho GTPase activating protein 24	**5: 102870089-102911460**	**3.321482**
Tmc1m4, Tmc1 modifier 4	5: 102870089-102911460	3.321482
Appd2, APP associated premature death 2	14: 17434991-17778674	3.254926
Rpl31-ps3, ribosomal protein L31, pseudogene 3	14: 11970057-12401283	3.219683
Olfr721-ps1, olfactory receptor 721, pseudogene 1	14: 15224397-15419224	3.192465
Olfr31, olfactory receptor 31	14: 14162704-15177633	3.162412
Rarb, retinoic acid receptor, beta	**14: 17260939-17434727**	**3.109579**
Top2b, Topoisomerase (DNA), beta II	**14: 17260939-17434727**	**3.109579**
Wbcq6, white blood cell quantitative locus 6	**14: 17260939-17434727**	**3.109579**
Nek10, NIMA (never in mitosis gene a)- related kinase 10	14: 15683725-15737034	3.084601
n-R5s44, nuclear encoded rRNA 5S 44	14: 9779720-9981564	3.059484
Il3ra, interleukin 3 receptor, alpha chain	**14: 13985651-15492174**	**3.024569**
Slc4a7, solute carrier family 4, sodium bicarbonate cotransporter, member 7	14: 11799713-15618059	2.995591
Evi5, ecotropic viral integration site 5	**5: 108163334-108178591**	**2.818136**
Ep400, E1A binding protein p400	**5: 111108675-111133204**	**2.759341**
Ptpn13, protein tyrosine phosphatase, non-receptor type 13	**5: 103855361-103866161**	**2.71364**
Idd21.1, insulin dependent diabetes susceptibility 21.1	18: 78923250-78937776	2.630284
Bmd8, bone mineral density 8	6: 80953210-80967127	2.614961
Efw, epididymal fat weight	6: 80953210-80967127	2.614961
Klhl8, kelch-like 8 (Drosophila)	5: 104324701-104326563	2.600348
Bmd39, bone mineral density 39	8: 118356974-118360978	2.582694
Tpi-rs9, triosephosphate isomerase related sequence 9	14: 10871174-18415403	2.569667
Ascc2, activating signal cointegrator 1 complex subunit 2	14: 9997616-18431455	2.548268
Caln1, calneuron 1	5: 130890171-130891984	2.488252
Vmn2r62, vomeronasal 2, receptor 62	7: 50006469-50091657	2.453735
Bmd3, bone mineral density 3	13: 3190342-3242611	2.450389
Tyw1, tRNA-yW synthesizing protein 1 homolog (S. cerevisiae)	5: 130758991-130829540	2.431364
Net1, neuroepithelial cell transforming gene 1	**13: 3876452-3899689**	**2.418577**
Noc4l, nucleolar complex associated 4 homolog (S. cerevisiae)	5: 111079377-111080089	2.409792
Ath8, atherosclerosis 8	4: 56766913-56778159	2.406824
Bmch2, bone mechanical trait 2	4: 56766913-56778159	2.406824
Ctrcts, cataract severity	4: 56766913-56778159	2.406824
Ikbkap, inhibitor of kappa light polypeptide enhancer in B-cells, kinase complex-associated protein	4: 56766913-56778159	2.406824
Skts-fp1, skin tumor susceptibility in FVB and PWK 1	4: 56766913-56778159	2.406824

GM top genes list

Gene	Region	-logp value
Ath13, atherosclerosis 13	14: 63145626-63173054	2.889386
Cosz2, cocaine seizure 2	14: 63145626-63173054	2.889386
Plast1, plasma plant sterol 1	14: 63145626-63173054	2.889386
Plast1b, plasma plant sterol 1b	14: 63145626-63173054	2.889386
Fbxl7, F-box and leucine-rich repeat protein 7	15: 26533596-26539153	2.705725
Defb43, defensin beta 43	14: 63614409-63639344	2.678805
Defb47, defensin beta 47	14: 63614409-63639344	2.678805
Fam124a, family with sequence similarity 124, member A	14: 63139934-63190101	2.470732
Atp8a2, ATPase, aminophospholipid transporter-like	14: 60475030-60613198	2.462052
Ints6, integrator complex subunit 6	14: 63310514-63363750	2.459213
Serpine3, serpin peptidase inhibitor, member 3	14: 63310514-63363750	2.459213
Ctsb, cathepsin B	**14: 63696395-63747802**	**2.425771**
Scara5, scavenger receptor class A, member 5 (putative)	14: 66279212-66323155	2.423693
Fbxo16, F-box protein 16	14: 65923363-66010722	2.420556
Zfp395, zinc finger protein 395	14: 65923363-66010722	2.420556
Gucy1b2, guanylate cyclase 1, soluble, beta 2	14: 63073236-63133525	2.408946
Msra, methionine sulfoxide reductase A	14: 64700581-64935824	2.394743
Prss51, protease, serine, 51	14: 64700581-64935824	2.394743
Prss52, protease, serine, 52	14: 64700581-64935824	2.394743
Prss55, protease, serine, 55	14: 64700581-64935824	2.394743
Zfp658, zinc finger protein 658	7: 50806886-50879931	2.296665
Zfp719, zinc finger protein 719	7: 50806886-50879931	2.296665
Zfp819, zinc finger protein 819	7: 50806886-50879931	2.296665
Gata4, GATA binding protein 4	**14: 63819697-63990229**	**2.290406**
Bmd4, bone mineral density 4	15: 100386574-100411741	2.286494
Pou6f1, POU domain, class 6, transcription factor 1	15: 100386574-100411741	2.286494
Blk, B lymphoid kinase	**14: 63813790-64091361**	**2.254468**
Fam167a, family with sequence similarity 167, member A	14: 63813790-64091361	2.254468
Dleu2, deleted in lymphocytic leukemia, 2	**14: 62190971-62280557**	**2.233419**
Kcnrg, potassium channel regulator	**14: 62190971-62280557**	**2.233419**
Mir15a, microRNA 15a	**14: 62190971-62280557**	**2.233419**
Mir16-1, microRNA 16-1	**14: 62190971-62280557**	**2.233419**
Trim13, tripartite motif-containing 13	**14: 62190971-62280557**	**2.233419**
Elp3, elongation protein 3 homolog (S. cerevisiae)	14: 66148441-66169246	2.214623
Kif13b, kinesin family member 13B	14: 65347007-65376483	2.193357
Arhgap6, Rho GTPase activating protein 6	X: 165319571-165322740	2.180174
Bmd5, bone mineral density 5	1: 123019155-123039644	2.144574
Bwq7, body weight, QTL 7	1: 123019155-123039644	2.144574
Scgq2, spontaneous crescentic glomerulonephritis QTL 2	1: 123019155-123039644	2.144574
Mtmr9, myotubularin related protein 9	14: 63809198-64260649	2.144055
Neil2, nei like 2 (E. coli)	14: 63809198-64260649	2.144055
Tdh, L-threonine dehydrogenase	14: 63809198-64260649	2.144055
Xkr6, X Kell blood group precursor related family member 6	14: 63809198-64260649	2.144055
Ap2a1, adaptor protein complex AP-2, alpha 1 subunit	7: 52178097-52178425	2.142233
Iglon5, IgLON family member 5	7: 50738756-50775032	2.120025
Cadps, Ca2+-dependent secretion activator	14: 13505483-13522770	2.101804
Defb30, defensin beta 30	14: 63593927-63750325	2.09575
Defb42, defensin beta 42	14: 63593927-63750325	2.09575
Defb48, defensin beta 48	14: 63593927-63750325	2.09575
Fecq3, fecundity QTL 3	1: 24822648-24829896	2.093422
Idd26, insulin dependent diabetes susceptibility 26	1: 24822648-24829896	2.093422
Lmbrd1, LMBR1 domain containing 1	1: 24822648-24829896	2.093422

E top genes list

Gene	Region	-logp value
Stk32b, serine/threonine kinase 32B	5: 38038420-38084656	2.849933
Ity2a, immunity to S. typhimurium 2a	11: 52093205-52150216	2.79202
Tcf7, transcription factor 7, T-cell specific	**11: 52093205-52150216**	**2.79202**
W6q3, weight 6 weeks QTL 3	11: 52093205-52150216	2.79202
Alpq3, alcohol preference QTL 3	9: 42048114-42065818	2.728445
Sc5d, sterol-C5-desaturase	9: 42048114-42065818	2.728445
Crmp1, collapsin response mediator protein 1	**5: 37681204-37711624**	**2.690689**
Evc, Ellis van Creveld gene homolog (human)	5: 37681204-37711624	2.690689
Blmpf2, bleomycin-induced pulmonary fibrosis 2	11: 54038026-54056774	2.532897
Cia40, collagen induced arthritis 40	11: 54038026-54056774	2.532897
Ckdbp2, chronic kidney disease blood pressure locus 2	11: 54038026-54056774	2.532897
Pregq1, pregnancy QTL 1	11: 54038026-54056774	2.532897
Elnv, epilepsy naïve	9: 34385442-34401249	2.527766
Igan3, IgA nephropathy 3	9: 34385442-34401249	2.527766
Athsq1, atherosclerosis susceptibility QTL 1	4: 142154832-142171686	2.46515
Bmd7, bone mineral density 7	4: 142154832-142171686	2.46515
Cytl1, cytokine-like 1	5: 38104702-38133786	2.435442
Synj1, synaptojanin 1	16: 90991665-91012590	2.432217
Bdlnq7, body length QTL 7	2: 160241262-160460912	2.431364
Bglu1, blood glucose level 1	2: 160241262-160460912	2.431364
Mob5, multigenic obesity 5	2: 160241262-160460912	2.431364
Prdt2, prion disease incubation time 2	2: 160241262-160460912	2.431364
Scgq3, spontaneous crescentic glomerulonephritis QTL 3	2: 160241262-160460912	2.431364
Cdc42se2, CDC42 small effector 2	11: 54532428-54574973	2.416867
Fstl4, follistatin-like 4	11: 52789869-52808156	2.368597
Evc2, Ellis van Creveld syndrome 2 homolog (human)	5: 37676733-37736901	2.344392
Arsj, arylsulfatase J	3: 126107801-126111371	2.298671
Ctrq2, C. trachomatis resistance QTL 2	3: 126107801-126111371	2.298671
Rapgef6, Rap guanine nucleotide exchange factor (GEF) 6	11: 54492977-54520054	2.28537
Ptprt, protein tyrosine phosphatase, receptor type, T	**2: 162384520-162390034**	**2.284619**
Jakmip1, janus kinase and microtubule interacting protein 1	**5: 37515261-37745440**	**2.280844**
Chd6, chromodomain helicase DNA binding protein 6	2: 160787704-160857202	2.279515
Fhit, fragile histidine triad gene	**14: 10402891-10416373**	**2.251435**
Bmd4, bone mineral density 4	15: 102961141-103060839	2.251233
Cbx5, chromobox homolog 5 (Drosophila HP1a)	**15: 102961141-103060839**	**2.251233**
Smug1, single-strand selective monofunctional uracil DNA glycosylase	15: 102961141-103060839	2.251233
Slc22a4, solute carrier family 22, member 4	**11: 53799692-53822305**	**2.250217**
Tbqt5, tibia bone quality traits 5	13: 107080581-107145390	2.241795
Alan2, alloantigen response 2	4: 141979409-141982891	2.240757
Cinda3, cytokine induced activation 3	4: 141979409-141982891	2.240757
Stx18, syntaxin 18	5: 38259984-38490902	2.22981
Lrrc3b, leucine rich repeat containing 3B	**14: 16181610-16207728**	**2.217484**
Ath13, atherosclerosis 13	14: 101291502-101501656	2.215726
Plast1, plasma plant sterol 1	14: 101291502-101501656	2.215726
Plast1b, plasma plant sterol 1b	14: 101291502-101501656	2.215726
Flt1, FMS-like tyrosine kinase 1	**5: 148421156-148422167**	**2.200029**
Slit3, slit homolog 3 (Drosophila)	11: 35100995-35120899	2.197969
Ptprg, protein tyrosine phosphatase, receptor type, G	**14: 12383660-12401283**	**2.193588**
Aaom1, autoimmune aoritis in MRL mice 1	4: 141360892-141385435	2.187286
Pregq4, pregnancy QTL 4	17: 67731385-67741554	2.172943

G top genes list

Gene	Region	-logp value
Camk2b, calcium/calmodulin-dependent protein kinase II, β	**11: 5864894-5891207**	**2.815092**
Ykt6, YKT6 homolog (S. Cerevisiae)	11: 5864894-5891207	2.815092
Bmd39, bone mineral density 39	8: 80875682-80881683	2.801206
Ttc29, tetratricopeptide repeat domain 29	8: 80875682-80881683	2.801206
Erbb2ip, Erbb2 interacting protein	**13: 104665228-104684576**	**2.794302**
Tbqt5, tibia bone quality traits 5	13: 104665228-104684576	2.794302
Nln, neurolysin (metallopeptidase M3 family)	13: 104701994-104824890	2.694254
Srsf12, serine/arginine-rich splicing factor 12	13: 104528844-104542789	2.686086
Pou4f2, POU domain, class 4, transcription factor 2	8: 80947042-81014484	2.613413
Bmd3, bone mineral density 3	13:10435947-10438947	2.499005
Mob5, multigenic obesity 5	2: 106553007-106556649	2.467799
Mpped2, metallophosphoesterase domain containing 2	2: 106553007-106556649	2.467799
Plast2b, plasma plant sterol 2b	2: 106553007-106556649	2.467799
T2dm2sa, type 2 diabetes mellitus 2 in SMXA RI mice	2: 106553007-106556649	2.467799
Tmc1m1, Tmc1 modifier 1	2: 106553007-106556649	2.467799
Slc10a7, solute carrier family 10, member 7	8: 81034594-81058787	2.46594
Rbmxrt, RNA binding motif protein, retrogene	8: 81029162-81029320	2.449382
Scgq2, spontaneous crescentic glomerulonephritis QTL 2	1: 126683785-126717845	2.332438
Bmd5, bone mineral density 5	1: 126683785-126717845	2.332438
Sxbq2, SGC/Knj cross B6 QTL 2	6: 24951661-25113230	2.312636
Ath13, atherosclerosis 13	14: 58830212-58836142	2.296665
Cosz2, cocaine seizure 2	14: 58830212-58836142	2.296665
Plast1, plasma plant sterol 1	14: 58830212-58836142	2.296665
Plast1b, plasma plant sterol 1b	14: 58830212-58836142	2.296665
Svtms, survival time modifier of Sod2	13: 117968579-117969103	2.276845
Opcml, opioid binding protein/cell adhesion molecule-like	9: 27911936-27975158	2.215064
Bmd1, bone mineral density 1	1: 171619669-171639814	2.206376
Rgs4, regulator of G-protein signaling 4	**1: 171619669-171639814**	**2.206376**
Scgq1, spontaneous crescentic glomerulonephritis QTL 1	1: 171619669-171639814	2.206376
Sle16, systematic lupus erythematosus susceptibility 16	1: 171619669-171639814	2.206376
Adamts6, a disintegrin-like and metallopeptidase	13: 105275519-105288870	2.178497
Slc13a1, solute carrier family 13, member 1	**6: 24067291-24276244**	**2.158614**
Nacad, NAC alpha domain containing	11: 6501551-6527323	2.138303
Snora5c, small nucleolar RNA, H/ACA box 5C	11: 6501551-6527323	2.138303
Tbrg4, transforming growth factor beta regulated gene 4	11: 6501551-6527323	2.138303
Kcnip1, Kv channel-interacting protein 1	11: 33621650-33649999	2.110309
W6q3, weight 6 weeks QTL 3	11: 33621650-33649999	2.110309
Dnm2, dynamin 2	9: 21242462-21309077	2.106644
Mir199a-1, microRNA 199a-1	9: 21242462-21309077	2.106644
Hcn1, hyperpolarization-activated, cyclic nucleotide-gated K+ 1	13: 118500852-118528940	2.106644
Plekha5, pleckstrin homology domain containing, member 5	6: 140330684-140381709	2.105794
Agnm2, autoimmune glomerulonephritis in MRL 2	4: 118439097-118439315	2.097489
Anxty, anxiety	4: 118439097-118439315	2.097489
Bmd7, bone mineral density 7	4: 118439097-118439315	2.097489
Dcdc5, doublecortin domain containing 5	2: 106132643-106160544	2.080386
Hyal4, hyaluronoglucosaminidase 4	6: 24597981-24886492	2.051474
Hyal5, hyaluronoglucosaminidase 5	6: 24597981-24886492	2.051474
Hyal6, hyaluronoglucosaminidase 6	6: 24597981-24886492	2.051474
Spam1, sperm adhesion molecule 1	6: 24597981-24886492	2.051474
Wasl, Wiskott-Aldrich syndrome-like (human)	**6: 24597981-24886492**	**2.051474**

M top genes list

Gene	Region	-logp value
Fhit, fragile histidine triad gene	**14: 11584520-11656529**	**2.94897**
Ctrq2, C. trachomatis resistance QTL 2	3: 131789521-131823292	2.714458
Dkk2, dickkopf homolog 2 (Xenopus laevis)	3: 131789521-131823292	2.714458
Dym, dymeclin	18: 75266222-75267209	2.693152
Idd21.1, insulin dependent diabetes susceptibility 21.1	18: 75266222-75267209	2.693152
Pgis1, proteoglycan induced spondylitis 1	18: 75266222-75267209	2.693152
Bmd4, bone mineral density 4	15: 56618365-56626586	2.660179
Mob5, multigenic obesity 5	2: 110016970-110020628	2.655399
Plast2b, plasma plant sterol 2b	2: 110016970-110020628	2.655399
T2dm2sa, type 2 diabetes mellitus 2 in SMXA RI mice	2: 110016970-110020628	2.655399
Tmc1m1, Tmc1 modifier 1	2: 110016970-110020628	2.655399
Dclre1a, DNA cross-link repair 1A, PSO2 homolog (S. cerevisiae)	19: 56596851-56621288	2.587244
Rpl21-ps4, ribosomal protein L21, pseudogene 4	14: 12042049-12162496	2.564197
Slit3, slit homolog 3 (Drosophila)	11: 35100995-35120899	2.520235
W6q3, weight 6 weeks QTL 3	11: 35100995-35120899	2.520235
Synpr, synaptoporin	14: 14202231-14220929	2.497986
Ptprg, protein tyrosine phosphatase, receptor type, G	**14: 12445127-12526023**	**2.492895**
Afap1l2, actin filament associated protein 1-like 2	19: 56936971-56992931	2.488735
Tdrd1, tudor domain containing 1	19: 56936971-56992931	2.488735
Vwa2, von Willebrand factor A domain containing 2	19: 56936971-56992931	2.488735
Lrrk2, leucine-rich repeat kinase 2	**15: 91386924-91588341**	**2.478729**
Slc2a13, solute carrier family 2 , member 13	15: 91386924-91588341	2.478729
Myo5b, myosin VB	18: 74863638-74889684	2.473403
Cadps, Ca2+-dependent secretion activator	14: 13563270-13578052	2.459644
Wdr67, WD repeat domain 67	15: 57796753-57816553	2.381197
Aod2, autoimmune ovarian dysgenesis 2	3: 52458253-52592669	2.37429
Ath13, atherosclerosis 13	14: 82146864-82154961	2.346516
Plast1, plasma plant sterol 1	14: 82146864-82154961	2.346516
Plast1b, plasma plant sterol 1b	14: 82146864-82154961	2.346516
Lrrc3b, leucine rich repeat containing 3B	**14: 16205186-16228725**	**2.341599**
Appd2, APP associated premature death 2	14: 17434991-17580486	2.290776
Nhlrc2, NHL repeat containing 2	19: 56570749-56660884	2.285557
n-R5s44, nuclear encoded rRNA 5S 44	14: 9779720-9874620	2.281791
Idd21.2, insulin dependent diabetes susceptibility 21.2	14: 14470385-14489374	2.267598
Gm3614, predicted gene 3614	14: 11969250-12401283	2.257278
Fezf2, Fez family zinc finger 2	14: 13174979-13196712	2.241588
Anxty, anxiety	4: 125536444-125587189	2.205249
Bmd7, bone mineral density 7	4: 125536444-125587189	2.205249
Ctrcts, cataract severity	4: 125536444-125587189	2.205249
Eae40, experimental allergic encephalomyelitis 40	4: 125536444-125587189	2.205249
Dcc, deleted in colorectal carcinoma	**18: 72369674-72489317**	**2.200258**
Dclre1a, DNA cross-link repair 1A, PSO2 homolog	19: 56556474-56716524	2.174883
Nek10, NIMA (never in mitosis gene a)- related kinase 10	14: 15737733-15748613	2.151523
Atrnl1, attractin like 1	19: 58171868-58183830	2.133273
Rthyd3, resistance to thymic deletion 3	14: 13316480-13423496	2.132206
Casp7, caspase 7	**19: 56510580-56536292**	**2.119476**
Bmd39, bone mineral density 39	8: 46044747-46343089	2.108621
F11, coagulation factor XI	**8: 46044747-46343089**	**2.108621**
Fat1, FAT tumor suppressor homolog 1 (Drosophila)	8: 46044747-46343089	2.108621
Mtnr1a, melatonin receptor 1A	8: 46044747-46343089	2.108621

Total top genes list

Gene	Region	-logp value
Fhit, fragile histidine triad gene	**14: 11584520-11656342**	**2.790107**
Ptprg, protein tyrosine phosphatase, receptor type, G	**14: 12838623-12859093**	**2.629132**
Slit3, slit homolog 3 (Drosophila)	11: 35100995-35120899	2.622682
W6q3, weight 6 weeks QTL 3	11: 35100995-35120899	2.622682
n-R5s44, nuclear encoded rRNA 5S 44	14: 9797430-9881986	2.509633
Synpr, synaptoporin	14: 14202231-14220929	2.488148
Cadps, Ca2+-dependent secretion activator	14: 13452033-13505602	2.469775
Dym, dymeclin	18: 75261748-75269685	2.411058
Idd21.1, insulin dependent diabetes susceptibility 21.1	18: 75261748-75269685	2.411058
Pgis1, proteoglycan induced spondylitis 1	18: 75261748-75269685	2.411058
Atxn7, ataxin 7	14: 14797756-14919236	2.400826
Prdt2, prion disease incubation time 2	2: 178866494-178868269	2.399079
Rpl21-ps4, ribosomal protein L21, pseudogene 4	14: 12042049-12079505	2.389314
Scgq3, spontaneous crescentic glomerulonephritis QTL 3	2: 164473694-164543019	2.387713
Spint4, serine protease inhibitor, Kunitz type 4	2: 164473694-164543019	2.387713
Wfdc10, WAP four-disulfide core domain 10	2: 164473694-164543019	2.387713
Wfdc11, WAP four-disulfide core domain 11	2: 164473694-164543019	2.387713
Wfdc13, WAP four-disulfide core domain 13	2: 164473694-164543019	2.387713
Wfdc9, WAP four-disulfide core domain 9	2: 164473694-164543019	2.387713
Rthyd3, resistance to thymic deletion 3	14: 13387864-13423496	2.386053
Tmc1m4, Tmc1 modifier 4	5: 104462697-104463002	2.382099
Fezf2, Fez family zinc finger 2	14: 13308624-12210361	2.32838
Thoc7, THO complex 7 homolog (Drosophila)	14: 14772525-14930217	2.314043
Bmd4, bone mineral density 4	15: 56618365-56626586	2.297213
Lrrc3b, leucine rich repeat containing 3B	**14: 16208056-16246773**	**2.285353**
Ity2a, immunity to S. typhimurium 2a	11: 52134589-52150216	2.281033
Ath13, atherosclerosis 13	14: 82146864-82154961	2.278372
Plast1, plasma plant sterol 1	14: 82146864-82154961	2.278372
Plast1b, plasma plant sterol 1b	14: 82146864-82154961	2.278372
Mob5, multigenic obesity 5	2: 110016970-110066102	2.257479
Plast2b, plasma plant sterol 2b	2: 110016970-110066102	2.257479
T2dm2sa, type 2 diabetes mellitus 2 in SMXA RI mice	2: 110016970-110066102	2.257479
Bglu1, blood glucose level 1	2: 164422011-164543944	2.250826
Wfdc16, WAP four-disulfide core domain 16	2: 164422011-164543944	2.250826
Wfdc6b, WAP four-disulfide core domain 6B	2: 164422011-164543944	2.250826
Wfdc8, WAP four-disulfide core domain 8	2: 164422011-164543944	2.250826
Olfr720, olfactory receptor 720	14: 14996243-15046024	2.24221
Alan2, alloantigen response 2	4: 141503560-141529979	2.234897
Athsq1, atherosclerosis susceptibility QTL 1	4: 141503560-141529979	2.234897
Bmd7, bone mineral density 7	4: 141503560-141529979	2.234897
Cinda3, cytokine induced activation 3	4: 141503560-141529979	2.234897
Fhad1, forkhead-associated (FHA) phosphopeptide binding domain 1	4: 141503560-141529979	2.234897
Lama1, laminin, alpha 1	17: 68051652-68076393	2.22981
Pregq4, pregnancy QTL 4	17: 68051652-68076393	2.22981
Psmd6, proteasome 26S subunit, non-ATPase, 6	14: 14725549-15046584	2.222066
Tbqt5, tibia bone quality traits 5	13: 107080581-107145390	2.205249
Appd2, APP associated premature death 2	14: 17565088-17626508	2.174883
Tmc1m1, Tmc1 modifier 1	2: 110016289-110086903	2.166578
Nek10, NIMA (never in mitosis gene a)- related kinase 10	14: 15683725-15694698	2.165838
Olfr31, olfactory receptor 31	14: 15106114-15169235	2.14145

Chapter 3: Omega 3 fatty acids reduce myeloid progenitor cell frequency in the bone marrow of mice and promote progenitor cell differentiation

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Abstract

Background

Omega 3 (n-3) fatty acids (FAs) have been found to inhibit proliferation, induce apoptosis, and promote differentiation in various cell types. The processes of cell survival, expansion, and differentiation are of key importance in the regulation of hematopoiesis. We investigated the role of n-3 FAs in controlling the frequency of various myeloid progenitor cells in the bone marrow of mice. Increased progenitor cell frequency and blocked differentiation are characteristics of hematopoietic disorders of the myeloid lineage, such as myeloproliferative diseases and myeloid leukemias.

Results

We found that increasing the proportion of n-3 FAs relative to the proportion of omega 6 (n-6) FAs in the diet caused increased differentiation and reduced the frequency of myeloid progenitor cells in the bone marrow of mice. Furthermore, increasing the proportion of n-3 FAs relative to the proportion of omega 6 (n-6) FAs in the diet had no adverse effect on peripheral white blood cell counts.

Conclusion

Our results indicate that n-3 FAs impact hematopoietic differentiation by reducing myeloid progenitor cell frequency in the bone marrow and promoting progenitor cell differentiation. Further exploration of this discovery could lead to the use of n-3 FAs as a therapeutic option for patients that have various disorders of hematopoiesis.

Introduction

n-3 and n-6 FAs are essential FAs that must be obtained from dietary sources. Recently, the ratio of n-3 to n-6 FAs consumed in the diet has been studied in relevance to cardiac health [1], neurological health [2], and cancer prevention [1]. n-3 FAs can compete with n-6 FAs for the same metabolic enzymes. n-3 and n-6 FAs are incorporated into cell membranes either directly or after elongation and desaturation by Δ4, Δ5, and Δ6 desaturases [3, 4]. n-3 FAs have greater affinity for the Δ4 and Δ6 desaturases than n-6 FAs [5-7]. Dietary linoleic acid (18:2, n-6) is generally considered to be the major source of tissue arachidonic acid (20:4, n-6) although meat fat can be a direct source of arachidonic acid [8]. All three major n-3 FAs, α linolenic acid (18:3), eicosapentanoic acid (20:5), and docosahexanoic acid (22:6), directly inhibit the production of arachidonic acid from linoleic acid [5]. Both arachidonic acid and eicosapentanoic acid can be cleaved from the cell membrane phospholipid stores by phospholipase A2 and acted on by cyclooxygenases (either the constitutive COX1 or the inducible COX2) to produce prostaglandin precursors that are isomerized by prostaglandin synthases to produce prostaglandins. In this manner, COXs convert arachidonic acid to form the 2-series prostaglandins that tend to be pro-proliferative and pro-inflammatory in most tissues [9]. Micromolar concentrations of prostaglandin E2 (PGE2) increase human myeloid progenitor cell proliferation [10]. However, COX activity on eicosapentanoic acid forms the 3 series prostaglandins that tend to have anti-proliferative and anti-inflammatory properties [9]. In addition to prostaglandins, leukotrienes and eicosanoids are formed from FAs through the activity of various lipoxygenases. These have been shown to have varying and sometimes controversial effects on either hematopoietic stem

cell or myeloid progenitor cell differentiation [10, 11]. Thus, a model system approach is needed to effectively dissect the net effect of dietary FAs on hematopoiesis *in vivo*. Abnormal hematopoiesis often involves expansion of immature cells of the myeloid lineage. In these cases, progenitor cell frequency could be expanded by increased proliferation, decreased apoptosis, or both. If n-3 FAs inhibit proliferation or induce apoptosis in myeloid progenitor cells, they could alleviate the cellular expansion that occurs in certain disorders of hematopoiesis. Another characteristic of these disorders is a block in differentiation. During normal hematopoiesis, a continuum of differentiation exists, such that long term hematopoietic stem cells progressively differentiate to produce terminally differentiated cells of the blood. Because n-3 FAs have been shown to promote differentiation [10], they may serve as a therapeutic tool [12-15] for disorders involving the inhibition of normal differentiation.

Our results indicate that the frequency of myeloid progenitors in the bone marrow of mice fed fish oil diets is two-fold less than in those fed corn oil diets. Our data also indicate that this reduction of progenitor cells does not have an adverse effect on the number of circulating white blood cells in the periphery, even after prolonged dietary change. These results suggest a possible role for n-3 FAs as therapeutic agents in disorders involving expansion of myeloid progenitors, such as the myeloid leukemias.

Materials and methods

Animals

Mice used in this study were FVB X sv129F1 hybrid mice. FVB mice were of a colony maintained by W. Elaine Hardman's laboratory at Marshall University. They were initially attained from Jeffrey E. Green's laboratory (Lab of Cell Regulation and

Carcinogenesis, National Cancer Institute, National Institute of Health, Bethesda, MD). Sv129 mice were purchased from Charles River Laboratories (Wilmington, MA). Only male mice were used to avoid cyclic changes in hormones. Mice were housed in the AAALAC accredited animal facilities of the Marshall University School of Medicine. All animal use and care was approved by the Marshall University Institutional Animal Care and Use Committee. The mice were housed 3 to 4 in a cage and individually numbered for identification.

Diet

The base diet was an American Institute of Nutrition (AIN)-76A diet modified by substitution of 5% sucrose for 5% more oils to contain a total of 10% w/w oil (Tables 3.1 and 3.2). The fish oil diet contained 3.65% n-3 FAs and 1.3% n-6 FAs. The corn oil diet contained 0.1% n-3 FAs and 6.1% n-6 FAs. Diets were prepared in the Marshall University School of Medicine animal diet prep room. Diet composition is shown in Table 3.1 and was formulated to be isocaloric, isonutrient and relevant to human consumption. The AIN-76A diet is adequate for the nutritional support of the mice [28]. The dry ingredients of the diet were obtained in bulk from MP Biomedicals (Solon, Ohio), sugar, corn and canola oil were purchased locally (100% canola oil, 100% corn oil, no additives or preservatives). The n-3 supplement (OmegaRx Liquid) was purchased from Zone Labs, Danvers, MA. Batches of diet were prepared as needed, about every two weeks. The diet mixture was pressed into trays and cut into small squares. Individual cage sized portions (25–30 g) were stored in sealed containers at -20°C to prevent oxidation of the fat and bacterial growth in the food. Mice had free access to food and water and were fed fresh food 5 days per week. Food removed from cages was discarded.

Colony forming cell (CFC) assays

FVB X sv129 F1hybrid mice were fed either a fish oil diet (n = 5) or a corn oil diet (n = 5) from weaning until 60 days old. CFC assays were then performed upon bone marrow isolated from these mice as previously discussed [29, 30]. Briefly, bone marrow was harvested by flushing from the femurs with Iscove's Modified Dulbecco's Medium (IMDM) and cells were counted and seeded in 4 well plates at a density determined empirically by a pilot study conducted with a broad and consistent range of seeding densities. After linear response of colony production to seeding density was ensured by the pilot study, seeding densities of 2.09×10^4 (fish oil) and 7.75×10^3 (corn oil) cells/well were chosen to produce 10–30 colonies for each well. Bone marrow was cultured in 1% semi-solid Methocult M3434 (StemCell Technologies), supplemented with 0.4% autochthonous sera. Cells were incubated for 6–7 days to allow colony formation. Colonies with a minimum cell number of 20 were scored as positive using an inverted microscope at 40X magnification. Colonies were counted based on morphological features that are associated with each progenitor type. Student's t type tests were used to detect differences between the experimental groups (n = 5).

White blood cell counts

White blood cell counts were performed on EDTA anticoagulated peripheral blood from tail veins of corn and fish oil fed female mice aged 115 days. Fish and corn oil diets were instituted after weaning. Student's t type tests were used to detect differences between the experimental groups.

Results

High levels of n-3 FAs alter the character of the myeloid progenitor cell compartment in the bone marrow

Progenitors for myeloid cells in bone marrow that can be assessed by CFC assays include granulocyte erythrocyte macrophage megakaryocyte (GEMM) progenitors, granulocyte macrophage (GM) progenitors, erythrocyte (E) progenitors, granulocyte (G) progenitors, and macrophage (M) progenitors. Only these progenitors have the necessary growth factors and proliferation capacity to produce colonies in the CFC assay. Morphology of these colony types is shown in Figure 3.1. Mice were fed fish oil (high level n-3 FAs) or corn oil (low level n-3 FAs) containing diets (Tables 3.1 and 3.2). Mice that were fed the fish oil diet had a significantly lower ($p = 0.010$) frequency of total myeloid progenitor cells ($9.78 \pm 0.46 \times 10^{-4}$) than those mice fed the corn oil diet ($2.10 \pm 0.55 \times 10^{-3}$) (Fig. 3.2A). These results indicate that n-3 FAs were inducing a reduction in total myeloid progenitors in bone marrow. GM, G, and M progenitors were significantly less ($p < 0.05$) frequent in the bone marrow of mice fed the fish oil diet than those fed the corn oil diet (Fig. 3.2A). Interestingly, E progenitors did not change in their frequency with diet, indicating n-3 FAs preferentially affected levels of GM progenitors and their daughter cells (see Fig. 3.1). There was also an apparently larger frequency of GEMM progenitors, which are early progenitors, in corn oil ($5.16 \pm 4.32 \times 10^{-5}$) vs. fish oil ($1.20 \pm 1.20 \times 10^{-5}$) fed mice. Although this was a greater than 4 fold difference in frequency, the difference was not statistically significant ($p = 0.11$) and the rarity of this progenitor relative to others made it difficult to accurately assay with this technique.

Analysis of the proportions of progenitor cells that make up the myeloid progenitor cell compartment in these mice revealed that mice fed a fish oil diet had a much different proportion of early and late stage progenitors compared to those fed a corn oil diet ($p = 0.00015$). There was a greater percentage of later stage progenitor cells (G, M, E progenitors) in fish oil diet fed mice ($94.9 \pm 1.6\%$) when compared to mice fed a corn oil diet ($71.3 \pm 4.8\%$) (Fig. 3.2B). Conversely, there was a greater proportion of earlier stage progenitors (GEMM and GM progenitors) in corn oil fed mice ($28.7 \pm 4.8\%$) vs. fish oil fed mice ($5.15 \pm 1.6\%$). Thus, our data indicate that the bone marrow of mice fed a high level of n-3 FAs had a more differentiated myeloid progenitor cell compartment than that of mice fed a diet containing mostly n-6 FAs.

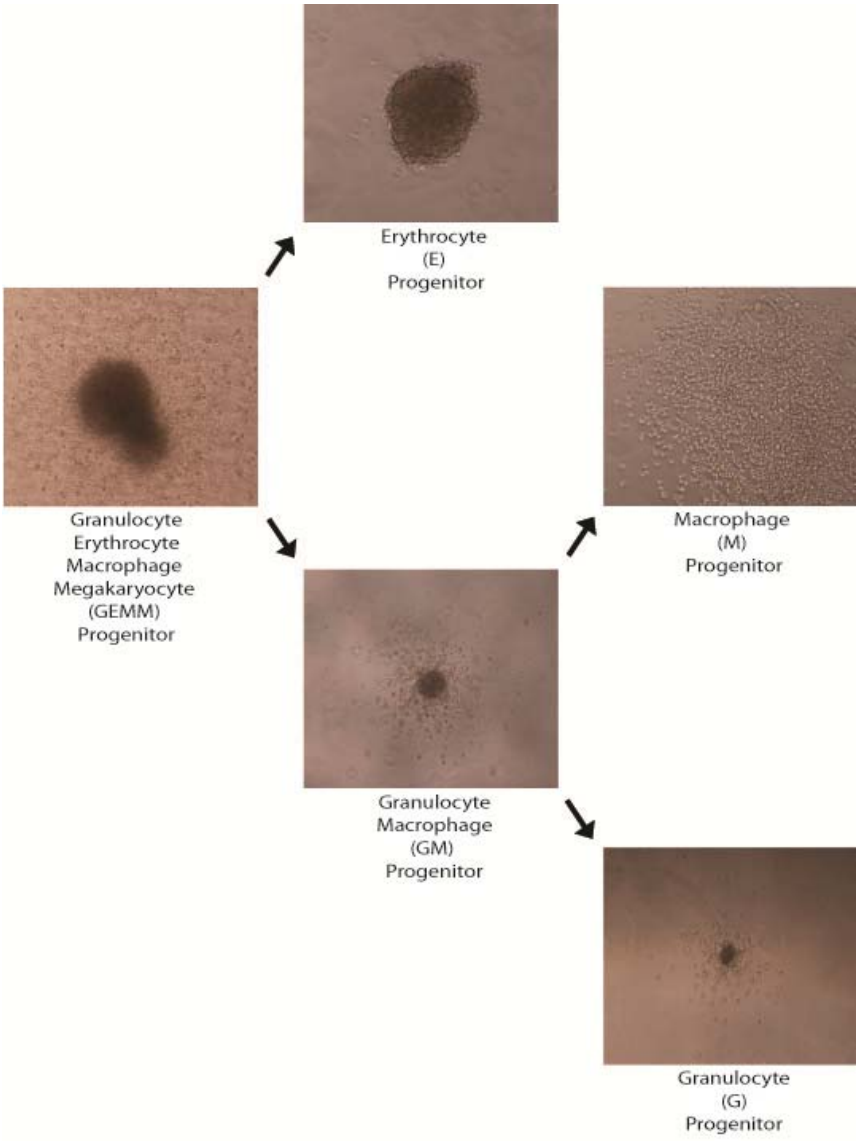

Figure 3.1. Hematopoiesis differentiation continuum – myeloid progenitor morphology. Hematopoiesis exists as a continuum of differentiation. Colony morphology representing the various myeloid progenitor cells is shown (magnification = 100×) in a differentiation hierarchy beginning with the least differentiated progenitor and continuing to the most differentiated progenitor. Abbreviations: Granulocyte erythrocyte macrophage megakaryocyte (GEMM), granulocyte macrophage (GM), erythrocyte (E), granulocyte (G), macrophage (M).

Table 3.1: Modified AIN-76A Diet Composition.

	Diet composition	
Ingredient	**% of weight**	**Amount/100 g**
Casein (protein)	20%	20 g
Sucrose	45%	45 g
Corn starch (carbs)	15%	15 g
Alphacel (fiber)	5%	5 g
Choline bitartrate	0.2%	0.2 g
DL-methionine	0.3%	0.3 g
Mineral mix	3.5%	3.5 g
Vitamin mix	1.0%	1 g
Fat	10%	10 g
Total	100%	100 g
Total fat		10 g
Total protein		20 g
Total carbohydrate		60 g

The base diet is an AIN-76A diet modified by substitution of 5% sucrose for 5% more oils to contain a total of 10% w/w oil. The mouse food recipe contains 10% fat in each diet supplied through these oils.

Table 3.2: Compositions of dietary fats (approximate %).

	Saturated fatty acids	Linoleic acid (omega 6)	Total omega 3	Monounsaturated fatty acids
Corn oil[a]	13	61	1	26
Canola oil[a]	6	20	10	62
n-3 supp[b]	9	6	63	21

The corn oil diet is the low omega 3:omega 6 fatty acid diet containing 10% w/w corn oil as the source of all fat. The fish oil diet is our high omega 3:omega 6 fatty acids diet containing 5% w/w canola oil and 5% w/w omega 3 fatty acid supplement. The corn oil diet contains 0.1% omega 3 fatty acids and 6.1% omega 6 fatty acids, which is a relative ratio of omega 6 to omega 3 of 61: 1. The fish oil diet contains 3.65% omega 3 fatty acids and 1.3% omega 6 fatty acids, which is a relative ratio of 1: 2.8.

[a]From: http://www.nal.usda.gov/fnic/foodcomp/search

[b]From: Manufacture's certified analyses.

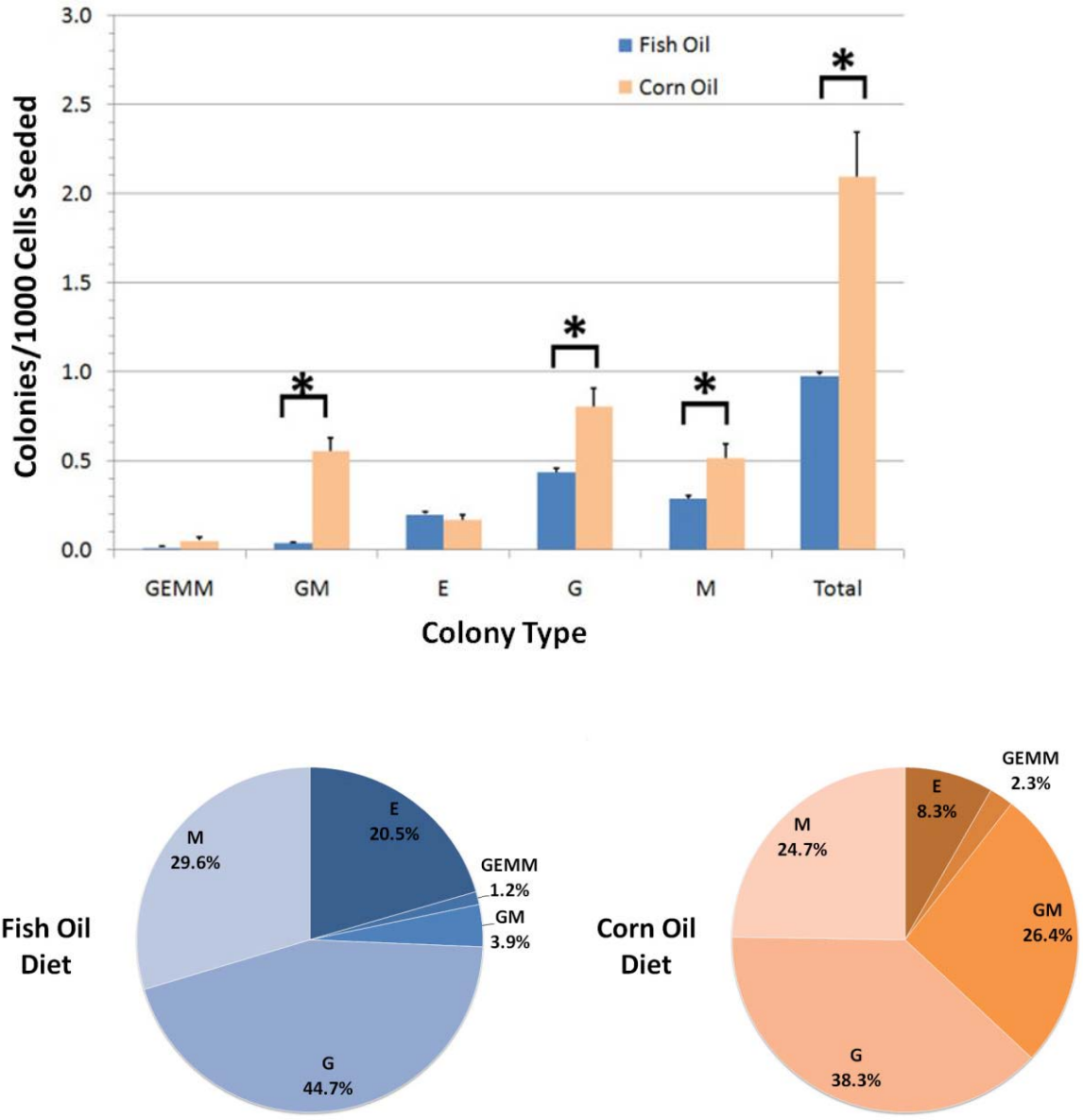

Figure 3.2: Fish oil diets cause changes in the steady state levels of myeloid progenitors in the bone marrow. A. Colony forming cell (CFC) assay results of bone marrow from mice fed corn oil (high n-6) vs. fish oil (high n-3) diets until 60 days of age. Colonies formed represent the presence of a progenitor cell in which the type of colony determines which progenitor cell type is present. Comparisons marked with (*) were found to be significantly different ($p < 0.05$). Error bars represent SEMs ($n = 5$). B-C.

Data from A in which each colony type is shown as a proportion of total progenitor cell frequency for each diet.

High levels of n-3 FAs do not have an adverse effect on white blood cell production in the peripheral blood

To account for the concern that a lower progenitor cell frequency or a greater proportion of differentiated progenitor cells in the bone marrow may cause a reduction in white blood cells in peripheral blood and therefore a weakened immune system, we performed white blood cell (WBC) counts on mice fed these diets from weaning to 115 days. Results indicated no significant differences in peripheral WBC counts between corn oil fed mice (14,825 WBC/μl) and fish oil fed mice (13,760 WBC/μl) (Fig. 3.3). Thus, fish oil diets induce a homeostatic condition of decreased progenitor cell frequency in the myeloid compartment of the bone marrow without adverse effects on WBC production.

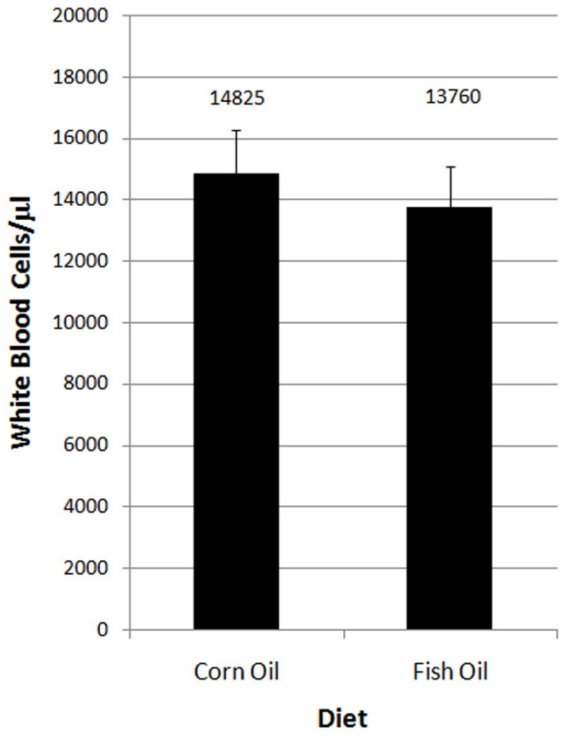

Figure 3.3: Fish oil diets do not cause adverse reduction of white blood cell counts in the peripheral blood. White blood cell (WBC) counts in WBC/µl from female mice on the two diets until 115 days of age (n = 4 for fish oil, n = 5 for corn oil, p = 0.602). Error bars represent SEMs.

Discussion

Our results indicate that n-3 FAs reduce total myeloid progenitor cell frequency and promote differentiation of specific progenitor cell types in the bone marrow of mice. Furthermore, this reduction of progenitors does not have an adverse effect on levels of circulating white blood cells. Our data indicate that n-3 FA effects are particular to granulocyte and macrophage progenitor cells rather than erythrocyte maturation. The

effects of n-3 FAs may occur as early in maturation as the GEMM progenitor stage and then be propagated to the GM and later progenitor stages. While our results indicate a difference at the GM progenitor stage, they are also suggestive, although inconclusive, of a difference at the GEMM stage. We are currently examining this question with ongoing experiments to look at the earliest stem and progenitor cell stages of hematopoiesis.

Examination of the effects of n-3 FAs on hematopoiesis could lead to potential therapeutic options for patients with disorders of the blood. Future work would include investigating the mechanisms of action for these events. Mechanisms by which n-3 FAs have been shown to inhibit proliferation, induce apoptosis, and promote differentiation in many cancers include the regulation of signaling pathways and gene expression by peroxisome proliferator receptor activator γ (PPARγ), for which n-3 FAs are natural ligands [16]. Another mechanism of action of n-3 FAs includes inhibition of COX2, which is upregulated in various cancers and is known to have proproliferative and antiapoptotic effects [17-25]. Furthermore, the combination of activating PPARγ and inhibition of COX2 expression has recently been shown to inhibit proliferation and induce apoptosis in pancreatic cancer [26]. We plan to explore these, among other mechanisms, using our animal model.

Regardless of the mechanisms by which n-3 FAs reduce and differentiate myeloid progenitor cells in the bone marrow of mice, these dietary agents serve as a promising option for therapy. The inhibitory effect of n-3 FAs on immune system function [10, 27] has led to the use of fish oils that are high in these FAs in the management of several inflammatory and autoimmune diseases [27]. Suppression of n-6 derived eicosanoids has been proposed as a strategy for chemoprevention and as an adjunct for treatment of

cancer [12-15]. Our results suggest that using n-3 FAs to more rapidly differentiate myeloid progenitor cells may slow progression of disorders of hematopoiesis, including leukemias, and restore a more normal myeloid progenitor cell compartment in the bone marrow.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MEV was responsible for the large part of data acquisition. MEV and VES analyzed data and wrote this manuscript. WEH provided the mice with appropriate diets and intellectual contributions on study design. VES and MEV provided intellectual contribution on study design as well. VES coordinated the project.

Acknowledgments

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Chapter 4: A high omega-3 fatty acid diet has different effects on early and late stage myeloid progenitors

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Lipids [In Press]

Abstract

The effects of the polyunsaturated omega-3 (n-3) and omega-6 (n-6) fatty acids (FAs) on hematopoiesis are complex in that both FA forms are processed into leukotrienes, eicosanoids, and prostaglandins, which can have independent effects. These FAs have antagonistic effects in that n-6 FA prostaglandins tend to be pro-proliferative and pro-inflammatory, whereas the effects of n-3 FA prostaglandins are the opposite. We have previously shown that diets high in n-3 FAs reduce the size of the middle to later stage myeloid progenitor compartment in FVB X sv129 F1hybrid mice. To assay the effects of high n-3 FA diets on earlier stages of myelopoiesis, we fed C57BL/6J mice diets high in n-3 FAs or at levels of n-3:n-6 FAs similar to western diets and assayed the effects on myelopoiesis with flow cytometry and colony forming cell (CFC) assays. Results indicate an expansion of the common myeloid progenitor cell compartment in high n-3 FA diets, which does not persist into later stages where the number of progenitor cells is actually lower in high n-3 FA fed animals. Investigations *in vitro* with the hematopoietic stem cell line EML-clone 1 indicate that cells cultured with eicosapentanoic acid (n-3 FA) or arachidonic acid (n-6 FA) have no differences in cell viability but that arachidonic acid more rapidly produces progenitors with low levels of the macrophage developmental marker, F4/80.

Key words: Omega fatty acids, EPA, DHA, immunology, stem cells, progenitor cells, bone marrow, nutrition

Introduction

The examination of omega-3 (n-3) and omega-6 (n-6) fatty acids (FAs) effects on hematopoiesis is important to patient care. The inhibitory effect of n-3 FAs on inflammation has led to the use of fish oils that are high in these FAs in the management of several inflammatory and autoimmune diseases [1]. n-3 FAs affect hematopoietic differentiation by influencing myeloid progenitor cells [2]. n-3 FAs are known to affect immune system function by reducing several aspects of neutrophil, monocyte, and lymphocyte function [1]. Suppression of n-6 derived eicosanoids has been proposed as a strategy for chemoprevention and as an adjunct for treatment of cancer [3-6].

n-3 and n-6 FAs are incorporated into cell membranes either directly or after elongation and then desaturation by Δ6 and Δ5 desaturases. Dietary linoleic acid (LNA, 18 carbons, n-6 FA) is generally considered to be the major source of tissue arachidonic acid (ARA, 20 carbons, n-6 FA) although meat fat can be a direct source of ARA [7]. n-3 FAs have greater affinity for the Δ5 and Δ6 desaturases than n-6 FAs. Consequently, increasing dietary intake of n-3 FAs reduces the desaturation of LNA and reduces the production of ARA [8]. All three major n-3 FAs – α linolenic acid (ALA, 18:3, n-3), eicosapentanoic acid (EPA, 20:5, n-3), and docosahexanoic acid (DHA, 22:6, n-3) – directly inhibit the production of ARA from LNA [8].

Both ARA and EPA can be cleaved from the cell membrane phospholipids stores by phospholipase A2 and acted on by cyclooxygenases (either the constitutive COX1 or the inducible COX2) to produce prostaglandin precursors which are isomerized by prostaglandin synthases to produce prostaglandins. COX activity on ARA forms the 2-series prostaglandins that tend to be pro-proliferative and pro-inflammatory in most

tissues [9]. Micromolar concentrations of prostaglandin E2 increase human myeloid progenitor cell proliferation [2]. However, COX activity on EPA forms the 3 series prostaglandins that tend to have anti-proliferative and anti-inflammatory properties [9]. In addition to prostaglandins, leukotrienes and eicosanoids are formed from FAs through activity of various lipoxygenases. These have been shown to have varying and sometimes controversial effects on either hematopoietic stem cell or myeloid progenitor cell differentiation [2, 10]. A model system approach is needed to effectively dissect the net effect of dietary FAs on hematopoiesis *in vivo*.

In this study, we examined the effects of n-3 and n-6 FAs *in vivo* in the mouse, with an analysis at the level of stem and progenitor subtypes. Our results indicate that, compared to diets rich in n-6 FAs, diets rich in n-3 FAs induce lower levels of later stage myeloid progenitor cells in mice but that there is a higher frequency of the earliest stage myeloid progenitor cells in these mice. Our *in vitro* results indicate that aspects of the *in vivo* effects of n-3 and n-6 FAs can be modeled using the EML cell culture system to ascertain the mechanisms involved.

Materials and methods

Animals

C57BL/6J mice used in this study were purchased from The Jackson Laboratory (Bar Harbor, ME). Only male mice were used to avoid cyclic changes in hormones. Mice were housed in the AAALAC accredited animal facilities of the Marshall University School of Medicine. All animal use and care was approved by the Marshall University Institutional Animal Care and Use Committee. The mice were housed 3 to 4 in a cage and

individually numbered for identification. Mice were fed either a fish oil diet (n=8) or a corn oil diet (n=8) from 6 weeks of age until 20 wks of age (100 days on diet).

Diet

The base diet was an American Institute of Nutrition (AIN)-76A diet modified by substitution of 5% sucrose for 5% more oils to contain a total of 10% w/w oil (Tables 4.1 and 4.2). The fish oil diet contained 3.65% n-3 FAs and 1.3% n-6 FAs. The corn oil diet contained 0.1% n-3 FAs and 6.1% n-6 FAs. Diets were prepared in the Marshall University School of Medicine animal diet prep room. Diet composition is shown in Table 4.1 and was formulated to be isocaloric, isonutrient and relevant to human consumption. The AIN-76A diet is adequate for the nutritional support of the mice [11]. The dry ingredients of the diet were obtained in bulk from MP Biomedicals (Solon, Ohio): sugar, corn and canola oil were purchased locally (100% canola oil, 100% corn oil, no additives or preservatives). The n-3 supplement (OmegaRx Liquid) was purchased from Zone Labs, Danvers, MA. Batches of diet were prepared as needed, about every two weeks. The diet mixture was pressed into trays. Food (25-30 g) was stored in sealed containers at -20°C to prevent oxidation of the fat and bacterial growth in the food. Mice had free access to food and water and were fed fresh food 5 days per week. Food removed from cages was discarded.

Colony forming cell (CFC) assays

CFC assays were performed upon bone marrow isolated from C57BL/6 mice as previously discussed [12, 13]. Briefly, bone marrow was harvested by flushing from the

femurs with Iscove's modified Dulbecco's medium and cells were counted and seeded in 4 well plates in at a density determined empirically by a pilot study conducted with a broad and consistent range of seeding densities. After linear response of colony production to seeding density was ensured by the pilot study, seeding densities were chosen to produce 10-30 colonies for each well (4-well plates). Bone marrow was cultured in 1% semi-solid Methocult M3434 (StemCell Technologies) supplemented with 0.4% autochthonous sera. Cells were incubated for 6-7 days to allow colony formation. Colonies with a minimum cell number of 20 were scored as positive using an inverted microscope at 40X magnification. Colonies were counted based on morphological features that are associated with each progenitor type.

Flow cytometry

The preparation of bone marrow for flow cytometry was performed as previously described [12]. Erythroid myeloid lymphoid (EML) cells were prepared by washing twice with FACS buffer (PBS supplemented with 0.5% bovine serum albumin and 2mM EDTA) and collecting by centrifugation. Thereafter, samples were incubated with 2% autochthonous sera obtained from cardiac puncture just before marrow harvest to prevent nonspecific binding by blocking the Fc receptors for 30 minutes at 4°C. The cells were washed again and labeled with antibodies for 30 minutes on ice. The following antibodies were used in the bone marrow studies with Streptavidin-Pacific Blue to detect biotinylated antibodies: PE-Cy7 conjugated Sca-1 (clone D7, eBiosciences #25-5981-81), APC-eFluor 750 conjugated CD117 (clone 2B8, eBiosciences #47-1171-80), biotinylated lineage panel (BD Biosciences #559971), biotinylated IL-7Rα (clone B12-1, BD

Biosciences #555288), APC conjugated FCRγ (clone 93, eBiosciences #17-0161-81), and PE conjugated CD34 (clone RAM34, BD Biosciences 551387). The following antibodies were used in labeling the EML-clone1 cells in labeling pairs with Streptavidin-APC (BD Biosciences #554067) to detect biotinylated antibodies: biotinylated Ly6G/C (clone RB6-8CS, BD Biosciences #553125) + PE conjugated CD117 (clone 2B8, BD Biosciences #553355), biotinylated CD11b (clone M1/70, BD Biosciences #553309) + PE conjugated Sca-1 (clone D7, BD Biosciences #553108), and biotinylated CD45 (clone RA3-6B2, BD Biosciences #553086) + PE conjugated F4/80 (clone BM8, Caltag #MF48004). Data acquisition was performed using BD FACS Aria I sorter and data analysis and compensation was performed using FlowJo v. 7.6 software (Treestar, Ashland, OR) with super-enhanced Dmax subtraction analysis for determination of small differences in overlapping histograms. Algorithms for super-enhanced Dmax analysis are used to calculate the percentage of positive cells found in the sample and not the control by allowing for a quantitative subtraction of non-specific fluorescence.

In vitro culture and differentiation

EML C1 cells were the kind gift of Dr. Schickwann Tsai (University of Utah, Salt Lake City, UT) and were maintained in Iscove's modified Dulbecco medium (IMDM,) supplemented with 20% horse serum (American Type culture collection, ATCC, Manassas, VA) and 10% baby hamster kidney (BHK)/MKL cell conditioned medium [14]. For differentiation studies, EML cells were induced to differentiate into myeloid cells with 10μM all-*trans* retinoic acid (ATRA; Sigma, St. Louis, MO, USA), 10% BHK cell conditioned medium (source of stem cell factor) and 15% WEHI (monocyte cell line)

cell conditioned medium (source of interleukin-3α) for three days. EML-clone1 cells were seeded at $2x10^5$ cells/mL and cultured for 24 hours in 60μM FAs in standard growth medium (20% horse serum, 70% Dulbecco's modified eagle medium, and 10% BHK cell conditioned medium). After 24 hours cells were placed in differentiation medium with 60μM FAs at $2.0x10^5$ cells/mL. Cell counts were performed using trypan blue at 24, 48, 72, and 96 hours. After 96 hours cells were processed for flow cytometry analysis.

Fatty acid metabolism studies

Cells were seeded at $2x10^5$ cells/mL and treated with vehicle control of ethanol or 60μM FAs. The ethanol vehicle control was added in the same concentrations as FAs. Cell counts were performed at 24, 48, 72, and 96 hours using trypan blue. At each time point, half the total volume of the culture was taken as a sample and replaced with untreated growth media. Each sample was centrifuged (500xg), the supernatant removed, and the pellet stored at -20°C until analyzed by gas chromatography. At the time of gas chromatography, cells were homogenized in 0.1% butylated hydroxytoluene in 70% methanol/distilled water to prevent FA oxidation. Lipids were extracted with chloroform/methanol and methylated. Methylated lipids were separated and identified using gas chromatography as previously published [15]. FA methyl ester standards (Nu-Chek-Prep, Elysian, MN) were used for peak identification. The FA methyl esters were reported as the percent of the total methylated FAs (area under the curve).

Statistical analysis

Statistical analyses were run using SAS software release 9.2 (SAS Institute Inc. Cary, NC). Student's t-tests were used to detect differences between the experimental groups (corn oil versus fish oil diet) of colony forming cells (n=8 for each group) and between the experimental groups (corn oil versus fish oil diet) in flow cytometry assays (n = 8 for each group). To determine statistical significance in gas chromatography experiments and differentiation studies the levels of FAs of a particular type were analyzed using either one-way analysis of variance (ANOVA) or by one-way Kruskal-Wallis analysis of variance on ranks. Dunnett multiple comparison tests were used for testing if any treatments are significantly different from a single control for all main effects means in the MEANS statement.

Results

Fish oil diets induce changes in the frequency of various myeloid progenitor cell types in the bone marrow

In our previous investigations of the effect of fish oil diets on murine hematopoiesis, we found a down-regulation in the frequency of myeloid progenitor cells in bone marrow in mice fed fish oil diets compared to those on corn oil diets[16]. Other investigators have reported that bone marrow of rodents readily changes composition based upon dietary FA sources [17]. However, our investigations using CFC assays limited our ability to assay the frequency of more immature cell types, such as the common myeloid progenitor (CMP, same as GEMM) and hematopoietic stem cell (HSC). In order to assay the effects of high n-3 FA diets on these cell types, we verified

that the effects seen in FVB X sv129 F_1 hybrid animals were also seen in C57BL/6 mice in which flow cytometry markers for examining these rare cell types have been verified [18]. In the experiments presented here, mice were fed diets rich in n-3 FAs (fish oil diet) or rich in n-6 FAs (corn oil diet) from 6 weeks of age until 20 wks of age (Tables 4.1 and 4.2, 100 days on diet). Bone marrow was then harvested and analyzed by colony forming cell assay and flow cytometry. There was no difference in density of cells in the bone marrow (Fig. 4.1A). Our results (Fig. 4.1B) indicated an overall lower frequency of mid to late stage progenitors when mice were fed n-3 FA rich diets compared to those that were fed n-6 FA rich diets. There was a significantly lower (26%, p-value < 0.01) overall myeloid progenitor cell frequency, with significant reductions (p-value < 0.05) in colony forming unit granulocyte-macrophage (CFU-GM) and colony forming unit macrophage (CFU-M).

When we examined the relative contributions of each progenitor cell type to the overall myeloid progenitor cell pool in the bone marrow (Fig. 4.1C), we found that there is a significant reduction in CFU-GM proportion in mice on the fish oil diet (p-value < 0.05). The comparison of proportions of subtypes of myeloid progenitors is different from what we observed in FVB X sv129 F1 hybrid mice [16], where we observed an overall shift to more later stage progenitor types [16]. Thus, though there was a reduction in myeloid progenitor cell frequencies in the marrow and a two-fold reduction in proportion of CFU-GM, there was little shift in proportions of the other progenitor subtypes. This difference may be due to particular characteristics of the C57BL/6 inbred strain that are not seen in F_1 hybrid animals, possibly including the lessening of homozygous recessive mutations in hybrid animals. In a previous publication, we have

shown that mice vary significantly in myeloid progenitor cell frequencies [12]. These data indicate that C57BL/6 mice responded to high n-3 FA diets with a reduction in overall myeloid progenitor cell frequencies and significantly altering the proportions of the CFU-GM present.

Table 4.1: Modified AIN-76A Diet Composition.

	Diet composition	
Ingredient	**% of weight**	**Amount/100 g**
Casein (protein)	20%	20 g
Sucrose	45%	45 g
Corn starch (carbs)	15%	15 g
Alphacel (fiber)	5%	5 g
Choline bitartrate	0.2%	0.2 g
DL-methionine	0.3%	0.3 g
Mineral mix	3.5%	3.5 g
Vitamin mix	1.0%	1 g
Fat	10%	10 g
Total	100%	100 g
Total fat		10 g
Total protein		20 g
Total carbohydrate		60 g

The base diet is an AIN-76A diet modified by substitution of 5% sucrose for 5% more oils to contain a total of 10% w/w oil. The mouse food recipe contains 10% fat in each diet supplied through these oils.

Table 4.2: Compositions of dietary fats (approximate %).

	Saturated fatty acids	Linoleic acid (omega 6)	Total omega 3	Monounsaturated fatty acids
Corn oil[a]	13	61	1	26
Canola oil[a]	6	20	10	62
n-3 supp.[b]	9	6	63	21

The corn oil diet is the low omega 3:omega 6 fatty acid diet containing 10% w/w corn oil as the source of all fat. The fish oil diet is our high omega 3:omega 6 fatty acids diet containing 5% w/w canola oil and 5% w/w omega 3 fatty acid supplement. The corn oil diet contains 0.1% omega 3 fatty acids and 6.1% omega 6 fatty acids, which is a relative ratio of omega 6 to omega 3 of 61: 1. The fish oil diet contains 3.65% omega 3 fatty acids and 1.3% omega 6 fatty acids, which is a relative ratio of 1: 2.8.

[a]From: http://www.nal.usda.gov/fnic/foodcomp/search

[b]From: Manufacture's certified analyses.

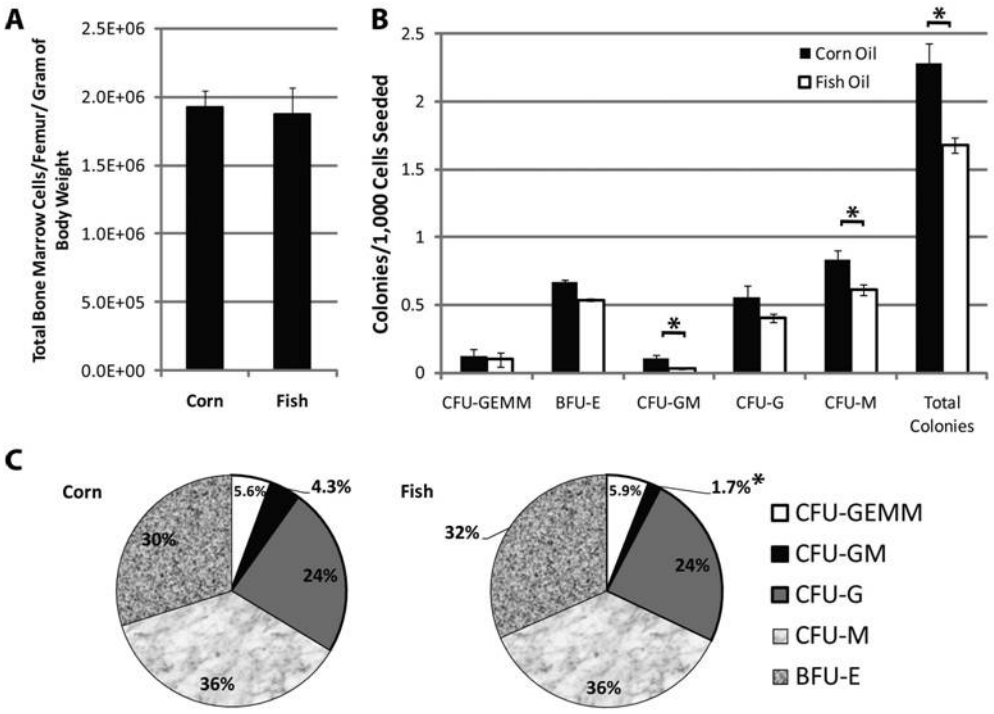

Figure 4.1: Reduction in middle to latter stage myeloid progenitor cell types by high n-3 diet. Mice were fed either corn oil or fish oil diets for 100 days followed by harvesting of bone marrow from femurs. A. The number of cells harvested from the mice on the different diets was similar. B. Colony forming cell assays were performed to enumerate the frequency of middle to latter stage progenitor types. The frequency of each progenitor cell type is shown. C. The relative distribution of progenitor types in the total progenitors assayed is shown. Significant differences as measured by Student's t-tests (p-value < 0.05) are indicated by *. Error bars represent SEM (n=8). SEMs in (C) for the corn oil diet data are BFU-E: 2.4%, CFU-GEMM: 0.8%, CFU-GM: 1.1%, CFU-G: 2.7%, CFU-M: 1.8% and for the fish oil diet are BFU-E: 2.4%, CFU-GEMM: 0.6%, CFU-GM: 0.5%, CFU-G: 2.1%, CFU-M: 2.1%. Abbreviations are: colony forming unit (CFU), blast forming unit (BFU), granulocyte erythrocyte monocyte macrophage (GEMM), erythrocyte (E), granulocyte monocyte (GM), granulocyte (G), and macrophage (M).

The frequency of the common myeloid progenitor fraction is increased in mice fed fish oil diets

In order to assay the frequencies of earlier stage stem and progenitors cells in mice fed corn and fish oil diets, flow cytometry studies were conducted using established gating parameters from the Weissman lab [18] for the assay of HSCs, CMPs, granulocyte-macrophage progenitors (GMPs), and megakaryocyte-erythrocyte progenitors (MEPs) in C57BL/6 mice (Fig. 4.2A). Bone marrow cells were first gated by excluding high and low forward scatter cells along with those labeled positively for one of the differentiation antigens (refer to Table 4.3) in the lineage panel or IL-7Rα, which marks lymphoid cells. This population of FSC^{mid}IL-7R$\alpha^{-}Lin^{-}$ cells was further gated into stem (FSC^{mid}IL-7R$\alpha^{-}Lin^{-}Sca\text{-}1^{+}$c-$Kit^{+}$) and progenitor ($FSC^{mid}$IL-7R$\alpha^{-}Lin^{-}Sca\text{-}1^{-}$c-$Kit^{+}$) cell fractions by expression of c-Kit and Sca-1 as shown. The progenitor pool was also analyzed and gated based upon the expression of CD34 and FcγR to delineate CMPs (FSC^{mid}IL-7R$\alpha^{-}Lin^{-}Sca\text{-}1^{+}$c-$Kit^{+}Fc\gamma R^{lo}CD34^{+}$), GMPs ($FSC^{mid}$IL-7R$\alpha^{-}Lin^{-}Sca\text{-}1^{+}$c-$Kit^{+}Fc\gamma R^{hi}CD34^{+}$), and MEPs ($FSC^{mid}$IL-7R$\alpha^{-}Lin^{-}Sca\text{-}1^{+}$c-$Kit^{+}Fc\gamma R^{lo}CD34^{-}$). These data show a 50% increase (p-value = 0.01) in the frequency of the CMP fraction in mice fed fish oil diets (Fig. 4.2B). There were no significant differences in frequency of HSC, GMP, or MEP fractions indicated in these studies. These data suggest that the frequency of the CMP has been increased by administration of a diet containing high levels of n-3 FAs.

Table 4.3: Flow cytometry markers for gating populations of stem and early myeloid progenitor cells.

Flow Cytometry Marker	Cell Population Positive for Marker
Lineage panel	Differentiated cells
IL-7Ra	Lymphocyte progenitors
Sca-1	Stem cells
C-kit	Stem and progenitor cells
FcγR	MEPs and CMPs - low positive GMPs - high positive
CD34	GMPs and CMPs

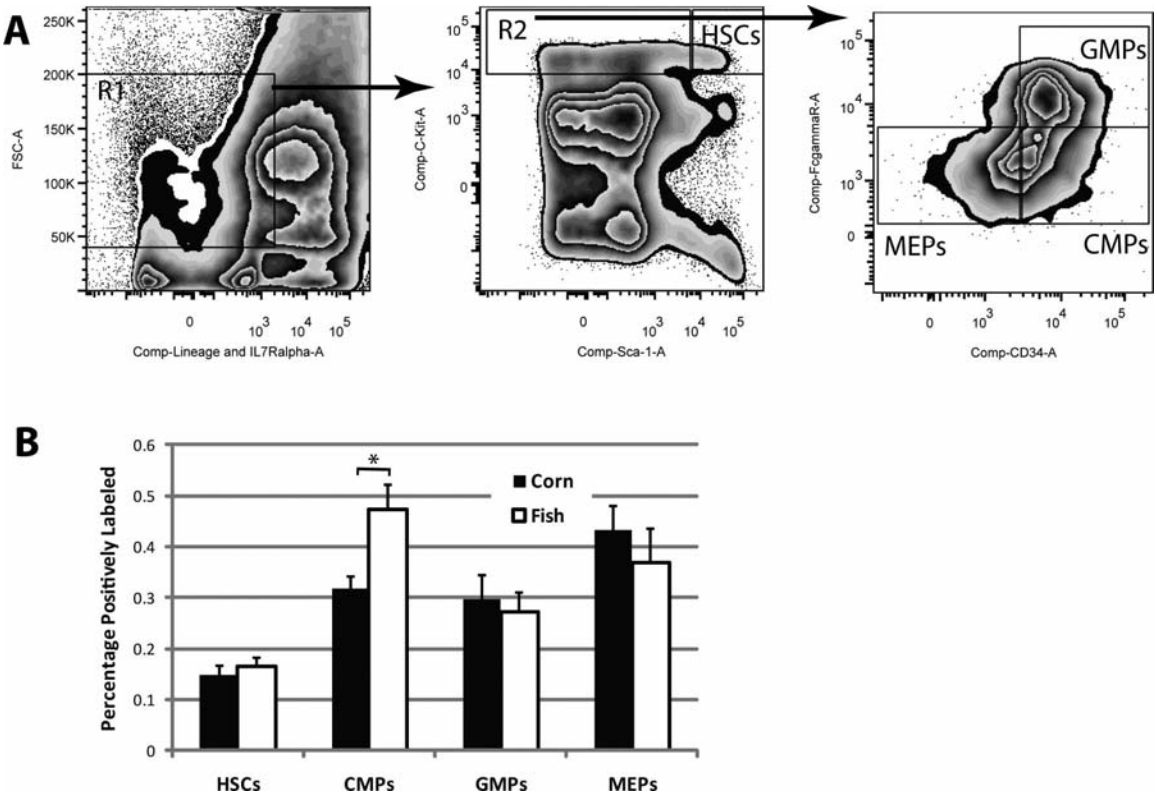

Figure 4.2: Increase in early myeloid progenitors by high n-3 diets. Mice were fed either corn oil or fish oil diets for 100 days followed by harvesting of bone marrow from femurs. A. Representative plots are displayed to show gating parameters. Bone marrow cells were analyzed by flow cytometry first by FSC X lineage panel + IL7Rα (left panel). R1 represents the population of undifferentiated cells of the myeloid lineage.

This population was further analyzed by C-kit and Sca-1 expression, allowing for the gating of the HSC and R2 populations. R2 represents early progenitor cell populations. The R2 population was further analyzed and gated into CMP, GMP, and MEP populations based on FcγR X CD34 expression. B. The percentage of the total marrow cells labeling positive for each fraction are shown. Significant differences as measured by Student's t-tests (p-value <0.05) are indicated by *. Error bars represent SEM (n=8). Abbreviations are: hematopoietic stem cells (HSCs), common myeloid progenitors (CMPs), granulocyte macrophage progenitors (GMPs), and megakaryocyte erythrocyte progenitors (MEPs).

FAs applied to EML cell culture medium are incorporated and processed

In order to study the observed effects of n-3 vs. n-6 FAs on early myeloid progenitor cells in more detail, we examined the effects of the polyunsaturated FAs EPA and ARA on EML cells in culture. We chose to compare these two FAs because EPA (20:5, n-3) is the FAs most molecularly similar to ARA (20 carbons, n-6 FA) available. DHA (22:6, n-3) has two more carbons in the chain. The EML cell line is a stem cell factor dependent multipotent cell line with erythroid (E), myeloid (M), and lymphoid (L) potential. It was established from DBA/2 mouse bone marrow infected with a retroviral vector (LRARα403SN) harboring a dominant negative retinoic acid receptor [14]. It is a suspension cell line consisting of mostly blast like cells with 20-30% hand mirror shaped cells. EML cells serve as an excellent model to study hematopoietic differentiation. It can be induced to differentiate toward granulocyte/monocyte progenitors by high concentration of all-*trans* retinoic acid (ATRA) in the presence of interleukin-3 (IL-3).

To verify the incorporation and processing by EML cells of FAs placed in the culture medium, we spiked the culture medium with 60μM ARA (Fig. 4.3A) or 60μM EPA (Fig. 4.3B) and analyzed the levels of various FAs present in the cells over the course of four days. Cells were analyzed with gas chromatography daily to determine the uptake and processing of FAs. The major FAs in untreated EML cell membranes are steric acid, oleic acid, LNA, and ARA (time 0). There are only faint traces of n-3 FAs such as ALA, EPA, docosapentanoic acid (DPA), and DHA in EML cell membranes under normal culturing conditions (time 0). In both cultures, the percentage of the major constituent of cell membranes (steric acid) is reduced as this is replaced by either ARA or EPA (Figs. 4.3A and 4.3B, 24 hours). Thus, FAs supplied in culture medium were being incorporated into cellular membranes and this occurs within 24 hours. Supplementation of ARA in the medium caused an increase in the already substantial levels of ARA in cellular membranes (Fig. 4.3A). There was a significant (p-value < 0.05) two-fold increase in ARA at 24 hours that drops to a 66% increase at 48 hours and was maintained for the remainder of the 4 days. The addition of EPA to the culture medium caused a significant (p-value < 0.001) increase in the n-3 FA EPA and elevated levels (p-value significant before correction for multiple comparisons) of the EPA elongation metabolite DPA, with a corresponding significant (p-value < 0.001) decrease in ARA by 24 hours (Fig. 4.3B). Interestingly, there was no increase in DHA, which is the Δ4 desaturase metabolite of DPA, indicating EML cells do not have this enzymatic activity. Thus, addition of FAs to the cell medium resulted in incorporation of FAs into cellular membranes and processing by 24 hours.

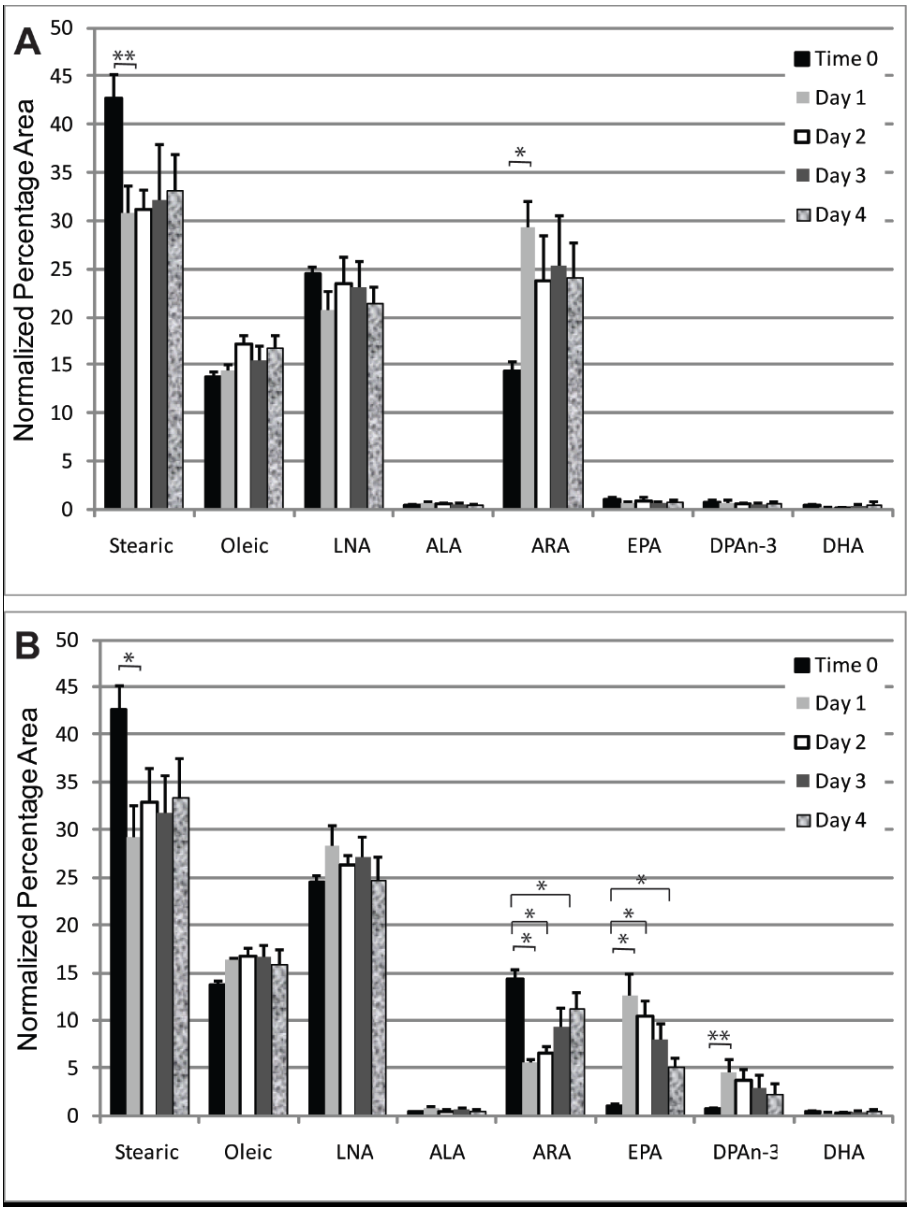

Figure 4.3: EML cells take up and metabolize FAs. EML cells were placed in growth medium containing 60μM arachidonic (A) or eicosapentanoic (B) acid. The uptake and processing of these FAs was assayed over the course of four days. Samples were taken just before addition of the FAs (t = 0), 24 hours, 48 hours, 72 hours, and 96 hours for analysis by gas chromatography. The gas chromatography data for each FA is shown above for the five data points in temporal order. Data displayed are the results of three

independent experiments with the mean and SEM displayed. Abbreviations: linoleic acid (LNA), alpha-linolenic acid (ALA), arachidonic acid (ARA), eicosapentanoic acid (EPA), docosapentanoic acid (DPA), and docosahexanoic acid (DHA). (*) indicate statistically significant (p-value < 0.05) differences based on one-way ANOVA test or Kruskal-Wallis test with Dunnett multiple comparisons adjustment. (**) indicate statistically significant (p-value < 0.05) differences based on Student's t-test or Mann-Whitney rank sum test without multiple comparison adjustment.

Incubation of EML cells with FAs has effects on immunophenotype and cell proliferation

To determine if the effects of n-3 vs. n-6 FAs seen in our *in vivo* experiment are due to viability or proliferation differences induced by the FAs, we analyzed the effect of n-3 and n-6 FAs on EML cells in culture (Fig 4.4A) for two days, as we have shown that the greatest levels of incorporation of FAs supplied through the culture medium occur at 24 hours according to the gas chromatography. EML cells in their native state are a model for hematopoietic stem cells. We found that cultures treated with 60μM ARA had significantly reduced (p-value < 0.05) cell counts after one day compared to the vehicle control. EPA treated cultures had slightly lower cell counts at day one, but the difference was not statistically significant. Neither of the treated cultures had statistically significant differences from the control at day 2, indicating that the effects of treatment with ARA were transitory. These results are consistent with the *in vivo* results, in which we saw no difference in the stem cell compartment of fish oil (high n-3) vs. corn oil (high n-6) fed mice.

To observe cell autonomous effects of n-3 vs. n-6 FAs on cell viability and proliferation during GMP myeloid progenitor cell differentiation, we primed EML cells for 24 hours with 60μM EPA or ARA and then induced differentiation into macrophage/granulocytic lineages with ATRA, stem cell factor (SCF), and IL-3. After 24, 48, and 72 hours in the differentiation medium, cell counts were performed on these EML cell cultures. Viability was measured by trypan blue dye exclusion (Fig. 4.4B). The viability of the cell cultures was very similar with the various treatments at each stage with a fairly consistent viability as the cultures differentiated (measured by non-stained cells/total cells, data not presented). There were no differences in viability when a one-way ANOVA tests were run. All cultures were proliferating robustly with a doubling time starting at 18 hours and shortening as differentiation proceeded (data not presented). The major finding of this study was a significant reduction compared to control (p-value < 0.05) in cell counts in the EPA treatment group at days 2 (11% reduction) and 3 (21% reduction) with a suggestive reduction at day 1 (Fig. 4.4B). No reduction in viability of the cultures at these time points suggests that the difference was due to cell proliferation. These results are consistent with our *in vivo* results that show a higher frequency of middle-later stage myeloid progenitors with high n-6 diets.

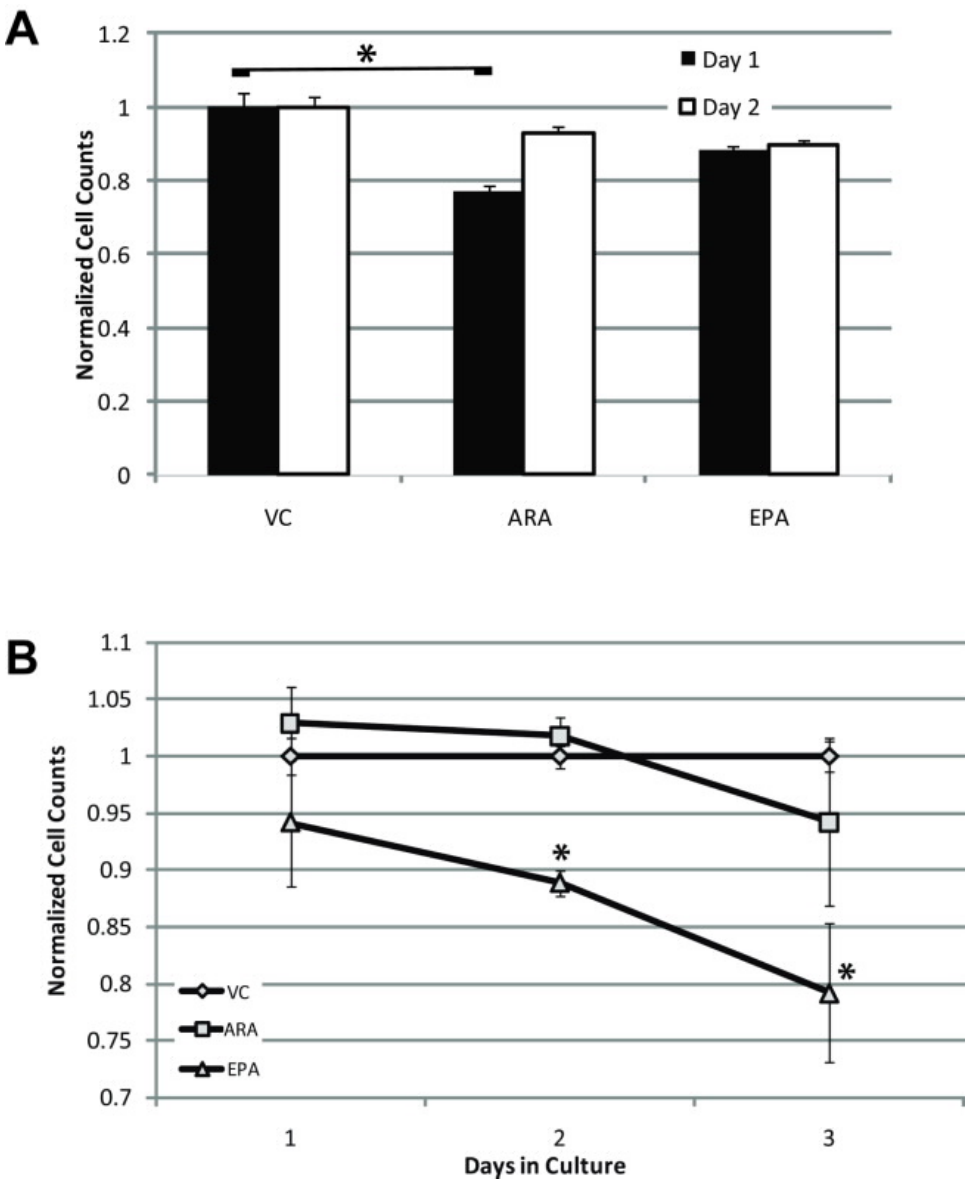

Figure 4.4: *In vitro* culture of EML cells shows differences in cell proliferation between n-3 vs. n-6 treated cultures. A. EML cells in standard culture were treated with either vehicle control, arachidonic acid (n-6), or eicosapentanoic acid (n-3) for 48 hours with cell counts performed at 24 and 48 hours. B. EML cells were cultured in either vehicle control, arachidonic acid (n-6), or eicosapentanoic acid (n-3) for 24 hours then induced to differentiate for another 72 hours down myeloid lineages. Data displayed are

the results of two (A) or three (B) independent experiments with the mean and SEM displayed of culture counts normalized to the vehicle control. (*) indicate statistically significant (p-value < 0.05) difference based on one-way ANOVA test with Dunnett multiple comparisons adjustment.

To observe cell autonomous effects of n-3 vs. n-6 FAs on myeloid progenitor cell differentiation we primed EML cells for 24 hours with 60μM EPA or ARA and then induced differentiation into macrophage/granulocytic lineages with ATRA, SCF, and IL-3. After 72 hours in the differentiation medium, EML cells were immunophenotyped for the early markers (refer to Table 4.4) Sca-1 and CD117, as well as the differentiation markers Ly6G, CD11b, CD45, and F4/80 (Fig. 4.5B). There were no significant decreases in Sca-1 levels (the first sign of differentiation in this model) with treatment, though the decrease with ARA treatment is suggestive (p-value significant before correction for multiple comparisons, but not after the appropriate Dunnett multiple comparison correction) when comparing treatments to vehicle control. There was a significant (p-value < 0.05) difference in the percentage of cells labeling with the F4/80 antigen in the ARA treatment group compared to the vehicle control. The sizeable increase in F4/80 positive cells coupled with the suggestive decrease for the Sca-1 antigen in the treatments with ARA compared to vehicle (Fig. 4.5B) indicates the production of a granulocyte/macrophage progenitor population with ARA treatment. These two results are consistent in that there is a lower frequency of the marker for stem cells and an increase in frequency of the differentiation marker F4/80. This result is also consistent with the *in vivo* data that found an increase in production of macrophage

progenitor subtypes at a later stage in the differentiation continuum of hematopoiesis (Fig.4.1B). The expression of F4/80 appears to be at a low abundance on a per cell basis in both ARA and EPA treated samples but much more prevalent in ARA treated samples (Fig. 4.5C). Thus, our model suggests that the differences in the common myeloid progenitor compartment in our *in vivo* experiment are not due to changes in rate of differentiation in the bone marrow and that n-6 FA supplementation produces a shift to macrophage/granulocytic lineages as seen *in vivo*.

Table 4.4: Flow cytometry markers for gating populations at various stages in the differentiation continuum

Flow Cytometry Marker	**Cell Population Positive for Marker**
Sca-1	Stem cells
C-kit (CD117)	Stem and progenitor cells
F4/80	Early macrophages
Ly6G	Granulocytes
CD11b	Macrophages
CD45	B-cells

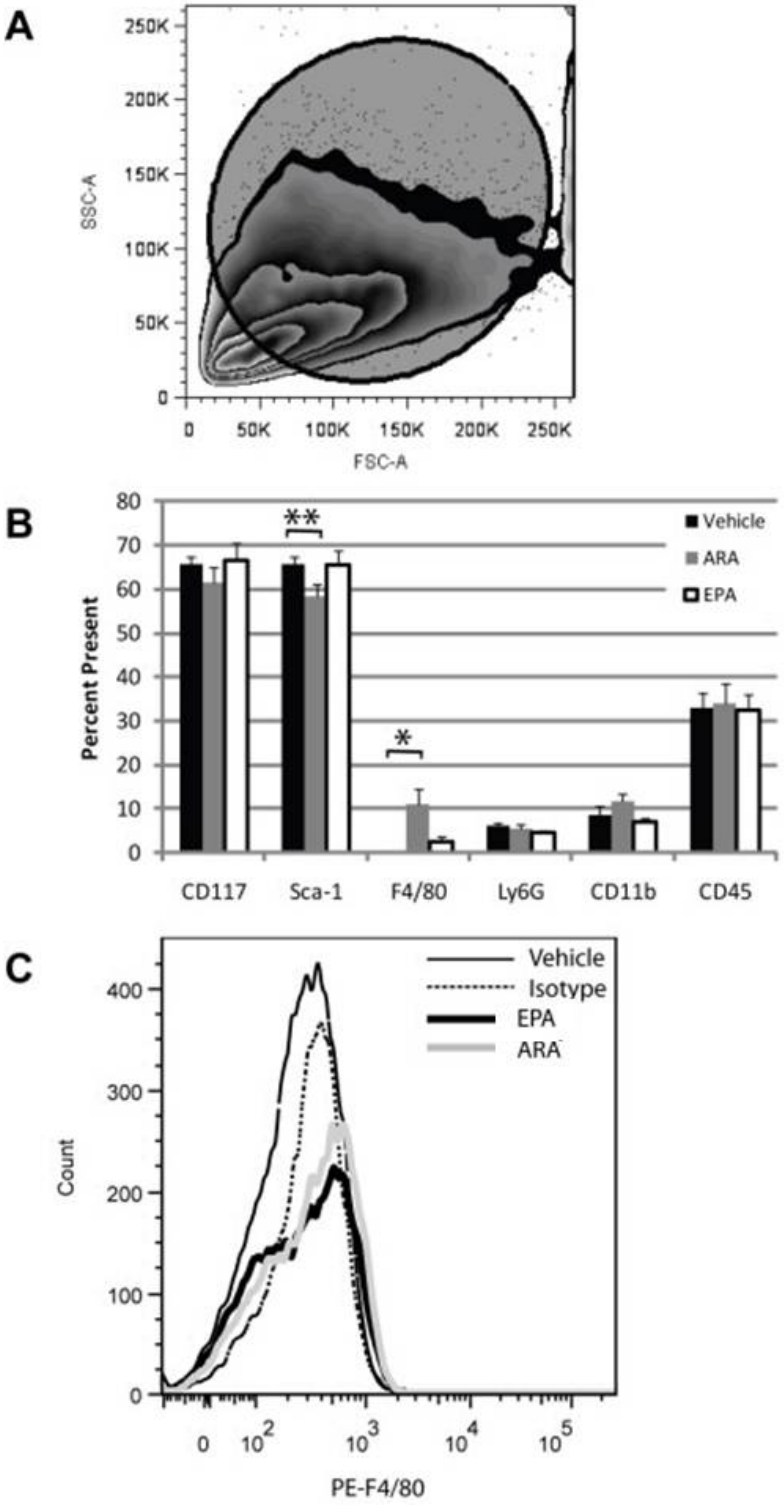
A
250K
200K
150K
100K
50K
0
SSC-A
0
50K
100K
150K
200K
250K
FSC-A
B
80
70
60
50
40
30
20
10
0
Percent Present
**
*
Vehicle
ARA
EPA
CD117
Sca-1
F4/80
Ly6G
CD11b
CD45
C
400
300
200
100
0
Count
Vehicle
Isotype
EPA
ARA
0
10^2
10^3
10^4
10^5
PE-F4/80

Figure 4.5: No significant differences in myeloid progenitor cell maturation rate between n-3 and n-6 FAs. EML cells were cultured in either vehicle control, arachidonic acid (n-6), or eicosapentanoic acid (n-3) for 24 hours then induced to differentiate for another 72 hours down myeloid lineages. A. Flow cytometry was performed analyzing the larger FSC population of cells. B. The percentage of cells displaying various markers of differentiation is shown for each culture type. C. Representative histograms are displayed for the isotype control (black, narrow line), vehicle control (dotted line), EPA treated cultures (gray line), and ARA treated cultures (black, thick line). Both FA treated cultures display a small shift over to the right indicating a small increase in fluorescence of the F4/80 antigen, but the ARA treated cultures show a greater shift (more cells present in the higher fluorescence channels). Data represent the results from five independent experiments with the mean and SEM displayed. (*) indicates statistically significant (p-value < 0.05) difference based on one-way Kruskal-Wallis analysis of variance on ranks followed by Dunnett test. (**) indicates statistically significant (p-value < 0.05) difference based on Student's t-test without multiple comparison adjustment.

Discussion

Our primary objective of this study was to determine if the reduction of later myeloid progenitor subtypes by diets high in n-3 FAs seen previously [12] was the continuation of differences induced at earlier stages of myeloid progenitor development. The data from these experiments indicate that this is not the case. The effect of lowering the frequency of myeloid progenitors of the later stages in mice fed n-3 FA rich diet was

recapitulated in the present study, but we did not see a lowering of the stem/early progenitor cell fractions in the bone marrow. Conversely, we observed an increase in the common myeloid progenitor cell fraction with flow cytometry. Thus, the effects of n-3 FAs on stem/progenitor cell biology in bone marrow are not a simple lowering of the overall frequency of these cell types. Determination of the exact mechanism of n-3 FA effects will entail studies at multiple levels of maturation in bone marrow. Possible mechanisms include effects through metabolites such as leukotrienes, resolvins, incorporation into cell signaling molecules such as hedgehog, or production of prostaglandins. Our data suggest that these mechanisms are eventually triggering a slowed differentiation between the early and middle myeloid progenitor phases resulting in an increase in early progenitors and a decrease in later stage progenitors in high n-3 diets. Understanding the mechanisms involved will enable targeted therapies for hematopoietic disorders involving problematic differentiation.

Our *in vivo* studies indicate that diets rich in n-3 FAs when compared to n-6 FAs have an effect of increasing the frequency of CMPs but no effect on HSCs (Fig 4.2). The changes seen in early stem/progenitor cell biology are then reversed, in that diets rich in n-6 FAs produce an increased number of later stage progenitor cells of the granulocyte and macrophage lineage (Fig 4.1). The results of our stem cell culture model found no lasting effects on stem cell viability or proliferation when cultured in n-3 vs. n-6 FAs (Fig. 4.4A), similar to that seen *in vivo*. It is interesting that we didn't see an increase in HSCs with the corn oil diet containing high n-6 FA levels considering the recent results indicating one of the products of n-6 FAs, prostaglandin E2, can increase HSC production *in vivo* [19-22]. This may be due to the complex metabolism of FAs and the

use of them in biomolecules or to the genetics of the C57BL/6 model. We found a low level expression of F4/80 antigen upon treatment with ARA, characteristic of macrophage progenitor development [23-26], consistent with these progenitors being greater *in vivo* in corn oil fed mice compared to fish oil fed mice (Fig. 4.1). No other significant changes were seen in differentiation state upon treatment with ARA or EPA in comparison to vehicle controls. We observed a lower proliferation rate during differentiation of EML cells while cultured in EPA compared to vehicle controls that wasn't seen with ARA treatment. This finding is consistent with an overall lowering of middle-later stage myeloid progenitor cell numbers in mice fed fish oil diets relative to corn oil diets (Fig.4.1). An important consideration in extrapolating these *in vitro* experiments to our *in vivo* results is that the EML cell model only takes into account cell autonomous factors. The stem cell niche is an important part of stem cell biology and it is not recapitulated in this model.

These data indicate that the effect of n-3 FAs in the diet of mice on reducing later stage myeloid progenitors is not a simple reduction of HSCs in the bone marrow relative to n-6 FA fed mice. n-3 FAs appear to have a diverse effect depending upon the stage of progenitor cell development. Understanding the mechanisms involved will allow a more targeted approach to using n-3 FAs clinically in differentiation therapies. We are currently investigating whether the use of n-3 FAs as a mechanism of induced differentiation is applicable to reduction of the progression of chronic myelogenous leukemia (CML) to the lethal blast crisis phase in a murine model as well as clinical trials with B-cell malignancies ([27] and unpublished results).

Authors' contributions

MEV was responsible for the *in vivo* data acquisition. MEV and VES analyzed data and wrote this manuscript. WEH provided intellectual contributions on study design, and the mice with appropriate diets. VES and MEV provided intellectual contribution on study design as well. VES coordinated the project. YD offered statistical expertise.

Acknowledgments

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Chapter 5: Discussion

Our research supports our hypotheses that genetic components as well as environmental components, specifically dietary fatty acids (FAs), regulate progenitor cell frequency. By understanding how hematopoiesis is influenced, we may better understand why it goes awry in hematological malignancies. In chapter 2, *in silico* mapping suggests that genes involved in the processes of lipid metabolism and bone remodeling play a key role in regulating myeloid progenitor cell frequency. In chapters 3 and 4, we show that the ratio of omega-3 (n-3) to omega-6 (n-6) FAs also plays a key role in this process. Both genetic and environmental components should be considered in attempts to identify mechanisms by which dysregulated hematopoiesis may lead to myeloproliferative diseases, myelodysplastic syndromes, and acute myelogenous leukemia (AML).

Identifying potential biomarkers and/or risk factors for AML prognosis is of value as alterations in the effects of these may give patients increased survival and lessened recurrence rate. Our data collectively suggest that FA content in the diet, metabolism, and bone properties are key regulators of hematopoiesis. Our *in vivo* and *in vitro* studies revealed that FA content in the diet has significant influence on myeloid progenitor cell frequency. Our genetics studies suggest that the way in which FAs are processed by the body also influences myeloid cell frequency. As insulin affects fat stores, it is not surprising that patterns of insulin related genes presented in chromosomal regions of high -logp values in our *in silico* study as well. Insulin stops the use of fat as an energy source and has a direct effect on FA synthesis in the body. Therefore, lack of control of insulin levels can have a profound effect on fat stores in the body [1]. Fat affects bone mineral density and bone volume [2]. The properties of the bone are part of the microenvironment

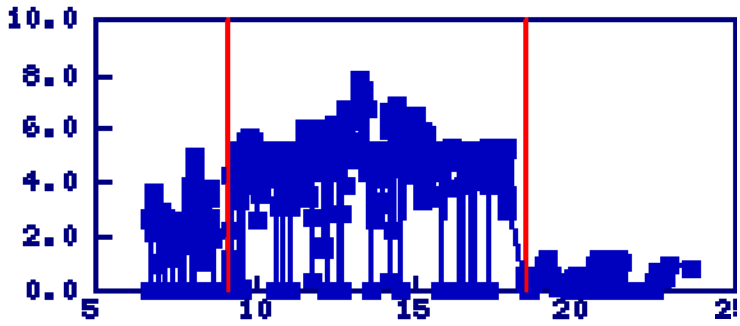
for hematopoiesis. Our research presents genes of interest as potential new biomarkers and risk factors in the areas of metabolism and bone properties for AML. The support for association of genes identified in our analysis is strong; however, causation must first be established in our system.

In silico mapping studies

In silico mapping across 27 strains of inbred mice revealed patterns of genes located in chromosomal regions of the highest -logp values. Use of this method for identification of chromosomal loci containing genes of interest in regulating myeloid progenitor cell frequency was successful, as made evident by the high -logp value associated with the region of chromosome 14 containing interleukin 3 receptor alpha (*IL3-Rα*). This region served as a control for our study since the region presented in a previous study, and it is known that the *IL3-Rα* gene has a 5bp deletion in some mouse strains that results in lowered myeloid progenitor cell frequency [3, 4]. Additionally, loci in mouse chromosome 11 presented in our study are syntenic with the region of human chromosome 5 that undergoes deletion in a myelodysplastic syndrome called 5q- syndrome [5]. Additional genes contained within regions found in our study overlap with more traditional quantitative trait loci (QTL) studies for importance in stem and progenitor populations (Table 5.1) [6,7]. Our studies, however, show greater resolution. Though our results showed several smaller peaks of significance in chromosome 14, theses peaks span a large range, which is similar to the results found in previous traditional QTL studies (Fig. 5.1). A schematic representative of our resulting genes of interest is show in Figure 5.2.

Table 5.1: *In silico* mapping overlaps traditional QTL studies

Previous QTLs	**Peaks of Interest in our *In Silico* Study**
4(37.6-48.5 and 66-78.5cM)	4(42.13-45.76cM and 74.75-75.77cM)
9(23-27cM)	9(23.07-24.07cM)
14 (broad range)	14(Several peaks within)



Chromosome 14 9.17-18.48

p-value 0.001-0.005 LOD 7.84

Figure 5.1: Chromosome 14 contains a wide range of high peaks. The 9.17-18.48Mb region of chromosome 14 has a LOD (logarithm (base 10) of odds) score of 7.84. This image was created using data from *in silico* mapping in Chapter 2 (GEMM phenotype).

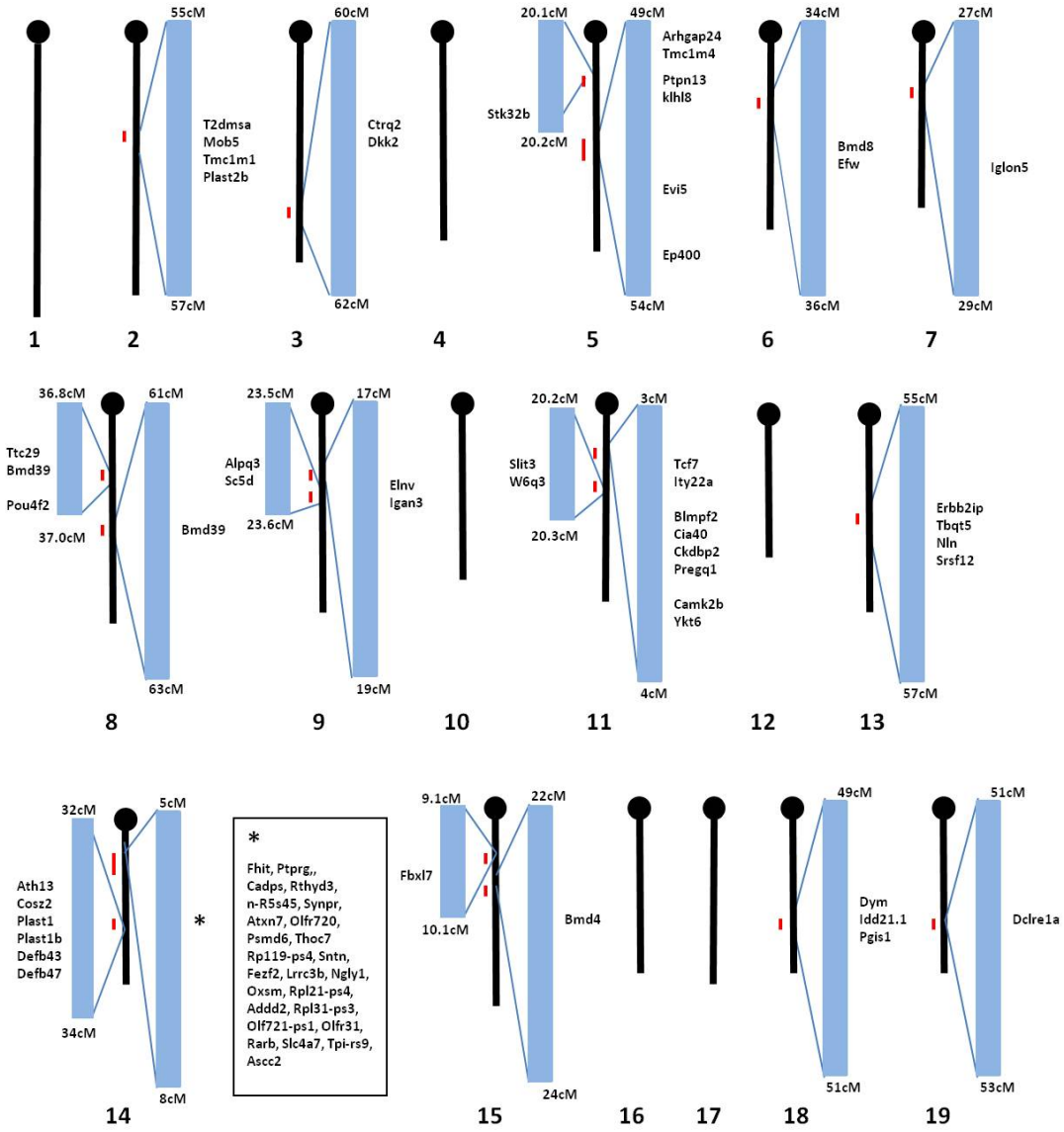


Figure 5.2: Schematic summary of resulting genes of interest from *in silico* mapping. Representative examples of mouse chromosomes 1-19 are shown. Regions of interest along chromosomes are indicated with red lines and blue bars. The GEMM phenotype has associations of interest with regions on chromosomes 5, 6, 7, 8, 14, and 18. The GM phenotype has associations of interest with regions on chromosomes 14 and 15. The E phenotype has associations of interest with regions on chromosomes 5, 9, and 11. The G phenotype has associations of interest with regions on chromosomes 8, 11, and 13. The

M phenotype has associations of interest with regions on chromosomes 2, 3, 11, 14, 15, 18, and 19. The total phenotype has associations of interest with regions on chromosomes 11 and 14.

In our goals of identifying loci associated with myeloid progenitor cell frequency as it relates to hematological malignancies, signaling pathway analysis was performed. Ingenuity Pathway Analysis (IPA) results showed the following pathways to be in the most significant for the top 50 genes found within chromosomal loci of the highest -logp values: gene expression, cancer, hematological disease, tumor morphology, genetic disorder, cell morphology, cell development, and cell cycle. As these are all important factors for leukemia, this further supports our method. In our literature searches, 30% of these top genes of interest were involved in cancer. Fifteen percent were associated specifically with leukemia. Eleven percent of genes were associated with hematopoiesis. Signaling networks from IPA also included many metabolic networks as well as reproductive system disease, and others. In completing literature searches on these genes as well, we found that 20% of genes had known association with dietary fat, FA uptake and oxidation, lipid metabolism, and atherosclerosis susceptibility. Atherosclerosis is a condition in which artery walls thicken as the result of a build-up of fatty materials. Nineteen percent of the top genes had association with insulin or diabetes. Eighteen percent had associations with bone properties. Many calcium and estradiol signaling genes presented in these top genes lists as well. This may be why the reproductive system disease presented as a top signaling pathway in IPA, as β-estradiol is in the center of this network and is known to be vitally important in bone properties [8].

Additional nodes or connection points in pathways of significance in IPA were transforming growth factor beta 1 (*Tgfβ1*) and hepatocyte nuclear factor 4 alpha (*Hnf4α*). *Tgfβ1* is known to be important to the process of hematopoiesis as it is a critical regulator for *in vivo* homeostasis of hematopoietic stem cells (HSCs), especially for their homing potential [9]. Furthermore, it has significant effects on bone. It functions to couple bone resorption and formation. *Tgfβ1* released during bone resorption coordinates bone formation by inducing migration of bone marrow stromal cells to bone resorptive sites [10]. It also works to suppress osteoclast apoptosis [11]. *Hnf4α* is involved in coordinating the expression of genes involved in FA transport and metabolism [12]. Hnf4α regulated genes are also correlated with femur strength [13]. It has also been shown to serve as a molecular switch in transdifferentiation of hematopoietic cells to hepatocytes *in vitro* by decreasing myeloid colony-forming potential and increasing expression of hepatocyte-specific genes and proteins [14].

Given these results and understanding of the relationships between insulin, metabolism, and bone properties, it will be interesting to determine if these processes, and the particular genes that presented in the chromosomal loci with the highest -logp values, in fact regulate hematopoiesis, with particular polymorphisms conferring susceptibility to hematological malignancies. Many insulin and diabetes associated genes in our study have been shown to have a major role in metabolism and bone remodeling [15-22]. As dietary fat content has been shown to regulate bone mineral density and bone volume, and as several QTLs for body size (body weight, length, and adipose mass) correlate with those identified for skeletal traits, there is evidence to suggest that many QTLs may regulate bone properties via their effect on body size [2,23]. Because the

microenvironment, including osteoclasts and osteoblasts, is highly involved in regulating hematopoiesis, it makes sense that the following process may be in effect (Fig. 5.3) [24]. Fat in the diet and how the body metabolizes and/or stores it along with the role of insulin in regulating fat stores and FA synthesis in the body, determine bone mineral density, which is the result of the work of competing osteoblasts and osteoclasts, each of which have effects on hematopoiesis [25-28]. Additionally, osteoclasts regulate insulin signaling in osteoblasts, which control whole body glucose homeostasis [29]. Furthermore, within the hematopoietic niche, marrow fat and bone mineral density are inversely related. Accelerated marrow adipogenesis has been associated with aging and, interestingly, diabetes mellitus and osteoporosis [30]. To address the importance of this component of the niche, Figure 5.4 shows the prevalence of adipocytes in a normal section of long bone.

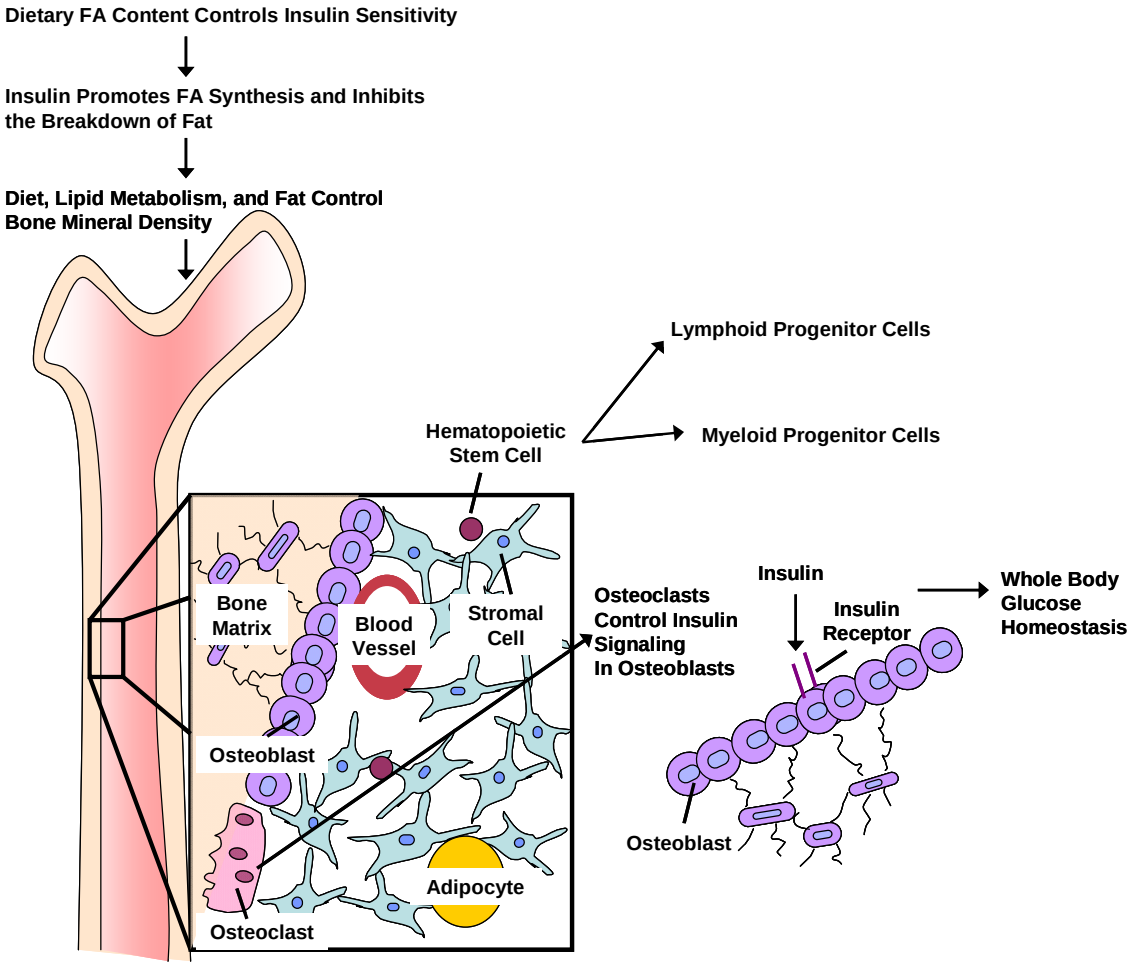


Figure 5.3: Schematic for relationships of dietary fatty acids, insulin, metabolism, bone properties, and hematopoiesis. Omega FA content in the diet is involved in controlling glucose levels and regulating insulin sensitivity. Insulin stops use of fat as an energy source and promotes synthesis of FAs. Fat from the diet and lipid metabolism regulate bone mineral density among other bone properties. Osteoblast and osteoclast work against one another in controlling mineralization of the bone. Osteoclasts regulate insulin signaling in osteoblasts, which regulate whole body glucose homeostasis. The microenvironment regulates hematopoiesis.

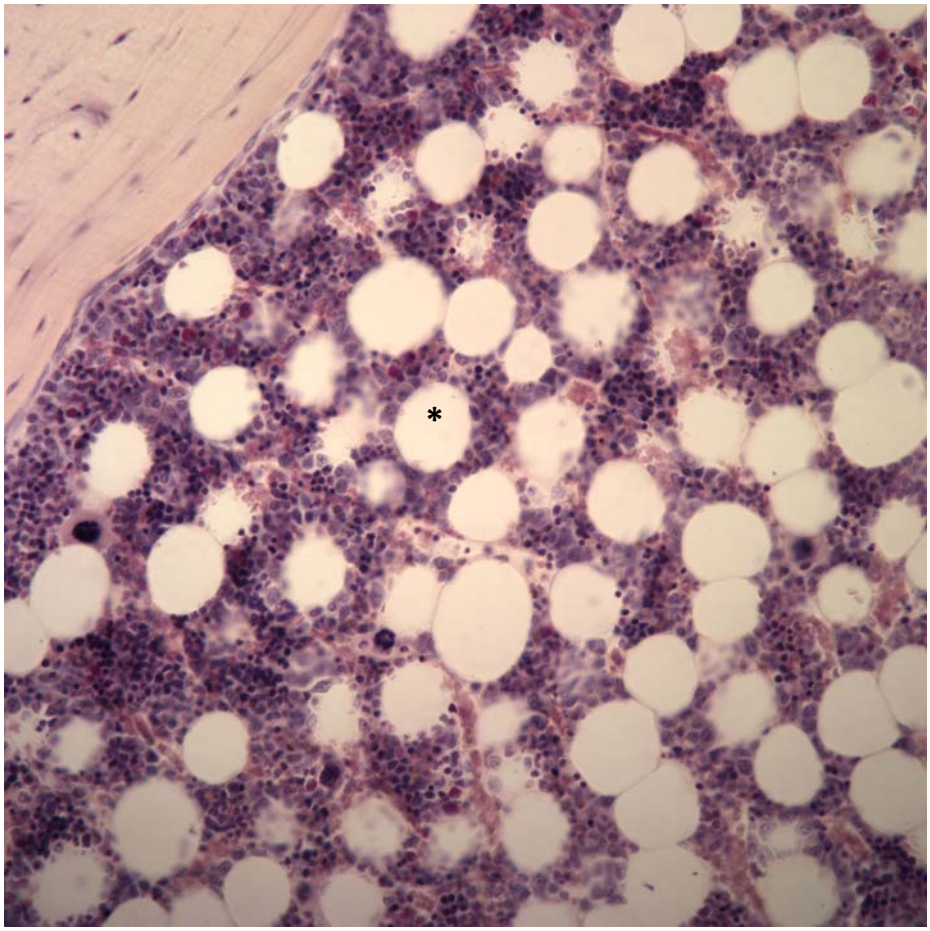


Figure 5.4: Hematopoietic niche histology. This section of long bone illustrates the prevalence of adipocytes (large circular cells, example shown with asterisk) within the hematopoietic niche. This photo was taken using a Nikon Eclipse 80i microscope fitted with the SPOT Pursuit CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI) at 20X magnification.

Dietary fatty acid studies

Corroborating the results of our *in silico* mapping studies, our studies of the effect of the diet on hematopoiesis revealed that dietary FA content significantly affects myeloid progenitor cell frequency. In our study of FVB X sv129 F1hybrid mice, we

found that a diet with higher n-3 FA content reduces total myeloid progenitor cell frequency and promotes differentiation of specific progenitor cell types in the bone marrow without having an adverse effect on levels of circulating white blood cells. Our data indicate that n-3 FA effects are particular to granulocyte and macrophage progenitor cells rather than erythrocyte maturation. Our results indicated a difference at the granulocyte macrophage (GM) progenitor stage and were suggestive, but inconclusive, of a difference at the granulocyte erythrocyte macrophage megakaryocyte (GEMM) stage. Our results presented the following question: is the reduction in middle and late stage progenitors by diets rich in n-3 FAs a continuation of differences induced at earlier stages of myeloid progenitor development?

Our second FA study addressed this question by investigating effects of dietary FAs at the earliest stem and progenitor cell stages of hematopoiesis. We examined the effects of n-3 and n-6 FAs *in vivo* in C57BL/6 mice, with an analysis at the level of stem and progenitor subtypes. Our results were consistent with the previous study in that compared to diets rich in n-6 FAs, diets rich in n-3 FAs induce lower levels of later stage myeloid progenitor cells in mice. We found, however, that reduction in late stage progenitor cells was not a continuation of any reduction in earlier progenitors or stem cells. Using flow cytometry, we found the common myeloid progenitor fraction was instead increased in these mice, suggesting that there are differentiation mechanisms responsible for an increase in early progenitors and a decrease in later stage progenitors in high n-3 diets. There was no difference in HSCs.

We performed *in vitro* studies as well to examine if the *in vivo* effects of n-3 and n-6 FAs could be modeled using the EML cell culture system to more readily ascertain

mechanisms involved. We found no lasting effects on stem cell viability or proliferation when cultured in n-3 vs. n-6 FAs, similar to that seen *in vivo.* We did find a low level expression of F4/80 antigen upon treatment with arachidonic acid (ARA) treatment, characteristic of macrophage progenitor development, and consistent with these progenitors being greater *in vivo* in corn oil fed mice compared to fish oil fed mice. No other significant changes were seen in differentiation state upon treatment with ARA or eicosapentanoic acid (EPA) in comparison to vehicle controls. Consistent with an overall lowering of middle-later stage myeloid progenitor cell numbers in mice fed fish oil diets relative to corn oil diets, in our *in vitro* model, we observed a lower proliferation rate during differentiation of cells cultured in EPA compared to vehicle controls that wasn't seen with ARA treatment. Our conclusion was that the EML cell culture model was able to accurately model some aspects of FA effects on early hematopoietic stem and progenitor biology.

It is important, however, to realize that the EML cell model only takes into account cell autonomous factors. The hematopoietic microenvironment is an important part of stem cell and progenitor cell biology and this is not recapitulated in this model. As noted previously, dietary fat content has significant effects on bone mineral density, and osteoclasts and osteoblasts have been shown to affect hematopoiesis. A model without these factors present can give us only a limited idea of what is taking place at the whole organism level. Further research into the mechanisms controlling this complex regulation of stem and progenitor cell frequency by FAs may provide key information to target therapies to hematopoietic disorders.

In silico mapping and dietary fatty acid studies

We have found that there are chromosomal loci associated with myeloid progenitor frequency and that these loci present interesting patterns when examining the genes within them. We have found that dietary n-3 to n-6 FA ratios play a significant role in controlling progenitor cell frequency. After discovering these answers to the questions originally proposed, new questions are presented. What possible mechanisms exist that combine dietary FA content with patterns of metabolic pathways and bone properties for controlling myeloid frequency?

In attempting to answer this question, I am proposing new hypotheses. As n-3 FAs have been associated with protective functions for osteoblastogenesis and they decrease osteoclastogenesis and osteoclast differentiation, I hypothesize that osteoblasts, protected by dietary n-3 FAs, subsequently upregulate granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and interleukin 6 (IL-6), which are responsible for the differentiation of hematopoietic cells toward the monocyte and granulocyte lineages [26, 31-32]. If this is true, as these cytokines work to differentiate these cells, there would be fewer cells in progenitor stages than those in n-6 FA rich diets. Because these cytokines promote granulocyte and monocyte differentiation, there would be no decrease in erythrocyte progenitors than when compared to n-6 FA rich diets, which would corroborate the results in our dietary FA studies (Fig.5.5). In order to address the result of increased common myeloid progenitors (CMPs) in mice fed n-3 FA rich diets, I additionally hypothesize that the push of HSCs toward the myeloid lineage by the presence of these cytokines creates an initial increase in CMPs when compared to a diet rich in n-6 FAs in which HSCs are

producing an even mix of common lymphocyte progenitors and CMPs. Additionally, the n-6 FA metabolite prostaglandin E2 (PGE2) significantly lowers macrophage colony stimulating factor (M-CSF) secretion by bone marrow stromal cells, further supporting this idea [33].

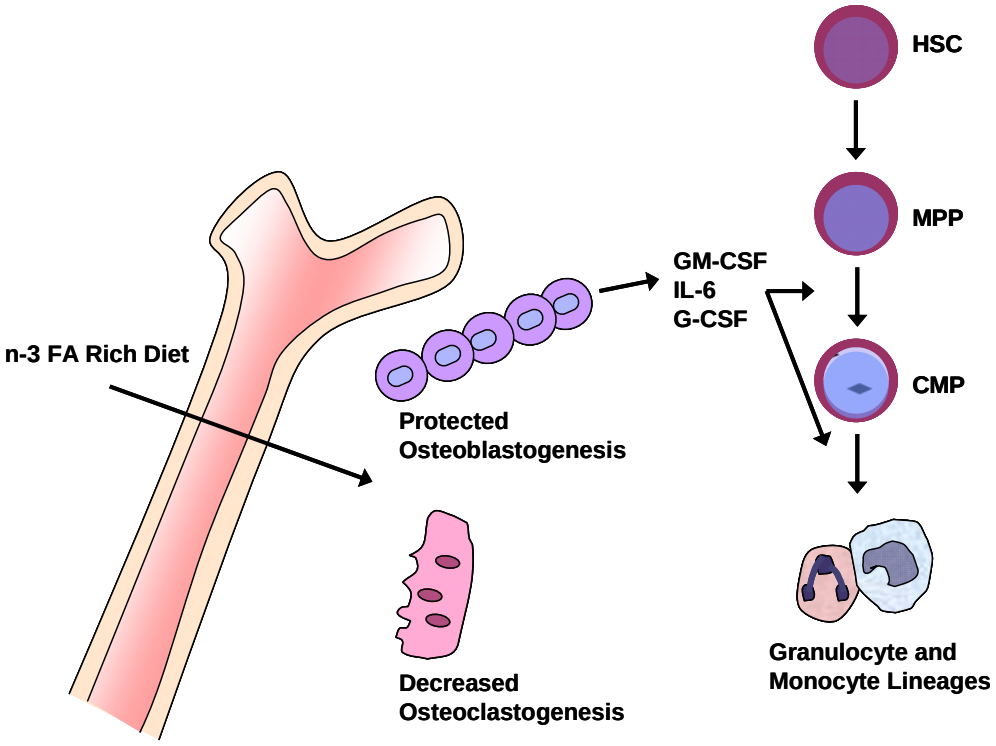


Figure 5.5: Influence of n-3 FA rich diet on myeloid progenitor cell frequency. n-3 FAs protect osteoblastogenesis and prevent osteoclastogenesis. Cytokines released by the osteoblasts stimulate multi-potential progenitors (MPPs) to differentiate to the myeloid lineage and continue to differentiate these cells into granulocytes and monocytes.

This finding also presents the idea that if cytokines that support differentiation of these progenitors are being suppressed in n-6 rich FA diets, these progenitor cells are given more time in this stage to acquire mutations. Current theory for the acquiring of leukemia suggests that failure of progenitors to differentiate causes prolonged

proliferation, thereby increasing the chances that these cells will accumulate events contributing to cancer progression [34]. On a side note, IL-6, is additionally produced in the working muscle during physical activity and has been proposed to act as an energy sensor by enhancing glucose disposal, lipolysis, and fat oxidation [35]. Perhaps this accounts for an additional role of physical activity in regulating hematopoiesis.

Determination of the exact mechanism of n-3 and n-6 FA effects will entail studies at multiple levels of maturation in bone marrow. Mechanisms by which n-3 FAs have been shown to inhibit proliferation, induce apoptosis, and promote differentiation in many cancers include the regulation of signaling pathways and gene expression by peroxisome proliferator receptor activator γ (PPARγ), for which n-3 FAs are natural ligands [36]. Ligand activation of the PPARγ-RXR heterodimer complex enhances insulin sensitivity. Insulin levels affect fat stores, which in turn affect bone composition. PPARγ has also been shown to control bone formation and bone mass through the modulation of bone marrow stromal cell differentiation [37]. Another mechanism of action of n-3 FAs includes inhibition of cyclooxygenase 2 (COX2), which is upregulated in various cancers and is known to have proproliferative and antiapoptotic effects [38-46]. COX2 was shown to significantly increase the rate of osteoclast formation in breast cancer studies [47]. Additional possible mechanisms include effects through metabolites such as leukotrienes, resolvins, incorporation into cell signaling molecules such as hedgehog, or production of prostaglandins.

Overall, our studies show that dietary FAs affect myeloid progenitor cell frequency. They suggest that metabolism and bone properties regulate myeloid progenitor cell frequency as well. As dietary intake and insulin regulate fat stores in the

body, fat regulates bone mineral density, and bone microenvironment influences hematopoiesis, these processes and the genes that control them should be further investigated in regulating myeloid progenitor cell frequency as it is pertinent to hematological diseases. As diet is controllable and bone mineral density is fairly easy and inexpensive to assess, these have great potential to serve as new therapies and risk prognosis indicators in AML.

Future directions for our lab will include determining if genes located in chromosomal regions with high -logp values are in fact associated with changes in myeloid progenitor cell frequency. Our lab will identify these genes through deep sequencing and determine if evidence supports a causal relationship to myeloid progenitor frequency in congenic strains. Our lab will also determine if the particular pathways that emerged the most (bone properties and metabolic processes) are biomarkers for AML progression risk by assaying mice for parameters associated with these pathways and determining correlation with myeloid progenitor cell frequency. Additionally, the validation of the role of these genes and pathways in human hematopoiesis and AML progression will take place. Bone marrow from AML patients and normal individuals will be analyzed to determine if the genes and pathways identified in mice translate to humans. Experiments involving the novel hypotheses presented in regard to the overall relationships of the FA content in the diet, its effects on bone, and therefore cytokines secreted in the hematopoietic microenvironment may also be pursued. Upon subjection to corn or fish oil diet, mice will be assessed for parameters of bone composition, cytokines in the hematopoietic microenvironment, and hematopoietic stem and progenitor cell frequencies, including common lymphoid progenitors. Once

correlation has been established, further investigation into mechanisms of action such as PPARγ activation or COX2 inhibition will pursued.

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