Modeling Gene Therapy of Beta-Thalassemia; An Evaluation of Sub-Lethal Myeloablation and Bone Marrow Transplantation as a Curative Approach

By

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ABSTRACT

Beta-Thalassemia (β-Thalassemia) is a serious disease of the blood that leads to significant morbidity and mortality world-wide. It is caused by a heritable defect in hemoglobin synthesis that leads to accelerated red blood cell destruction, anemia and a plethora of secondary sequelae. While β-Thalassemia can be treated on an out-patient basis, effective control of disease symptoms via blood transfusion is burdensome, both emotionally and financially. Traditionally, the only curative measure has been bone marrow transplantation from a compatible donor. However, recent advances in the field of retroviral gene therapy have made the long-term correction of β-Thalassemia by autologous hematopoietic stem cell (HSC) transplant, a very real possibility. Even so, the risk of death associated with bone marrow transplantation procedures is still unacceptably high in many cases. The ultimate goal of the work described in this thesis was to explore the use of sub-lethal myeloablation in combination with bone marrow transplantation as a curative approach to β-Thalassemia in the $Hbb^{th(d3)}/Hbb^{th(d3)}$ mouse model.

The results described in this thesis confirm the potential of hematopoietic stem cells to engraft in normal mice (B6C3 and C57BL/6J) in both the completely unirradiated and sub-lethally irradiated setting. Engraftment levels in the sub-lethally irradiated setting were linearly related to preparative radiation doses over the range 200 cGy (Rads per minute) to 500 cGy. In addition, engraftment levels were found to demonstrate a curvilinear relationship to the number of cells transplanted over the range 1×10^4 to 1×10^8 cells. Increasing radiation doses from 200 cGy to 300 cGy had the effect of increasing the maximal level at which engraftment levels plateau, decreasing the cell dose at which engraftment efficiencies begin to taper-off and decreasing the threshold after which

engraftment levels increase linearly with cell doses. A mathematically-based model has been proposed to explain this relationship. It has been postulated that observed levels of engraftment are dependent upon the ratio of donor to recipient HSCs within the animal. As an alternative to decreasing the number of recipient HSCs by increasing preparative radiation doses, an attempt was made to increase the number of HSCs in the transplant inocula, by treating donors with 5-Fluorouracil prior to bone marrow harvest. However, this approach provided no additional engraftment advantage over bone marrow from untreated controls. Finally, mouse models of β-Thalassemia were transplanted with 3×10^6 normal cells following sub-lethal radiation doses over the range 100 cGy to 500 cGy. In an attempt to model the conditions of gene therapy where genetic correction of HSCs can be as low as 60%, these cells were competed with 2×10^6 syngeneic thalassemic cells. Mice treated with as little as 300 cGy showed signs of a therapeutic effect with marked improvements in all blood indices assessed. In addition, there was evidence of erythroid-specific amplification of normal red blood cells within thalassemic mice treated with preparative radiation doses of 300 cGy or higher. These results demonstrate that autologous bone marrow transplantation in combination with retroviral gene therapy techniques may be curative for β-Thalassemia in the sub-lethally irradiated setting with as few as 3×10^6 corrected cells and 300 cGy of preparative radiation.

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ABBREVIATIONS

AML	Acute Myelogenous Leukemia
APC	Allophycocyanin
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BM	Bone Marrow
BMT	Bone Marrow Transplant
BMTs	Bone Marrow Transplants
B-cells	B-Lymphocytes
CD	Cluster of Differentiation
cGy	centiGray
CFU-GM	Colony Forming Unit-Granulocyte/Monocyte
CFU-S	Colony Forming Unit-Spleen
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myelogenous Leukemia
CRU	Competitive Repopulating Unit
DLI	Donor Lymphocyte Infusion
DNA	Deoxyribonucleic Acid
FACS	Fluorescence Activated Cell Sorting
F-dUMP	5-fluoro-2'-deoxyuridine-5'-phosphate
Fig.	Figure
FITC	Fluoroscein isothiocyanate
F1	First filial generation
GVHD	Graft-Versus-Host Disease
Hb	Hemoglobin
HbA	Hemoglobin A
HbA_2	Hemoglobin A2
HbF	Hemoglobin F
HbG_1	Hemoglobin Gower1
HbG_2	Hemoglobin Gower2
HbP	Hemoglobin Portland
HCt	Hematocrit
HIV-1	Human Immuno-deficiency Virus-Type 1
HLA	Human Leukocyte Antigen
HPP-CFC	High Proliferative Potential-Colony Forming Cell
HSC	Hematopoietic Stem Cell
HSCs	Hematopoietic Stem Cells
HSCT	Hematopoietic Stem Cell Transplant
IL	Interleukin
kb	kilobases
LCR	Locus Control Region
LTC-IC	Long Term Culture-Initiating Cell
Ly-5	CD45
mAb	monoclonal Antibody
mAbs	monoclonal Antibodies
MDD	Major Double Deletion
MM	Multiple Myeloma
MHC	Major Histocompatibility Complex
NHL	Non-Hodgkins Lymphoma
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
Rads/min	Rads per minute
RBC	Red Blood Cell
RBCs	Red Blood Cells
rpm	revolutions per minute
RU	Repopulating Unit
SA-APC	Streptavidin-APC
SCD	Sickle Cell Disease
SCF	Stem Cell Factor
TBI	Total Body Irradiation
T-cells	T-Lymphocytes
VCAM-1	Vascular Cell Adhesion Molecule-1
VSV-G	Vesicular Stomatitis Virus-G protein
WBCs	White Blood Cells
α-globin	alpha-globin
β-globin	beta-globin

β^M	beta-major
β^m	beta-minor
δ-globin	delta-globin
Δ–MGMT	mutant O6-methylguanine DNA-methyl transferase
ε-globin	epsilon-globin
γ^A-globin	gamma A-globin
γ^G-globin	gamma G-globin
ζ-globin	zeta-globin
^oC	degrees Celsius
2% PBS	PBS with 2% Fetal Calf Serum by volume
2X	2 times recommended final concentration
3X	3 times recommended final concentration
3'	3-primed
5'	5-primed
5-FU	5-Fluorouracil

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CHAPTER 1 INTRODUCTION

1.1 Overview

Like all warm-blooded mammals, humans rely on a closed circulatory system to transport gases, nutrients and waste to and from the various tissues of the body. The circulatory system itself is a complex network of arteries, capillaries and veins through which blood is pumped via the heart. Within the blood are suspended the billions of so-called blood cells that make life possible. Among these are the red blood cells (RBCs) or erythrocytes that carry out the key role of transporting oxygen and carbon dioxide to and from the tissues of the body. The ability to carry gases is conferred upon RBCs primarily by virtue of the hundreds of millions of hemoglobin molecules packed into each and every cell. Hemoglobin is a globular protein that is made up of two different families of polypeptides collectively referred to as the globins. In adult humans, each hemoglobin molecule is a tetramer made up of two alpha-globin (α-globin) and two beta-globin (β-globin) polypeptides or chains, each of which is associated with a complex, iron-containing co-factor called heme. Ultimately, it is the heme component of each globin subunit that is responsible for the oxygen-carrying capacity of hemoglobin. As such, each hemoglobin protein is capable of binding four molecules of oxygen and each RBC is capable of transporting billions of molecules of oxygen with each pass of the lungs. Despite the overwhelming oxygen-carrying capacity of each RBC, trillions (1×10^{12})of these cells are required to support aerobic respiration throughout the body. Due to the relatively short lifespan of RBCs within the circulation (~120 days), these cells must be produced in large numbers throughout life. Ultimately, this burden is carried by a relatively small number of

stem cells that reside primarily in the bone marrow (BM). These stem cells belong to a unique population of cells called hematopoietic stem cells (HSCs), which are capable of differentiating into any of the cells commonly found in the circulation, as well as others that extravasate into the tissues shortly after their release from the BM. In combination with their ability to undergo self-renewal divisions, the multi-lineage capacity of HSCs makes them a virtually limitless source of all blood cells.

Genetic disorders associated with impaired production or altered forms of the globin proteins constitute major causes of morbidity and mortality world-wide. Diseases such as Sickle Cell Disease (SCD), alpha-Thalassemia (α-Thalassemia) and beta-Thalassemia (β-Thalassemia), that are collectively referred to as hemoglobinopathies, are the most common. Despite their wide-spread prevalence, all three of the aforementioned hemoglobinopathies are monogenic disorders resulting from mutations in one or more of the globin genes.

In combination with the self-renewing and clonal nature of RBC production, their simple genetic cause makes the hemoglobinopathies, especially the thalassemias, attractive targets for gene therapy trials. In particular, retroviral gene therapy is especially well-suited to the task since these viruses demonstrate a marked natural affinity for blood cells and are capable of stably integrating exogenous deoxyribonucleic acid (DNA) into the genome (transduction). Together with the high accessibility of HSCs, the ability of retroviruses to integrate engineered genes into a cell's DNA, makes the long-term correction of hemoglobinopathies an exciting and very real possibility. However, there are several barriers that need to be overcome before retrovirally-based gene therapies can become a clinical reality. In particular, the toxicity of the treatment itself, which requires complete

destruction of the BM (myeloablation) and rescue from certain demise by the transplant of genetically engineered cells, is a major deterrent. The research described in this thesis was aimed at addressing the issue of transplant-related toxicity by exploring the use of sub-lethal condition regimens in combination with bone marrow transplants (BMTs), as a curative approach to treating β-Thalassemia. To this end, the efficacy of BMTs in the absence of recipient conditioning was explored. The subsequent success of this approach, spurred a more rigorous analysis of the relationship between preparative conditioning regimens of both BM donors and recipients, transplant cell doses and observed levels of engraftment within transplanted mice. Finally, in an attempt to model the use of sub-lethal conditioning regimens in the context of gene therapy protocols, mouse models of β-Thalassemia were given sub-lethal doses of radiation followed by BMTs. The efficacy of this approach was then assessed on the basis of clinical indicators of β-Thalassemia. The following introduction is divided into six main parts that serve to give the reader a more complete understanding of the clinical impetus behind the research, the basic hematological principles and tools that are employed in the research, the previous findings published in the scientific literature that have guided the research, and the designs and objectives of the research detailed in this thesis.

1.2 Beta-Thalassemia

1.2.1 A Historical Perspective of Thalassemia

Beta-Thalassemia was first described by Drs. Thomas B. Cooley and Pearl Lee in 1925. Originally this condition, which has since become known as Cooley's Anemia, was

described in five children who presented with a unique illness characterized by splenomegaly (enlarged spleen), hepatomegaly (enlarged liver), decreased osmotic fragility, moderate leukocytosis (increased numbers of leukocytes) and reticulocytosis (increased number of immature erythrocytes), skin and sclera discoloration and enlargement of the cranial and facial bones (Lin-Fu, 1981). All of the children were of Italian or Greek descent. Seven years later in 1932, Whipple and Bradford published the results of the first complete autopsy of an individual diagnosed with Cooley's Anemia (Whipple & Bradford, 1932). Shortly thereafter, they coined the term "thalassemia", a term that was derived from the Greek word for "sea" and that was indicative of the strong geographical predisposition of this disease (Whipple & Bradford, 1936). While the scientific literature was quickly flooded with reports of conditions resembling Cooley's Anemia, it was not until 1935 that its familial nature was recognized by Micheli et al. (Micheli et al., 1935). By 1944, the field had come to recognize the presence of two broad types of thalassemia. Those that were more severe in their pathology fell into the "Thalassemia-major" category, and those that were less severe fell into the "Thalassemia-minor" category (Valentine & Neel, 1944). In the following years, the terms "Thalassemia Intermedia" and "Thalassemia Minima" were coined, the former to describe those cases whose severity fell between Thalassemia-major and Thalassemia-minor, and the latter to describe those cases that were almost imperceptible. By the end of the 1940's, it was well-accepted that the clinical descriptors "Thalassemia-major" and "Thalassemia–minor" generally represented the homozygous and heterozygous forms of the Cooley's Anemia trait respectively. However, it was not until the discovery of Sickle Cell-Thalassemia double heterozygotes that Dr. Linus Pauling realized that it was a decrease in RBC adult hemoglobin A (HbA) levels that were responsible for

Thalassemia (Pauling, 1954). Finally, in 1957 Rhinesmith et al. described the two polypeptides making up human hemoglobin (Rhinesmith et al., 1957) and 2 years later the distinction between α- and β-Thalassemias was made (Ingram & Stretton, 1959).

1.2.2 Clinical Presentation of beta-Thalassemia

Since the first description of Cooley's Anemia in 1925, the clinical picture of β-Thalassemia has become considerably more detailed. It is now widely accepted that several different forms of β-Thalassemia exist. In general, the different forms are categorized into one of the four groups previously mentioned. These are 1) Thalassemia-major (Cooley's Anemia), 2) Thalassemia Intermedia, 3) Thalassemia-minor and 4) Thalassemia Minima. Within each of these categories, there is a continuum of severities, each with its own underlying causes and modifying factors. Clinically, patients presenting with some form of β-Thalassemia are usually lumped into one of two more simplistic categories. Those with reduced HbA are designated as having β^+-Thalassemia (pronounced "beta plus-Thalassemia) and those with no HbA are designated β^0-Thalassemia (pronounced "beta not-Thalassemia). β^0-Thalassemia is the most severe form of β-Thalassemia and individuals who have this condition typically present with marked anemia from infancy and hemoglobin levels ranging from 4 to 6 grams per deciliter (g/dL) of blood (Lin-Fu, 1981). Infants with β^0-Thalassemia are usually normal at birth, with symptoms appearing between 3 months and 1 year later. The first recognizable symptom is usually an increasingly pale complexion (pallor) with a slightly yellow tint (jaundice). The appearance of these features is often followed by a marked distention of the abdomen due to splenomegaly, poor feeding

habits and failure to thrive. Constipation, fever, diarrhea and fatigue are also common. As individuals with this condition get older, complaints of abdominal pain, shortness of breath upon exertion, and overall weakness are typical. Thickening of the facial bones (facies), and skull (bossing), squaring of the ribs, long bones and short bones of the hands and feet, growth retardation and failure to develop secondary sex characteristics are usually evident by early adolescence and irreversible damage to the liver, spleen, kidneys, heart and most other organs ensues by late adolescence. Death usually results between the ages of 15 and 20. For β^+-Thalassemia, the clinical picture can be similar in all respects or much less severe with any combination of the pathological findings present in β^0-Thalassemia, depending on the underlying causes, genotype of the individual and other modifying factors such as the level of compensatory Hemoglobin A_2 and Hemoglobin F production (see below for a description of the different types of hemoglobin).

1.2.3 Biochemical Etiology of beta-Thalassemia

Although there is a wide range of clinical severities associated with β-Thalassemia and an even wider array of genetic lesions responsible for β-Thalassemia, all forms of the disease ultimately result from a lack of functional hemoglobin and the presence of excess α-globin chains within the RBCs. In particular, the presence of excess α-globin triggers a cascade of events that can be directly correlated to the disease pathology associated with β-Thalassemia. Free α-globin chains in RBCs initiate a series of oxidation/reduction reactions that result in the catabolism of methemoglobin to hemichromes that precipitate on the RBC membrane and damage the cytoskeleton. In addition, free iron released from the break down of hemichromes forms aggregates of ferritin and/or hemosiderin and catalyzes

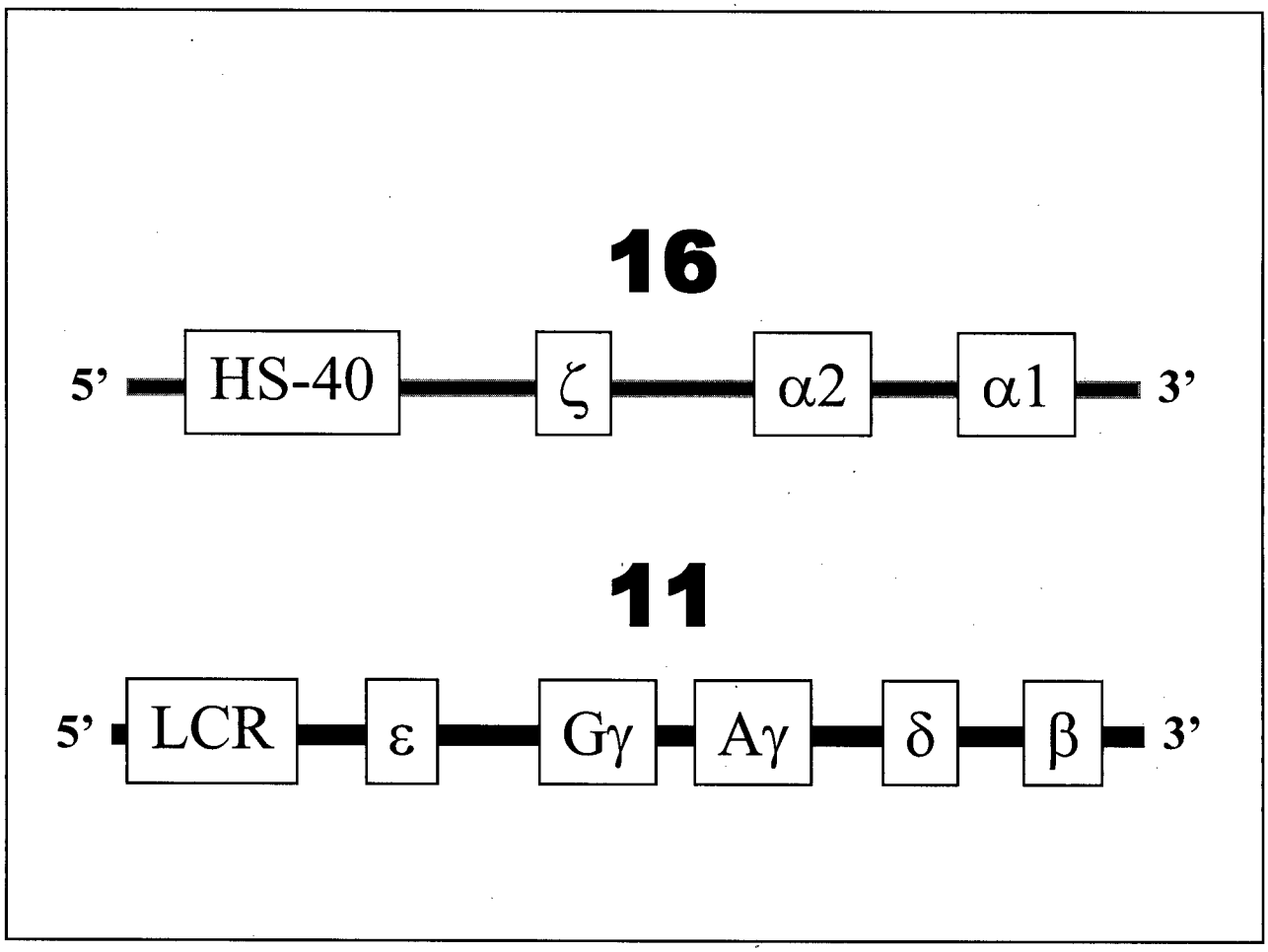
membrane protein and lipid peroxidation. Taken together, damage to the cytoskeleton and cell membrane increases fragility and decreases deformability, leading to accelerated hemolysis, apoptosis and immune clearance of the RBCs (Rund & Rachmilewitz, 2001). In turn, RBC destruction results in severe anemia and the release of free iron into the circulation. As iron saturates the serum transferrin pool, non-transferrin bound iron accumulates and is deposited in the heart, liver and endocrine system (Wonke, 2001). Besides the toxicity of the iron deposits themselves, there is evidence to suggest that organs are also damaged by thromboembolic events that are triggered by the presence of anionic phospholipids on the surface of thalassemic RBCs (Ruf et al., 1997). The combined effect of these two factors eventually results in organ failure. In 70% of β-Thalassemia related deaths, cardiac failure is causative (Wonke, 2001).

1.2.4 Molecular Etiology of beta-Thalassemia

Beta-thalassemias are genetic disorders that usually demonstrate autosomal recessive patterns of inheritance (Rund & Rachmilewitz, 2001) and are caused by reduced or aberrant expression of the β-globin genes that are needed to produce hemoglobin. Hemoglobin is a tetramer consisting of two alpha-like and two beta-like globin chains that are essential to its function. The alpha-like globins are encoded for on Chromosome 16 and the beta-like globins are encoded for on Chromosome 11 (Fig. 1). In mammals, the globin genes are arranged into two clusters, each of which is preceded by a Locus Control Region (LCR) that controls the temporal and tissue specificity of expression. During development the expression of alpha-like and beta-like globin genes is tightly regulated and different globins

Figure 1- Chromosomal Arrangement of the Globin Genes in Humans.

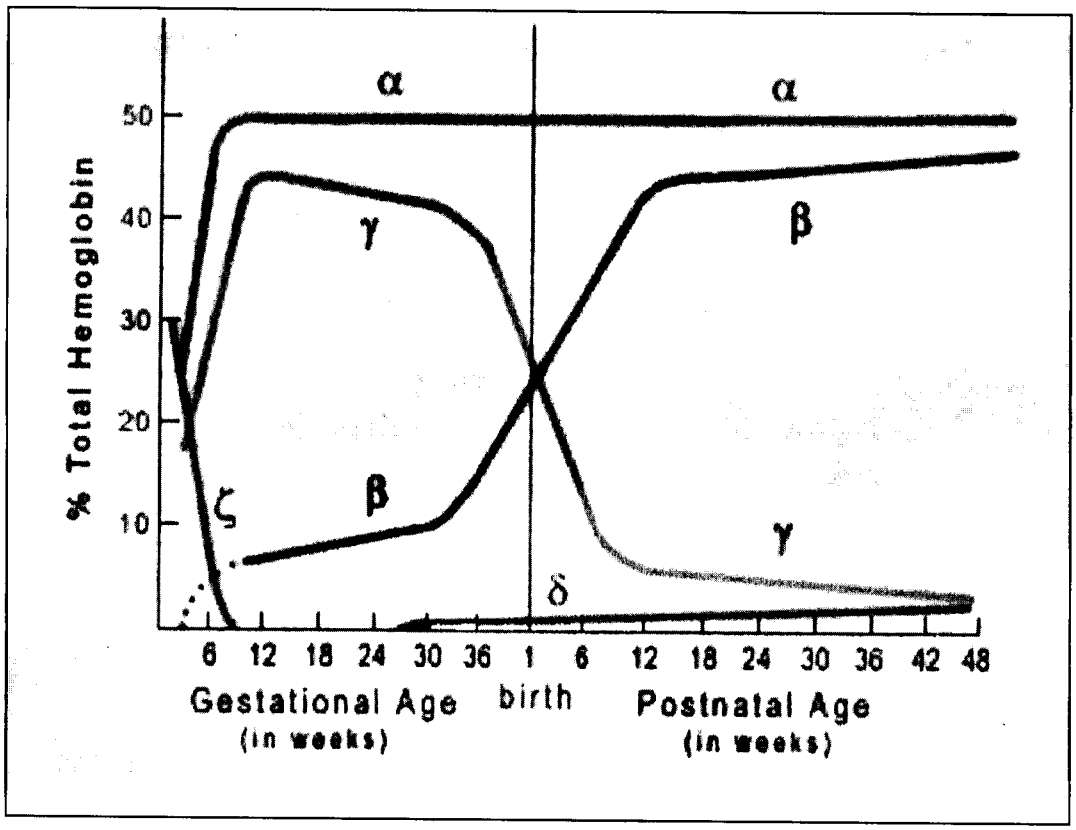
The α- and β-globin chains are arranged in order of their expression during development with the earliest globins situated at the most 5' end of each locus. Each locus includes a 5' Locus Control Region (LCR) that controls expression throughout development. There are three alpha-like globins on Chromosome 16 and five beta-like globins on Chromosome 11. The alpha-like globins are theta-globin (ζ), which is expressed early in embryonic life and two adult alpha-globins (α2 and α1) that are equally expressed thereafter. The beta-like globins are epsilon-globin (ε), which is expressed early in embryonic life, the two gamma-globins (Gγ and Aγ), which are expressed at high levels during the fetal stage and at low levels throughout life, and the two adult globins, delta-globin (δ) and beta-globin (β) that are expressed at low levels during the fetal stage and at higher, non-equal levels thereafter.


16
5'
HS-40
ζ
α2
α1
3'
11
5'
LCR
ε
Gγ
Aγ
δ
β
3'

are produced at different times depending on physiological needs (Fig. 2). The first beta-like globin to be expressed is epsilon-globin (ε-globin), which is needed along with the alpha-like theta-globin (ζ-globin) and α-globin for the production of Hemoglobin G_1 (HbG_1; $\zeta_2\varepsilon_2$) and Hemoglobin G_2 (HbG_2; $\alpha_2\varepsilon_2$), respectively. However this is expressed for only a very short time during the embryonic stage and is quickly replaced by the gamma-globins (γ^G-globin and γ^A-globin) during the fetal period. The function of the γ-globins is essential in the developing fetus, as they are needed to produce Hemoglobin F (HbF; $\alpha_2\gamma_2$) and Hemoglobin P (HbP; $\zeta_2\gamma_2$), two fetal varieties of hemoglobin with high oxygen affinity that are necessary to facilitate unloading of maternal oxygen stores at the placenta. Expression of the γ-globins continues until birth and declines rapidly thereafter as it is replaced almost entirely with delta-globin (δ-globin) and β-globin. With the exception of the embryonic stage that is characterized by ζ-globin expression, the alpha-like globin that is produced throughout life is α-globin. In adult humans, the vast majority (>95%) of hemoglobin contains two α- and two β-globin chains (HbA; $\alpha_2\beta_2$) and only a small proportion (~5%) contains either δ-globin (HbA_2; $\alpha_2\delta_2$) or γ-globin (HbF; $\alpha_2\gamma_2$). Therefore, it essential that β-globin and α-globin be expressed at approximately equal levels so that the two will tetramerize in a 1 to 1 ratio to form functional adult hemoglobin. Thalassemia per se, refers to the situation where this is not the case and either the α or β chains are in excess, due to unbalanced expression of one or the other. Therefore, β-Thalassemia is the condition in which an excess of α-globin exists. While an excess of α-globin is usually the result of decreased β-globin expression, cases of increased α-globin expression have also been described (Weatherall & Clegg, 1972). However, almost 200 distinct mutations that result

Figure 2- Developmental Switching of Human Globin Genes.

The expression of different human globin genes is tightly regulated during development (www.thalassemia.com).


α
α
50
40
30
20
10
% Total Hemoglobin
γ
β
ζ
β
γ
δ
6 12 18 24 30 36 1 6 12 18 24 30 36 42 48
Gestational Age
(in weeks)
birth
Postnatal Age
(in weeks)

in the deficient production of β-globin have now been reported (Huisman et al., 1998). These mutations can result in decreased transcription, compromised transcript stability, or decreased transcript translation. Among mutations responsible for β-Thalassemia, the vast majority are point mutations, with deletions being considerably less common (Rund & Rachmilewitz, 2001). In some rare cases, point mutations in the third exon of the β-globin gene have been reported as leading to an autosomal dominant form of β-Thalassemia.

1.2.5 Mouse Models of beta-Thalassemia

Genes for the murine α- and β-globin polypeptides are encoded for in complexes that are similar in structure to that seen in humans, with the notable absence of functional fetal β-like globins. There are however two embryonic β-globin like genes called bh1 and εy^2 that are expressed sequentially early in gestation and that have 90 % and 88% sequence similarity to the human γ-globin genes respectively (Ciavatta et al., 1995). The two β-globin genes that are normally expressed in adult mice are referred to as beta-1 (β1) and beta-2 (β2) and are encoded for close to the 3' end of the β-globin-like cluster on Chromosome 7. In nature, these genes are commonly arranged in haplotypes that encode for either two identical or two polymorphic forms of β-globin polypeptides. When two polymorphic forms of the β-gene are present, the more 5' gene is referred to as beta-major (β^M) globin and the more 3' as beta-minor (β^m) globin. In adult mice, the β^M-globin gene is responsible for the production of 80% of the β-globin chains while the β^m-globin gene is responsible for remaining 20% (Shehee et al., 1993).

Several mouse models of β-Thalassemia exist and have been described in the literature. Earliest among these was the Hbb^{th-1} model discovered by the group of Lewis in 1983 (Skow et al., 1983). The mutation responsible for the thalassemic phenotype of these mice was naturally occurring and was originally discovered in (C57Bl/6J x DBA/2J)F1 animals. The mutations itself was a 3.7 kilobase (kb) deletion that encompassed the entire β^M-globin locus including 5' and 3' flanking sequences. As such, mice homozygous for this mutation produced only β^m-globin and exhibited hypochromic (reduced hemoglobin in RBCs), microcytic (reduced size of RBCs) anemia with severe anisocytosis (irregular size), poikilocytosis (irregular shape), reticulocytosis and inclusion bodies in a high proportion of circulating RBCs. Analysis of globin chain synthesis indicated β-globin synthesis was about 75% of normal in these mice (Skow et al., 1983). The Hbb^{th-1} mutation was subsequently isolated on the C57Bl/6J background and was renamed $Hbb^{d3(th)}$. The $Hbb^{d3(th)}$/ $Hbb^{d3(th)}$ mice are hereafter referred to as Major Double Deletion (MDD) mice. The second mouse model of β-thalassemia to be described was created by the group of Smithies using insertional disruption of the β^M-globin gene in embryonic stem cells derived from inbred 129 mice (Shehee et al., 1993). Of the seven chimeras born to surrogate females, only three males transmitted the ES-derived genome to their offspring after being mated to C57Bl/6J females. The F1 offspring from this mating were heterozygous for the disrupted β^M gene and were phenotypically indistinguishable from normal controls. However, homozygotes obtained from mating heterozygotes and designated Hbb^{th-2}/Hbb^{th-2}, did not survive longer than 4 hours post-*partum*, indicating the lethality of this genotype. A third mouse model of β-Thalassemia was created by gene targeting in 1995, again by the group of Smithies in a manner that was similar to that used to create the Hbb^{th-2} models (Yang et al., 1995). In

mice with this mutation, which was designated Hbb^{th-3}, a complete deletion of both adult β-globin genes was accomplished, but again the mutation was lethal in the homozygous state, with all Hbb^{th-3}/Hbb^{th-3} mice dying before 12 hours post-*partum*. However unlike the Hbb^{th-2} models, mice heterozygous for the Hbb^{th-3} mutation demonstrated marked disease pathology that closely resembled that seen in human patients heterozygous for β-Thalassemia Major. When blood indices were analyzed, Hbb^{th-3} heterozygotes demonstrated phenotypes that were similar to $Hbb^{d3(th)}$ homozygotes (Table 1).

1.2.6 Curing beta-Thalassemia with Bone Marrow Transplants

While regular blood transfusions and effective iron chelation with Desferrioxamine can relieve many of the symptoms of β-Thalassemia, 50% of all patients are noncompliant (Wonke, 2001) and most will not survive into the fifth decade of life (Giardina & Grady, 2001). The only curative treatment available is BMT. The source of marrow used in BMTs for β-Thalassemia can come from one of two sources including a non-identical family member, or unrelated donors. BMTs that use BM from either of these sources are referred to as allogeneic transplants. As with any allogeneic transplant, one of the major barriers to curative BMT for β-Thalassemia has been a special type of graft rejection that presents clinically as Graft-Versus-Host-Disease (GVHD). While the elucidation of the immunological mechanisms underlying GVHD and Human Leukocyte Antigen (HLA)-haplotyping has greatly improved the success rate of BMTs, immune rejection still remains a major problem, as most patients do not have readily available HLA-matched (also known as Major Histocompatibility (MHC)-matched) donors. Even the use of BM from non-

Table 1- Phenotypic Comparison of Existent Mouse Models of Human β-Thalassemia with Human β^0-Thalassemia and β^0-Thalassemia Trait.

	Hct [%]	RBC [x 10^6/mm^3]	Nucleated [Cells/mm^3]	Hb [g/dL]	MCH [pg/cell]	MCV [fL]	Retics [%]
Normal Human (male)	47.0 +/- 5.0[a]	5.4 +/- 0.8	7000-10,000	16.0 +/- 2.0	29 +/- 2	87 +/- 5	1-2
Human β^0-Thalassemia Trait (heterzygote)	data unavailable	5.2 +/- 0.7 (3.9-6.7)[b]	data unavailable	11.6 +/- 1.5 (7.8-15.7)	22 +/- 2 (18-25)	74 +/- 5 (66-82)	data unavailable
Human β^0–Thalassemia (homozygote)	data unavailable	data unavailable	normal or slightly elevated	3-7	12-18	50-60	data unavailable
Normal Mice (Male)	47.4 +/- 0.7	11.8 +/- 0.4	5370 +/- 541	14.7 +/- 0.2	12.5 +/-0.4	40.5 +/- 1.1	4.6 +/- 0.5
Hbb^{th-3}/ + Mice	30.5 +/- 0.8	6.6 +/- 0.1	Not Done	10.9 +/- 0.3	17.6 +/- 0.4	43.0 +/- 0.5	30.3 +/- 0.9
$Hbb^{d3(th)}$/$Hbb^{d3(th)}$ Mice	37.4 +/- 0.5 (35-41)	10.2 +/-0.3 (8.5–12.3)	25870 +/- 5798 (9300–108200)	10.8 +/- 0.3 (8.9-12.6)	10.6 +/- 0.3 (7.6-12.6)	36.0 +/- 0.9 (29.3-42.9)	41.4 +/- 2.1 (26.6-62.2)

a- Standard error of the mean
b- range

identical family members (i.e.- siblings or parents) can cause problems, since patients are 50% HLA-matched with parents and only 25% of sibling pairs are HLA-identical. BMTs from relatives further removed than this or unrelated donors can cause problems even if they are completely matched for HLA antigens and ABO blood type, as several other minor histocompatibility genes are not usually even considered. As a consequence of the difficulties that can be encountered with BMTs, even when the donors are closely related individuals, total lymphomyeloablation and aggressive immuno-suppression are usually employed in order to ensure high levels of donor cell engraftment and to avoid GVHD. Since the success of this procedure is dependent upon the rescue from BM failure by transplanted HSCs, there is a significant chance of transplant-related mortality, which falls in the range of 5% to 42% depending on the patients age, pre-transplant disease progression and pathology, and the transplant protocol used (Lucarelli et al., 1993). As a result, the decision to subject a child to this treatment as a curative measure for β-Thalassemia becomes a difficult one to make, especially in light of the high probability of survival into mid-adulthood with regular blood transfusions and effective iron-chelation therapy. However, transfusion and chelation therapy is not an easily chosen alternative either. Besides the prohibitive cost, which can be in excess of $110,000 US per year (Wonke, 2001), this treatment is both physically and emotionally draining as transfusions occur bi-weekly and effective chelation requires painful subcutaneous administration for 9 to12 hours daily. Obviously, there is a dire need for alternative treatments.

1.2.7 Curing beta-Thalassemia Using Gene Therapy Techniques

As previously discussed, one of the major reasons behind preparative myeloablation prior to BMT is the assurance of high level donor cell engraftment and the avoidance of GVHD. In some cases where a patient is fortunate, the problem of GVHD can be significantly reduced by using transplant materials that are derived from an HLA-identical donor. Another solution to the problem of GVHD is to use syngeneic or even autologous material for transplantation. Such approaches have found utility in the treatment of malignant diseases such as leukemia and increasingly cord-blood stem cells of newborns are being preserved by parents in anticipation of such developments. However, in the case of inherited diseases, neither syngeneic nor autologous transplants have traditionally been of benefit. Over the past 2 decades however, gene therapy techniques have made autologous transplants a very real possibility for the curative treatment of inherited diseases. Inherited diseases of the blood are particularly amenable to this approach due to the clonal nature of hematopoiesis and the relative accessibility of HSCs. Traditionally, such treatments have been envisioned in the context of oncoretroviral vectors. Indeed, oncoretroviral vectors have been used successfully in the past to treat adenosine-deaminase deficiency and associated Severe Combined Immuno-deficiency Disease. However in the context of hemoglobinopathies where the selective advantage of genetically corrected cells is not so pronounced, oncoretrovirally-based gene therapy has been less successful. Despite the monogenic nature of β-Thalassemia and its potential cure through simple gene augmentation, successful transduction of sufficient numbers of HSCs to bring about curative results has been difficult and maintenance of long-term expression of ectopic β-globin constructs has been plagued by transgene rearrangements and silencing. However, the field

has progressed nonetheless and in recent years this progress has been greatly accelerated by the use of Human Immuno-deficiency Virus-Type 1 (HIV-1) based lentiviral vectors. Several aspects of these vectors including their ability to carry in excess of 8 kb of foreign DNA, their amenability to Vesicular Stomatitis Virus G-protein (VSV-G) pseudotyping and subsequent high titer concentration (>10^9 infectious units per milliliter) and their ability to faithfully transmit large β-globin constructs to quiescent HSCs, has made them the vehicle of choice in recent years. Indeed, lentiviral vectors carrying the human β-globin gene under the control of extensive LCR, enhancer and promoter elements, have recently been used to cure mouse models of SCD, β-Thalassemia and β-Thalassemia Intermedia (Pawliuk et al., 2001; May et al., 2000; May et al., 2002). Despite these recent successes however, much work remains to be done in regards to the safety and efficacy of such procedures in humans. To date, the cures of murine hemoglobinopathies have required lethal doses of preparative radiation to achieve success. Such a potentially lethal conditioning regimen in the context of a currently treatable disease such as β-Thalassemia is unacceptable. As such, sub-lethal BMT protocols are desperately needed before lentivirally-based gene therapy protocols can be translated into the clinical setting.

1.3 Hematopoiesis

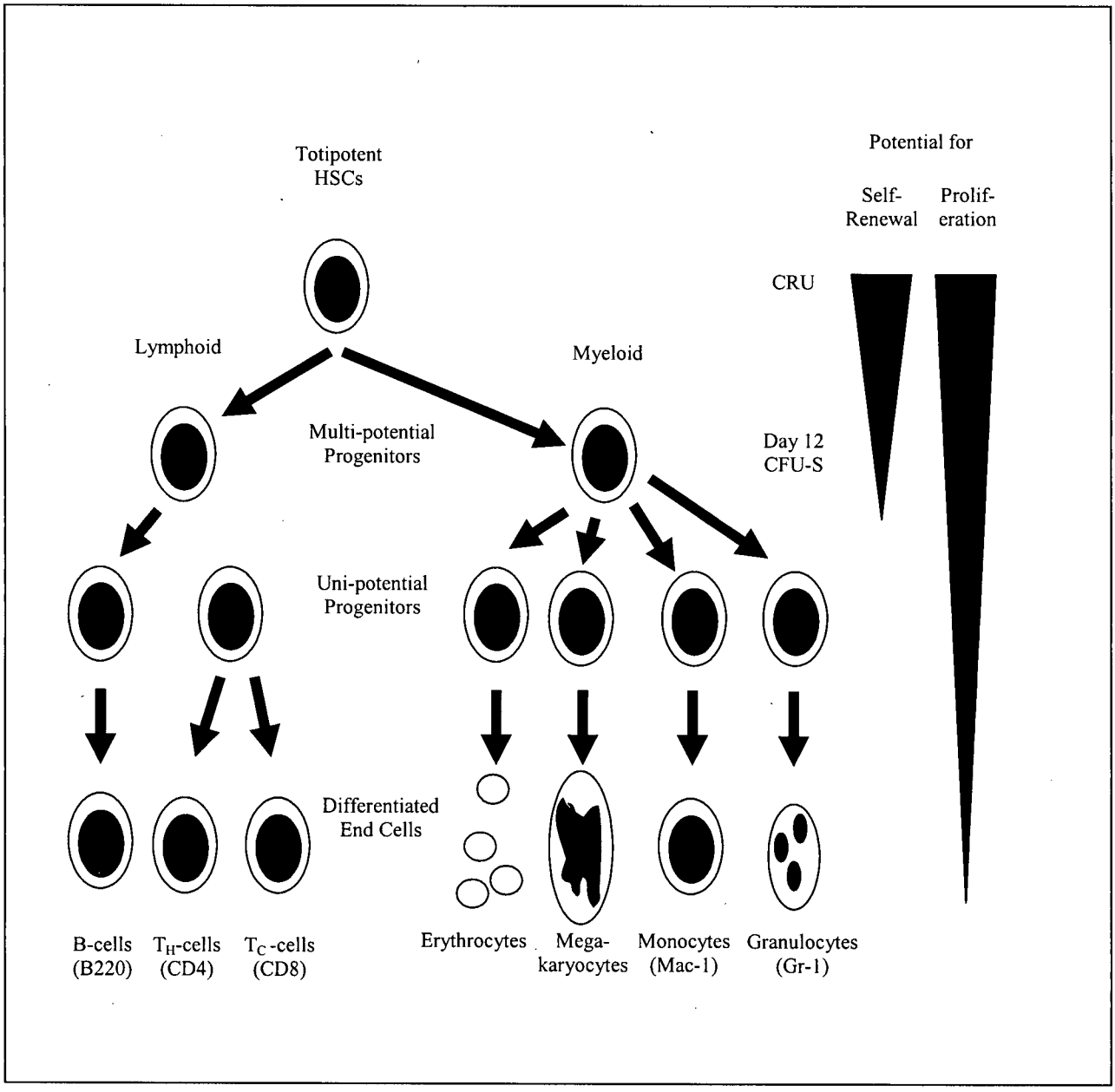
1.3.1 Overview of Hematopoietic Hierarchy

The life-long production of RBCs and indeed all blood cells, results from a process referred to as hematopoiesis. More specifically, hematopoiesis refers to the process by which HSCs divide and differentiate into more committed multi-potential cells, which in

turn undergo multiple divisions to give rise to the all the different cells found within the circulation (Fig. 3). Cells with such capabilities are said to be totipotent. As HSCs divide and differentiate, the cells produced in each stage of the hierarchy have progressively less self-renewal capacity and less ability to differentiate into the cells of other lineages. Furthermore, as cells divide and differentiate, the total number of cells increases exponentially. As a result, those cells with the most self-renewal and multi-lineage differentiation capacity are relatively few in number. It is these rare stem cells that are responsible for the life-long production of every blood cell within the body. While the hematopoietic hierarchy leading from primitive HSCs to differentiated end cells is commonly depicted as a multi-step process, the actual process is more continuous. Nonetheless, it is possible to distinguish cells at different stages of the hierarchy on the basis of their proliferation and differentiation potential, responses to different culture conditions and cell surface markers (Fig. 3). Using these indicators and marked cells, it has been shown that the two main branches of the hierarchy diverge very early in hematopoiesis. Totipotent HSCs are thought to give rise to multi-potential lymphoid and myeloid progenitors after only a few divisions. In turn, these multi-potential progenitors divide and differentiate into more mature, lineage restricted progenitors. It is these uni-potential cells that give direct rise to the differentiated end-stage cells that facilitate the many functions of the blood. Despite their common origin, cells within the myeloid lineages, which include the erythrocytes, macrophages, monocytes, granulocytes and megakaryocytes are produced in the BM, while cells of the lymphoid lineages, which include the B-lymphocytes (B-cells), T-lymphocytes (T-cells) and Natural Killer cells, are found in the BM, spleen, thymus and lymph nodes to varying degrees. While the distinctions between myeloid and lymphoid

Figure 3 – Organization of the Hematopoietic System.

The organization of the Hematopoietic system is hierarchical in nature, with more primitive cells towards the top and more differentiated cells nearer the bottom. The capacity for self-renewal and proliferation decreases as the cells become more differentiated. The functional end cells in each lineage are depicted at the bottom along with the pertinent cell surface marker for those types of cells. Some of the functional names associated with assays used to detect more primitive cells are shown along the right. HSCs, hematopoietic stem cells; CRU, competitive repopulating unit assay; Day 12 CFU-S, colony-forming unit-spleen cells that generate nodules on the spleen 12 days after injection. (modified from Thorsteinsdottir, 1997).


Totipotent
HSCs
Potential for
Self-
Renewal
Prolif-
eration
CRU
Lymphoid
Myeloid
Multi-potential
Progenitors
Day 12
CFU-S
Uni-potential
Progenitors
Differentiated
End Cells
B-cells
(B220)
T_H-cells
(CD4)
T_C-cells
(CD8)
Erythrocytes
Mega-
karyocytes
Monocytes
(Mac-1)
Granulocytes
(Gr-1)

lineages are now a central dogma of hematology, historical classification systems were more arbitrarily based on visible attributes such as color. As such, all of the cells lacking hemoglobin, namely the granulocytes, monocytes, B-lymphocytes, T-lymphocytes and NK cells, are referred to as White Blood Cells (WBCs) and all other cells, namely the erythrocytes, are referred to as Red Blood Cells (RBCs).

1.3.2 Hematopoietic Stem Cells

The idea that all blood cells within the body are descended from common "totipotent" cells has been upheld for almost 40 years. Early indications that such cells may exist came from studies in which the transplantation of chromosomally marked cells into lethally irradiated mice resulted in the long-term recovery of both lymphoid and myeloid cells that were also marked (Wu et al., 1968). Later, these findings were also confirmed by studies in which retrovirally marked cells were found to give rise to both lymphoid and myeloid cells that were also marked following BMT (Dick et al., 1985; Keller et al.; 1985, Capel et al., 1989). Since these studies, the existence of totipotent HSCs that are capable of self-renewal and multi-lineage differentiation has become a central dogma of hematology. Still to this day, the most rigorous definition of a HSC is "a cell capable of long-term repopulation of both lymphoid and myeloid lineages" (lymphomyelo-repopulation).

Qualitatively, proving the existence of HSCs requires only that the long-term derivation of both lymphoid and myeloid cells from a common cell be shown. However, quantitation of these cells has been much more difficult. One of the first methods of detecting and quantifying primitive hematopoietic cells *in vivo* was the Colony Forming Unit–Spleen (CFU-S) assay (Till & McCulloch, 1961). Originally, this assay was thought to

detect true HSCs that formed visible colonies on the spleen of lethally irradiated mice 8 to12 days after BMT. However, despite their ability to form large colonies ($10^5 - 10^7$ cells) consisting of cells from multiple hematopoietic lineages (Wu et al., 1968) and capacity for self-renewal, as demonstrated by the formation of spleen colonies in mice injected with spleen colony cells (secondary spleen colonies) (Siminovitch et al., 1963), the long-term lymphoid repopulating capacity of these cells remained debatable (Wu et al.,1968; Lala & Johnson, 1978; Paige et al., 1979) and was eventually disproved (Lepault et al., 1993). As such the CFU-S is no longer considered to be a HSC candidate as it fails the most fundamental test of long-term lymphomyelo-repopulation. Nonetheless, the CFU-S is widely accepted as a very primitive, though not a totipotent cell, whose detection is usually indicative of HSC presence. More recently other methods of detecting and quantitating HSCs have emerged. These include the Competitive Repopulating Unit (CRU) assay (Szilvassy et al., 1990), the Repopulating Unit (RU) assay (Harrison et al., 1993), the Long Term Culture-Initiating Cell (LTC-IC) assay (Sutherland et al., 1990), and the High Proliferative Potential-Colony Forming Cell (HPP-CFC) assay (Bradley, 1979). In mice, the CRU assay, which evaluates the survival of lethally irradiated recipients following the injection of limiting numbers of BM cells, has been the most widely used method of quantitating HSCs. Using this assay, the frequency of HSCs in the BM of a mouse (called CRUs) has been estimated to be about 1 in 1 x 10^4 nucleated cells (Szilvassy et al., 1990).

1.4 Hematopoietic Stem Cell Engraftment

1.4.1 Phenotype of Engraftable Hematopoietic Stem Cells

The totipotent, self-renewing cells known as HSCs are found primarily in the BM of adult mammals. However, studies in which the circulatory systems of two genetically distinguishable mice have been joined have shown that there is an exchange of HSCs between the two, suggesting that HSCs are capable of leaving the BM, traveling through the circulatory system and re-entering the BM on a constant basis (Wright & Wagers, 2001). In keeping with this finding, intravenously injected HSCs have been shown to travel through the circulatory system and migrate into stem cell "niches" within the BM in a process known as "homing" or "engraftment". Once homed to the BM or engrafted, these cells self-renew, divide and differentiate to produce all the cells of the blood, resulting in long-term lymphomyelo-repopulation by donor-derived cells.

Studies on the kinetics of homing have demonstrated that HSCs disappear from the circulation following BMT into murine recipients. In recent studies, secondary transplants of BM from unirradiated mice transplanted 24 hours earlier, have demonstrated that the primary donor engraftment levels of secondary recipients are unchanged at later time points, suggesting that transplanted HSCs home to the BM within 24 hours of injection (Bubnic & Keating, 2002). In support of these findings, the results of hydroxyurea-killing experiments have indicated that transplanted HSCs are initially quiescent, and thereafter become synchronized, with the majority entering the cell-cycle approximately 12 hours after injection (Nilsson et al., 1997). Since it would seem unlikely that normally adherent cells would be stimulated to divide while in the circulation, these results imply that homing may occur even more rapidly than the earliest time point tested by Bubnic & Keating. While

little is known about the mechanisms of this rapid and specific homing to the BM, the involvement of a number of different molecules such as β1 integrin and VCAM-1 has been implied. Perhaps most convincing of all, are studies using conditional β1 integrin knockouts, which have shown $\beta1^{-}/\beta1^{-}$ HSCs accumulate in the circulation following BM transplantation (Potocnik et al., 2000). While it is possible that the effects of β1 integrin deletion are the result of the absent or compromised function of molecules that are downstream from β1 integrin and not β1 integrin per se, this would seem unlikely in light of other findings. Indeed, β1 integrin has been shown to be expressed at high levels on most stem and progenitor cells and its down regulation following *ex vivo* expansion is associated with impaired homing of transplanted HSCs (Szilvassy et al., 2001).

1.4.2 Hematopoietic Stem Cell Engraftment in the Non-Myeloablative Setting

For many years, the success of HSC transplantation (HSCT) was believed to be dependent upon the creation of "niches of availability" within the BM compartment (Schofield et al., 1978). The most common method of achieving this has been total body irradiation (TBI). However, in recent years it has become apparent that preparative myeloablation is not necessary for the engraftment of HSCs within the murine system. The first evidence that suggested that engraftment in the absence of myeloablation could be possible, came as early as 1968 when Micklem et al. demonstrated 8.5% engraftment of T6T6 cells into the BM of unmanipulated CBA mice 3 months after the transplantation of 20 million cells (Micklem et al., 1968). Similar results were reported by Brecher et al. 14 years later when they observed 16% to 25% engraftment following the injection of 200 million male BM cells into unmanipulated female host mice (Brecher, 1982). Saxe et al. achieved

similar, albeit more varied, levels of engraftment that ranged from 0% to16% after the transplantation of 1 x 10^8 cells (Saxe et al., 1984). In addition, they demonstrated that engraftment levels were independent of dose scheduling, route of injection and age of the recipient. These studies showed that engraftment, as determined by chromosome analysis and biochemical differences, was stable up to 3 months post-transplant, the latest time point analyzed. Further, in 1993 the group of Quesenberry attained levels of BM chimerism as high as 48% at 12 months post-transplant, by injecting two tibia/femur equivalents of normal male BALB/cJ BM cells (~ 75 x 10^6 cells) into syngeneic female recipients (Stewart, 1993). A tibia/femur equivalent represents the total BM contained in the two hind long bones of a mouse. While these same experiments also demonstrated successful engraftment into both the spleen and the thymus, the proportion of male cells in these populations was considerably less. Nonetheless, these studies did lend support to the findings of previous studies, adding to the mounting evidence against the need for myeloablation to ensure successful engraftment of HSCs. Results published by Wu and Keating in the same year also confirmed the findings of Stewart et al., although on a much smaller scale and shorter time frame (Wu & Keating, 1993). Again using the BALB/cJ model system, Wu and Keating found that injecting 20 million normal male cells into unmanipulated female recipients resulted in 2% to10% male-derived cells in the BM 8 weeks post-transplant. However, perhaps more interestingly Wu and Keating also found that from 13% to 47% of Day 12 CFU-S, 0% to 14% of Colony Forming Unit-Granulocyte/Macrophage (CFU-GM) and 0% of peripheral blood (PB), spleen and thymus cells were donor-derived. The authors concluded that their results seemed to indicate that even though early hematopoietic

progenitors (and presumably HSCs) engraft at relatively high efficiency into the unirradiated host, there appears to be low tendency towards differentiation in this setting.

While the possibility of HSC engraftment in unconditioned mice has been appreciated for over 10 years, the mechanisms by which this occurs and the factors that dictate the extent of the established chimerism, remain to be definitively elucidated. However, several studies carried out in recent years have shed light on these two issues. In particular, Rao et al. attempted to describe the relationship between cell dose, transplant schedule and levels of engraftment (Rao et al., 1997). Key among their findings was that the level of BM chimerism increased from 11% to 39% with increasing cell dose over the range 40 million to 200 million cells. Corresponding ranges in the spleen and thymus however, were only 5% to 30% and 3% to 15% respectively. In addition, they reported that engraftment efficiency was highest on the first and second days of a 5 day transplant schedule and when the daily transplant dose was 10×10^6 million cells. Despite the latter findings, previous work had established that scheduling was not an important factor and that similar levels of engraftment were obtained when 200 million donor cells were given over 5 days or in a single dose (Ramshaw, 1995). In addition, the group of Quesenberry came up with several models of engraftment based on either augmentation (simple addition) or replacement of the endogenous BM by donor cells (Rao et al., 1997). While both of these models were based on generous assumptions such as 100% seeding efficiency of transplanted HSCs to the BM, the observed levels of BM chimerism either approached or exceeded the theoretical maxima in every case. Therefore, the authors reported that engrafted donor HSCs had a proliferative advantage over endogenous HSCs, a conclusion which is in direct conflict with results of Wu & Keating. In addition, the high levels of

engraftment observed in these experiments suggested that donor cells might be replacing rather than augmenting the host marrow. In order to prove this, Blomberg et al. attempted to completely replace the host BM by "mega-doses" of donor cells (Blomberg et al, 1998). However, despite transplanting up to 800 million cells, they found no significant increase in the levels of BM chimerism as compared to mice given only 200 million cells. However, the levels of chimerism in the spleen and thymus more than doubled. While these results were inconclusive with regards to distinguishing between replacement and augmentation models of engraftment, additional analysis of the BM and spleen showed that neither progenitor cell number nor total cellularity increased with increasing cell dose. The authors therefore concluded that host cells were being replaced and not augmented by donor cells.

1.4.2.1 Reducing Effective Cell Doses in the Non-Myeloablative Setting

The high rates of mortality following BMT are due to the inability of the BM to recover after lethal preparative regimens should donor-derived hematopoiesis not ensue. Therefore, the finding that HSC engraftment is not only possible, but also surprisingly efficient in the unirradiated setting, is one of great significance to the field. However, one of the major aspects of unconditioned BMTs that has impeded their translation to the clinical arena, has been the extremely high cell doses required to attain reasonable levels of chimerism. As a result, there has been a substantial amount of effort devoted to finding ways of decreasing effective cell doses without compromising therapeutic potential. In general, there are two ways to achieve this. The first way to reduce effective cell doses is to enrich the donor population for HSCs so that a greater number of cells capable of long-term reconstitution are transplanted in a given cell dose. The second way in which effective cell

doses may be decreased is to reduce the competition with transplanted cells by killing endogenous HSCs prior to transplantation. Each of these approaches has been explored in recent years.

1.4.2.1.1 Conditioning Transplant Donors with 5-Flurouracil

One cytoreductive drug that has been used as a means of stem cell enrichment for over a decade is the nucleotide analog 5-Fluorouracil (5-FU). The cytoreductive action of 5-FU, which specifically targets rapidly dividing cells, is thought to result from a build up of a metabolic derivative of 5-FU called 5-fluoro-2'-deoxyuridine-5'-phosphate (F-dUMP). F-dUMP blocks DNA synthesis by inhibiting the intracellular enzyme thymidylate synthetase, which ultimately leads to death as the cells progress through the synthesis phase of the cell-cycle (Lerner & Harrison, 1990). The use of this drug in experimental BMTs was first employed by Hodgson and Bradley (Hodgson & Bradley, 1979). More recently, studies by Lerner and Harrison demonstrated that 5-FU treatment of BM donors spared HSCs responsible for long-term repopulation in lethally irradiated mice (Lerner & Harrison, 1990). In these studies, they observed more than a ten-fold decrease in total BM cellularity without the loss of stem cell function after a single dose of 5-FU (150 mg/kg) was given to donor mice 5 days prior to BM harvest. It is now generally accepted that the frequency of CRU within BM treated 4 days prior with 5-FU is approximately 1 in 3000 (Szilvassy & Cory, 1993). More recently several groups have found it pertinent to explore the effects of 5-FU on engraftment potential in the non-myeloablated setting, since the primary transplants done by Lerner & Harrison were performed in recipient mice that had been lethally irradiated. In particular, one study by Stewart et al. that evaluated the effect of 5-FU on the engraftment

potential of HSCs, found that equal numbers of male HSCs, determined on the basis of tibia/femur equivalents, that had or had not been previously treated with 5-FU, competed unequally with endogenous female HSCs in unirradiated BALB/cJ mice (Stewart et al., 1993). In fact, BM that had been treated with 5-FU 6 days prior to harvest (Day-6 5-FU BM) resulted in an average 3.4-fold reduction in the observed level of chimerism at 12-months post-transplant as determined by Southern Blot analysis. Similar patterns of engraftment were observed for both the spleen and thymus. Therefore, treatment of donor HSCs with 5-FU is now generally accepted to result in an engraftment defect that is only evident in the non-myeloablated setting.

Another aspect of 5-FU treatment that is of importance, is its tendency to promote cycling of spared HSCs within the donor BM. The induction of cell-cycle is a direct physiologic response to the depletion of donor BM by the cytotoxic effects of 5-FU. In the context of globin gene therapy, this is a potentially important side-effect as post-infection mitotic division is necessary for oncoretroviral integration into the host genome, since the DNA is made accessible during this time. As such, the use of 5-FU to prepare BM donors for harvest has become a wide-spread practice in murine gene therapy trials, as it is thought to improve the transduction efficiency and thus the probability of success. However, recent studies have suggested that the induction of cell-cycle may in fact be responsible for the engraftment defects that are observed following 5-FU treatment of HSCs. In particular, the group of Quesenberry has confirmed and extended the previous findings of others, demonstrating a significant engraftment defect in 5-FU treated BM 1 to 6 days after administration (Ramshaw et al., 1995, D'Hondt et al., 2001). In addition they have shown that the engraftment defect is in fact transient and completely eliminated within 12 days of

5-FU treatment (D'Hondt et al., 2001). As such, they hypothesized that engraftment defects associated with Day-6 5-FU BM were reversible and related to the cell-cycle status of HSCs contained therein. In support of this hypothesis, they were able to demonstrate that about 27% of all engraftable HSCs within Day-6 5-FU BM are in active cycle, although more recent studies have found the proportion to be closer to 100% (D'Hondt, 2001). The reason for this discrepancy is not known. Nonetheless, these results indicate that actively cycling stem cells are capable of engraftment, but may be markedly defective in their ability to compete for BM niches in the non-myeloablated setting as a result of their active cell-cycle status. This notion is further supported by studies done by Habibian et al. in 1998 in which the engraftability of HSCs treated with interleukin-3 (IL-3), IL-6, IL-11 and Stem Cell Factor (SCF) from 24 to 48 hours was found to fluctuate over 2 to 4 hour periods with nadirs in engraftment occurring late in S-phase and early in G_2-phase of the cell-cycle. While this finding supports the idea that HSC engraftment defects are related to cell-cycle status, the fact that this experiment was done in lethally irradiated recipients makes the results difficult to apply in the non-myeloablated setting. Nonetheless, the fact remains that the treatment of BM donors with 5-FU 1 to 6 days prior to harvest results in a transient HSC engraftment defect that is observable in both the unirradiated and lethally irradiated setting. This defect is related to cell-cycle status at the time of transplant in the lethally-irradiated host. Whether or not the same is true of these cells in the non-myeloablated setting, remains to be seen.

1.4.2.1.2 Conditioning Transplant Recipients with Ionizing Radiation

The use of 5-FU as a means of enriching donor inocula for HSCs is only one way of reducing effective cell doses. Another alternative that has been recently explored, is

ionizing radiation. The use of ionizing radiation in preparing patients for BMT stems from its ability to release high energy electrons (ionize) from the phosphodiester bonds of the DNA backbone. This ionization results in breakage of the DNA and subsequent initiation of cellular repair mechanisms. In addition, autoionization of cytoplasmic water results in the production of intracellular oxidants that are toxic to the cell. At low doses, many cells are capable of repairing the damage induced by ionizing radiation and oxidation. However, as doses increase damage accumulates and at some critical level becomes irreparable. At this point, cellular suicide is initiated and cells undergo programmed cell death (apoptosis). Early studies using CFU-S as surrogate markers for HSC survival have indicated that radiation-induced cell death is approximately linearly related to radiation doses above 200 cGy, with about 97% of CFU-S being killed at this dose and 99.9% being killed at 500 cGy (Till & McCulloch, 1961). Previous studies that have examined the radiosensitivity of CFU-S have found that biological effectiveness increases with increasing dose rate (i.e.-Rads/min) over this range, although these effects are relatively small (Puro & Clark, 1972).

In comparison to the amount of literature describing the use of 5-FU as a cytoreductive agent in transplant donors, there exists a relative paucity of data concerning the use of low dose radiation (i.e.- < 500 centiGray) as a means of increasing engraftment levels in transplant recipients. Among the noteworthy, is a study done by van Os et al., in which levels of BM chimerism up to 41% were obtained following the injection of 1×10^6 cells into mice treated with as little as 200 centiGray (cGy) of ^{60}Co-γ radiation (van Os et al., 1993). Results obtained a number of years later by the group of Quesenberry, also demonstrated high levels of engraftment with the use of radiation doses even lower than van Os et al. (Stewart et al, 1998). With as little a 100 cGy of ^{137}Cs-γ radiation, they were able

to demonstrate levels of BM engraftment ranging between 10% and 100% following the infusion of 2 x 10^6 to 40 x 10^6 unfractionated BM cells. The ability of Stewart et al. to successfully use lower radiation doses cannot be explained by the differing sources of radiation as ^{60}Co-γ radiation is higher in energy than ^{137}Cs-γ radiation.

1.4.2.2 Non-myeloablative Transplants for the Treatment of Human Disease

In the past, HSCTs for the treatment of inherited human diseases have been carried out under conditions of recipient myeloablation. The primary reasons for this are two-fold. Firstly, past beliefs have been that it is necessary to create "niches of availability" within the BM cavity in order to obtain sustained engraftment. Secondly, myeloablation is an effective prophylactic against GVHD, a serious post-transplant immune reaction that can compromise engraftment sustainability and can lead to death. Despite the dogmatic nature of the foregoing reasons for myeloablation, recent findings have thrown into question its continued justification. In particular, several studies in mice have challenged the need for creating "niches of availability", and have instead proposed that donor to recipient HSC ratios are a more important factor (Stewart et al., 1993; Stewart et al., 1998; Stewart et al., 2001). In addition, advances in understanding of T-cell biology have led to efficient, yet transient immuno-suppressive treatments that can prevent the development of GVHD. As a result of these findings, clinical protocols that employ HSCTs in the absence of myeloablation have begun to recently emerge in the literature. While most of these protocols have been used in the context of treating hematologic malignancies, they set the stage for similar trials in the treatment of non-malignant diseases such as the hemoglobinopathies. Of particular interest is a recent study in which more than half of the patients (median age 56 years) treated for

hematologic malignancies, were treated on an entirely outpatient basis (McSweeney et al., 2001). In this study, patients were given only 200 cGy of preparative radiation prior to HLA-identical sibling HSCT. Post-transplant prophylactic treatment consisted of 35 days of cyclosporin to achieve target blood levels of 500 nanograms per milliliter (ng/mL), and donor lymphocyte infusion (DLI) for persistent malignancy, mixed hematopoietic chimerism or both. After a median follow-up of 417 days, 53% of the patients were in complete remission. In light of the fact that the median age of patients who have received HSCT for chronic myelocytic leukemia (CML), acute myelocytic leukemia (AML), chronic lymphocytic leukemia (CLL), multiple myeloma (MM) and non-Hodgkin lymphoma (NHL) is approximately 2 decades younger than the median age at diagnosis (Molina & Storb, 2000), the results of this study represent a significant advance in the treatment of hematologic malignancies in older patients, and lay a solid foundation for continued research into non-myeloablative HSCTs.

While there have now been a substantial number of patients successfully treated for hematologic malignancies under non-myeloablative conditions, little is known about the utility of non-myeloablative HSCTs in the context of non-malignant diseases. However, in one recent case reported by Krishnamurti et al., a young girl was successfully treated for homozygous SCD under non-myeloablative conditions, by transplanting HLA-A, -B, -C and –DR matched, but Rh mismatched sibling BM (Krishnamurti et al., 2001). The conditioning regimen consisted of 2 milligrams (mg) busulfan per kilogram (kg) of bodyweight every 12 hours for 2 days, 35 mg of fludarabin per square meter of body surface and 30 mg/kg of antithymocyte globulin for 5 days, and 500 cGy of total lymphoid irradiation delivered as a single dose with shielding of the liver, heart, lungs and ovaries. A total dose of 3.49×10^8

nucleated cells per kg was infused intravenously on day 0 and prophylaxis against GVHD consisting of mycophenolate mofetil and cyclosporin (blood levels > 200 ng/mL) was maintained for 45 and 100 days respectively. The authors reported no obvious toxic effects, transient myelosuppression and 67%, 75%, 96%, 97% and 100% PB chimerism on days 12, 21, 60, 100 and 360 respectively. The patient showed no signs of GVHD or clinical symptoms of SCD up to 14 months post-transplant, indicating that BMT following non-myeloablative conditioning can be well-tolerated, can result in stable engraftment and can ameliorate SCD on a long-term and perhaps permanent basis.

1.5 Experimental Objectives

The experiments detailed in this thesis were driven by an underlying need to make current globin gene therapy protocols more clinically relevant by reducing effective conditioning regimens and thus the associated risk of transplant-related mortality. While this was always the primary objective, several important sub-aims precipitated along the way as results were obtained. These sub-aims provided a solid foundation on which more important and resource consuming experiments could be planned. These sub-aims were as follows:

1) to establish a model system in which sub-lethal BMT regimens could be evaluated.
2) to define the relative importance and interacting dynamics of factors contributing to the results of sub-lethal BMT.
3) to assess the effects of stem cell enrichment on the outcome of sub-lethal BMT.
4) to cure β-Thalassemia in mice using sub-lethal preparative irradiation and BMT.

CHAPTER 2 MATERIALS AND METHODS

2.1 Mice

All mouse strains were obtained from the Jackson Laboratories and were bred and maintained in the Joint Animal Facility of the British Columbia Cancer Research Centre under pathogen free conditions, which were approved by the University of British Columbia's Animal Care Committee. Mice were given sterile water and mouse chow *ad libitum* and were kept under conditions of room temperature and equal light and darkness. All BMT donors and recipients were chosen based on their CD45 genotype with donors always being positive for the CD45.1 (Ly-5.1) allotype and recipients always being homozygous for the CD45.2 (Ly-5.2) allotype. Transplant donor/recipient pairs were either (C57BL/6-Ly-Pep3b x C3H/HeJ) F1 (PepC3) and (C57BL/6J x C3H/HeJ) F1 (B6C3), C57BL/6-Ly-Pep3b (Pep3b) and C57BL/6J (B6) or C57BL/6-Ly-Pep3b and C57BL/6J-$\beta^{Major\ -/-}$ (MDD).

2.1.1 Mouse Models of beta-Thalassemia

Thalassemic mice (MDD) were originally obtained from Drs. R.A. Popp and D.M. Popp and were subsequently bred and maintained in the Joint Animal Facility of the BC Cancer Research Centre. The mutation responsible for the thalassemic phenotype of the MDD mice used in the following experiments, is a large scale (~3.7 kb) deletion of the β^{M}-gene that originally occurred naturally in a DBA/2J male. The mutation was first discovered in (C57BL/6J x DBA/2J)F1 mice sired by the mutant male (Hbb^{th-1}/Hbb^{th-1}) and

was subsequently bred into the C57BL/6J background ($Hbb^{d3(th)}/Hbb^{d3(th)}$). These mice are referred to as MDD (Major Double Deletion) mice.

2.2 Murine Bone Marrow Transplants

2.2.1 Irradiation

All mice requiring pre-transplant irradiation were irradiated at a rate of 85 cGy per minute using a ^{137}Cs source of gamma radiation. Mice were contained in a Plexiglas container and were irradiated in groups of two to six mice depending on the size of the mice and on the size of the cohort dictated by the experimental design. All mice were irradiated at least 4 hours prior to transplant and were housed in the Joint Animal Facility procedures room until BMTs were complete.

2.2.2 5-Fluoruracil Treatment of Bone Marrow Donors

Mice that required treatment with 5-FU before BM harvest were given 150 mg/kg of 5-FU (Faulding Incorporated, Vaudreuil, Quebec) 4 days prior to sacrifice. 5-FU stock solutions were purchased at a concentration of 50 milligrams per milliliter (mg/mL) and were diluted to a concentration of 30 mg/mL with Dulbecco's Phosphate Buffered Saline (PBS) prior to use. Unless otherwise indicated, all solutions were obtained from StemCell Technologies Incorporated (Vancouver, Canada). The use of 5-FU at this concentration allowed for simple determination of injection volumes after weighing each mouse as simple multiplication of the animals measured weight (in grams) by five yields the required dose volume in microliters (μL). All doses of 5-FU were administered via tail vein injection

using a 26-gauge needle and a 1 cubic centimeter (cc) syringe. Injection volumes typically ranged from 100 μL to 125 μL.

2.2.3 Bone Marrow Harvest

All mice used as BM donors were between 6 and 8 weeks old and were killed by carbon dioxide asphyxiation immediately prior to use. Tibiae and femora were removed from the hind legs of each mouse under sterile conditions and were kept on ice. BM was harvested from each of the long bones following the removal of all tissues from the outside using forceps. The epiphyses of each bone was cut using surgical scissors and the BM was ejected with 2% Fetal Calf Serum in PBS (2% PBS) using a needle and syringe. In order to ensure complete recovery of BM inclusive of HSCs that are known to adhere to the endosteal surface, the inner surfaces of the bones were repeatedly scoured in a helical pattern with a 26-gauge needle for tibiae and a 22-gauge needle for femora and were then rinsed with ice-cold 2% PBS into a 15 mL polystyrene tube. All donor marrow from same sex animals was pooled and was kept on ice until all of the animals necessary for each experiment had been harvested. In order to ensure that the cells were in a single-cell suspension, the BM suspensions were repeatedly aspirated and ejected through a 22-gauge needle and large particulate matter was removed by transferring the resultant suspension to a clean tube using a 26-gauge needle and a 3 cc syringe. Resultant cell suspensions were kept on ice until enumeration.

2.2.4 Bone Marrow Enumeration

BM cell suspensions were enumerated using an Improved Neubauer Hemocytometer. Aliquots used for the purposes of enumeration were diluted with 3% acetic acid in order to obtain counts ranging between 100 and 250 nuclei per quadrant. Since dilution in 3% acetic acid lyses cell membranes but not nuclear membranes, and since the vast majority of erythrocytes are enucleated, these counts are indicative of WBC concentration prior to dilution. In cases where the concentration of cells within the BM suspension was too low for efficient injection, the suspensions were centrifuged at 1,200 revolutions per minute (rpm) for 7 minutes and were then resuspended in an appropriately smaller volume of 2% PBS. Resuspension was then followed by re-enumeration in the manner previously described. In those cases where cell concentration was too high to allow for an injection of at least 200 μL, BM suspensions were further diluted with 2% PBS. At least two duplicate dilutions were prepared and at least 400 nuclei were counted for each enumeration. The results of each enumeration were then averaged for a given sample. BM cell suspensions were kept on ice until injection into recipient mice.

2.2.5 Bone Marrow Injection

Following enumeration, BM suspensions were used for the purposes of BMT into recipient mice. In preparation for transplantation, recipient mice were heated briefly under a heating lamp in order to promote vasodilation and successful injection. Each mouse was then loaded into a Plexiglas holding chamber, and the tail was swabbed with alcohol pads in an attempt to minimize the chance of subsequent opportunistic infections in the mice, some of which were immuno-compromised following irradiation. Bone marrow suspensions were

resuspended immediately prior to transplantation and each mouse was given a volume-based dose of the BM suspension as was appropriate according to previous enumerations and experimental design. All injections were done via tail vein in volumes ranging from 100 μL to 1000 μL. In all transplants except those involving recipient mice given lethal doses of irradiation (i.e.- 900 cGy), male recipients received only male BM cells and female recipients received only female BM cells. Following transplantation, prophylactic antibiotic and hydrochloric acid was added to the water bottles in each cage of transplant recipients to prevent opportunistic infections, and mice were housed in the Joint Animal Facility until further analysis.

2.3 Analysis of Engraftment in Transplant Recipients

2.3.1 Bleeding Mice and Isolation of White Blood Cells

All transplant recipients were bled in preparation for Fluorescence Activated Cell Sorting (FACS) analysis of WBCs. In short, mice were warmed briefly under a heating lamp to promote vasodilation and then each mouse was loaded into a holding chamber. Tails were then sterilized by swabbing with alcohol and each animal was ear-marked for future identification while the tails were drying. Once the tails were completely dry, a shallow cut was made on the lateral side with a razor blade in order to puncture the tail vein. 200 μL of blood was then collected into heparinized disposable micropipettes (Fisherbrand, Nepean, Ontario, Canada), which were then blown out into ice-cold eppendorf tubes using a 26-gauge needle and a 1 cc syringe. Once all mice had been bled, RBCs were lysed by adding 1 mL of ice-cold ammonium chloride to each eppendorf tube. Samples were then

briefly vortexed to ensure that each was a single-cell suspension and then they were incubated on ice for 10 minutes. Following incubation, the samples were centrifuged at 5000 rpm for 7 minutes in order to pellet the unlysed WBCs. The red supernatant and coagulated hemoglobin were then aspirated using a Pasteur pipette and the remaining pellets were resuspended in 600 μL of 2% PBS.

2.3.2 Immuno-staining and Fluorescence Activated Cell Sorting Analysis

With the exception of control samples, each RBC-depleted PB sample was divided into six equal parts by pipetting 100 μL into each of six wells of a 96-well tissue culture plate. This was repeated for each experimental sample. For control samples, the volume pipetted into each well was decreased so as to allow for the preparation of staining controls. Once all samples had been divided, the 96-well plates were centrifuged at 1200 rpm for 7 minutes at 4 degrees Celsius (°C). The supernatants were then decanted by inverting the plates. This method of decanting leaves a small amount of supernatant behind in each well. This volume was found to be about 35 μL on average. Optimal immuno-staining then required the addition of 35 μL of stain preparations that were either twice as concentrated (2X) or three times as concentrated (3X) as needed, for single and double staining respectively. All antibodies used were purchased from BD Biosciences (Mississauga, Ontario, Canada). One aliquot of each sample was singly stained with fluorescently labeled rat anti-mouse monoclonal antibodies (mAbs) to the Ly-5.1 antigen. The remaining five aliquots of each sample were stained with biotinylated rat anti-mouse mAbs to the Ly-5.1 or Ly-5.2 antigen. This was done so as to facilitate double staining using streptavidin-allophycocyanin (SA-APC) and phycoerythrin (PE) labeled mAbs to the different blood

lineage markers. For aliquots that were stained with a single stain, 35 μL of a 2X mAb solution was added to each well. The stains used for this purpose were either PE-labeled or fluoroscein isothiocyanate (FITC)-labeled rat anti-mouse Ly-5.1. Both PE- and FITC-labeled anti-Ly5.1 mAb stock solutions were used at a final concentration of 1:1000. For aliquots that were double stained, 35 μL of a 2X solution of biotinylated anti-Ly5.1 or -Ly5.2 mAb was added to each well. Each well was then thoroughly mixed by repeated pipetting and then the plates were incubated on ice for 30 minutes. Following incubation, the plates were centrifuged at 1200 rpm for 7 minutes at 4°C. Supernatants were then decanted by inversion as previously described. All sample aliquots were then washed with 200 μL of 2% PBS and were then centrifuged again as before. This was repeated twice. After the last wash, the supernatants were decanted and 35 μL of a 3X SA-APC solution was added to the five aliquots of each sample that were previously stained with the biotinylated anti-Ly5 mAb. In addition, each of the five aliquots from each sample was stained with a different lineage stain used at an optimal final dilution (shown in brackets). Therefore, 35 μL of a 3X solution of PE-labeled rat anti-mouse B220 (1:1000), Mac-1 (1:2000), Gr-1 (1:2000), CD4 (1:1500) or CD8 (1:2000) mAbs was added to one aliquot for each sample as appropriate. All wells were thoroughly mixed and the plates were incubated in the dark on ice for 30 minutes. Following incubation all aliquots were washed twice with 2% PBS as previously described and were then resuspended in 200 μL of a 1 ug/mL solution of propidium iodide in 2% PBS. Each aliquot of each sample was then transferred to a mini-FACS tube and was then kept in the dark on ice until FACS analysis. All FACS analysis was carried out using a Becton Dickinson (BD) FACSCalibur (Sparks, MD, USA).

2.3.3 Determination of Blood Indices in Transplanted MDD Mice

With the exception of hematocrits determined 3 months following transplant, all blood indices were determined at the Vancouver Children's and Women's Hospital (Vancouver, Canada) from PB samples collected via tail vein puncture. Hematocrits determined after 3 months were obtained using a Clay Adams READACRIT Centrifuge (BD, Sparks, MD, USA) borrowed from Stem Cell Assay (Vancouver, BC, Canada). Peripheral blood samples were collected into heparinized micropipettes (Fisherbrand, Nepean, Ontario, Canada) via tail vein puncture and were kept on ice until analysis. Each sample was blown out into an ice-cold eppendorf tube and mixed briefly by hand prior to filling precalibrated mylar-wrapped capillary tubes (Clay Adams, BD, Sparks, MD, USA). Samples were then centrifuged for 5 minutes before assessing hematocrits using the scale located within the centrifuge.

2.4 Statistical Analyses

All data was assessed for statistical significance. Differences between means and linear regressions were assessed on the basis of two-tailed Student's t-tests and were deemed significant if the probability of the null hypothesis being true was less than or equal to 5%. In one case, the Chi-Squared test was used to assess the deviation of data points from an expected value.

CHAPTER 3 RESULTS

3.1 Hematopoietic Stem Cell Engraftment in the Unirradiated Host

Previous work by the group of Quesenberry (Centre for Stem Cell Biology, Roger Williams Medical Centre, Providence, RI, USA) has shown that high levels of chimerism are attainable in the absence of pre-transplant conditioning, disproving the long-standing dogma that it is necessary to create niches of availability within the BM cavity in order for transplanted HSCs to successfully engraft (Stewart, 1993; Ramshaw, 1995; Rao, 1997; Blomberg, 1998). In all of these studies, the assessment of engraftment levels was based on the detection of male BALB/cJ donor cells in female BALB/cJ recipients. In most cases, the method of engraftment assessment was based on the detection of male DNA sequences by Southern Blot analysis and chromosomal staining techniques. In an attempt to prove that the engraftment of true stem cells had occurred in these experiments, the authors analyzed cells taken from the BM, spleen and thymus and were able to detect male sequences in all. With the *a priori* knowledge that the BM consisted primarily of myeloid cells and that the spleen and thymus consisted primarily of lymphoid cells, the authors concluded that true HSC engraftment had taken place. Despite this evidence however, there existed some skepticism in regards to the high levels of engraftment obtained and a discordance between the levels of engraftment in the three tissues analyzed made it difficult to define the success of this approach.

In order to evaluate the ability of non-myeloablative BMTs to result in therapeutic levels of engraftment in MDD mice, it was decided that previous results in the completely unirradiated setting should be verified. Therefore, an experiment was designed with the

intention of achieving high levels of engraftment in the absence of pre-transplant conditioning. In past studies, it had been necessary to transplant "mega-doses" of BM cells in order to attain high-level engraftment. As such, I planned to inject 200 x 10^6 nucleated BM cells into each unconditioned recipient. Since the total complement of BM within a mouse is about 300 x 10^6 nucleated cells, this dose represents about two-thirds of a "mouse-equivalent". However, due to the laborious and resource consuming nature of harvesting mega-doses of BM, and the preliminary nature of the study, it was decided that only one mouse should be transplanted. In order to assess long-term levels of engraftment, the PB of transplant recipients was to be evaluated for donor-derived WBC content at 2 months and 4 months post-transplant. In addition, the lineage distribution of the donor-derived component of the WBC complement was to be assessed in order to verify true HSC engraftment as evidenced by lymphomyelo-reconstitution.

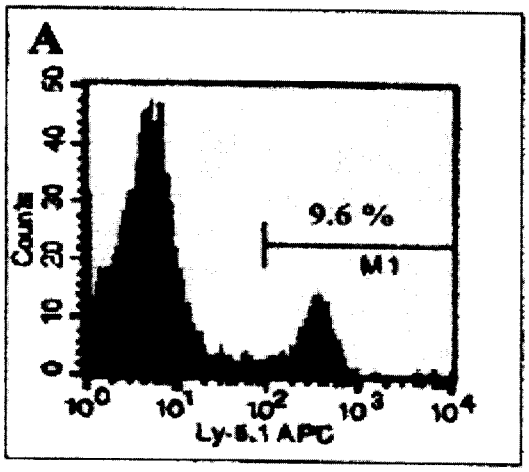
The BALB/cJ model system used by the group of Quesenberry was specifically selected because females of that strain show little or no immune response to the male H-Y antigen Smcy (Nilsson, 1997). In an attempt to similarly minimize the complications of immune rejection in this experiment, the congenic strains PepC3 and B6C3 were chosen to be donors and recipients respectively. Since the WBCs of PepC3 mice are heterozygous for the cell surface marker Ly5 (i.e.- Ly5.1/Ly5.2) and B6C3 mice carry only the Ly5.2 allotype, the presence of Ly5.1 positive cells within recipient mice is indicative of donor engraftment. Subsequently, BM was harvested from the femora of 12 male PepC3 donors, and 205 x 10^6 unfractionated donor BM cells were infused in a single dose of 1 mL into one completely unconditioned male B6C3 recipient. As a control, a second male B6C3 mouse was injected with 1 mL of 2% PBS. After 10 weeks, the mice were bled and their WBCs

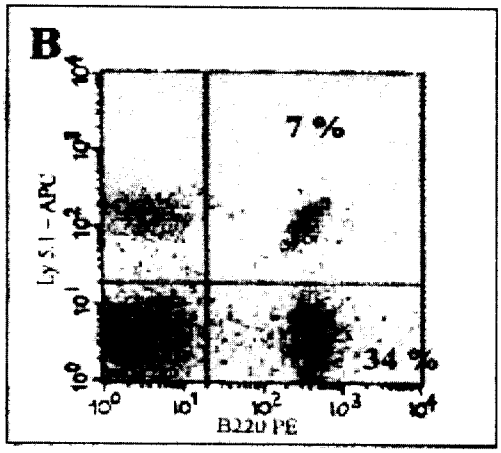
were analyzed by FACS for the presence of Ly5.1 surface antigens. In addition, five different blood lineages were assessed for the presence of Ly5.1 antigens in combination with the appropriate lineage markers (Fig.4). The overall proportion of donor-derived WBCs in the recipient mouse was found to be 9.6%. In addition, evidence of donor-derived hematopoiesis was found to be present in all WBC lineages with 16% of B-cells, 18.7% of granulocytes, 18.4% of monocytes and 4.5% of T-cells staining positive for Ly5.1. Donor-derived hematopoiesis was found to be stable up to 6 months post-transplant and was found to have increased slightly to 12.8 % (Fig. 5). This increase was reflected in four of the five lineages analyzed and was especially pronounced in T-cell lineage, which increased 4.5-fold over the 4 months between successive analyses.

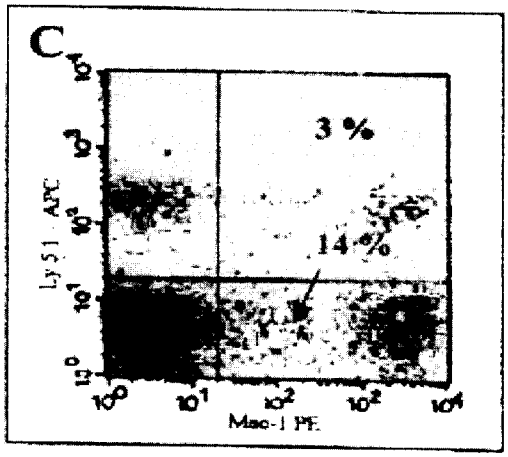
The results of the present experiment unequivocally demonstrate the ability of transplanted murine HSCs to engraft in completely unconditioned hosts. In support of the findings of others, this engraftment was found to be multi-lineage and detectable at the level of differentiated PB cells, albeit at greatly reduced levels to those previously reported. On the basis of the transplant dose that exceeded two-thirds of a mouse equivalent, the levels of chimerism were expected to be in the range of 40% to 60% according to additive or replacement models of engraftment respectively. The reason for this discrepancy is not known. It is possible that the analysis of engraftment levels in the BM overestimates the level of PB chimerism. This possibility is supported by the results of previous studies (Wu & Keating, 1993). However, since an analysis of the BM was not done on this mouse, it is difficult to say whether or not the low-levels of engraftment are the result of a low tendency of transplanted HSCs to differentiate or not. Nonetheless, the results of the present study

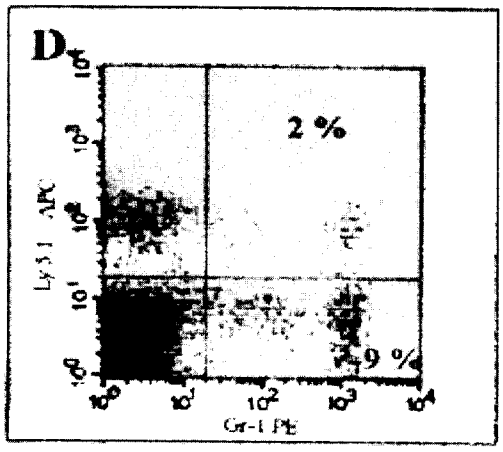
Figure 4 – FACS Profiles of WBCs Drawn and Stained with Fluorescently Labeled Antibodies to Various Cell Surface Antigens 10 Weeks After the Injection of 205 x 10^6 Unfractionated BM Cells Into an Unirradiated Mouse.

A) Recipient WBCs stained with APC-labeled anti-Ly5.1 mAbs. B) Recipient WBCs double-stained with APC-labeled anti-Ly5.1 mAbs and PE-labeled mAbs against the B-cell surface antigen B220. C) Recipient WBCs double-stained with APC-labeled anti-Ly5.1 mAbs and PE-labeled mAbs against the monocyte surface antigen Mac-1. D) Recipient WBCs double-stained with APC-labeled anti-Ly5.1 mAbs and PE-labeled mAbs against the granulocyte surface antigen Gr-1. E) Recipient WBCs triple-stained with APC-labeled anti-Ly5.1 mAbs and PE-labeled mAbs against the T-cell surface antigens CD4 and CD8. The proportion of cells in each pertinent region is indicated.


A
Counts
9.6 %
M1
Ly-5.1 APC


B
7 %
34 %
Ly 5.1 - APC
B220 PE


C
3 %
14 %
Ly 5.1 APC
Mac-1 PE


D
2 %
9 %
Ly 5.1 APC
Gr-1 PE

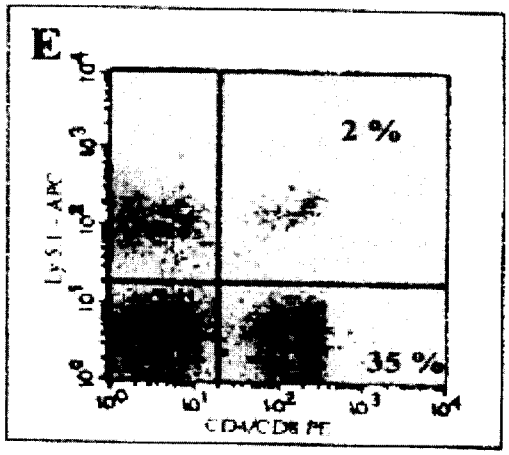
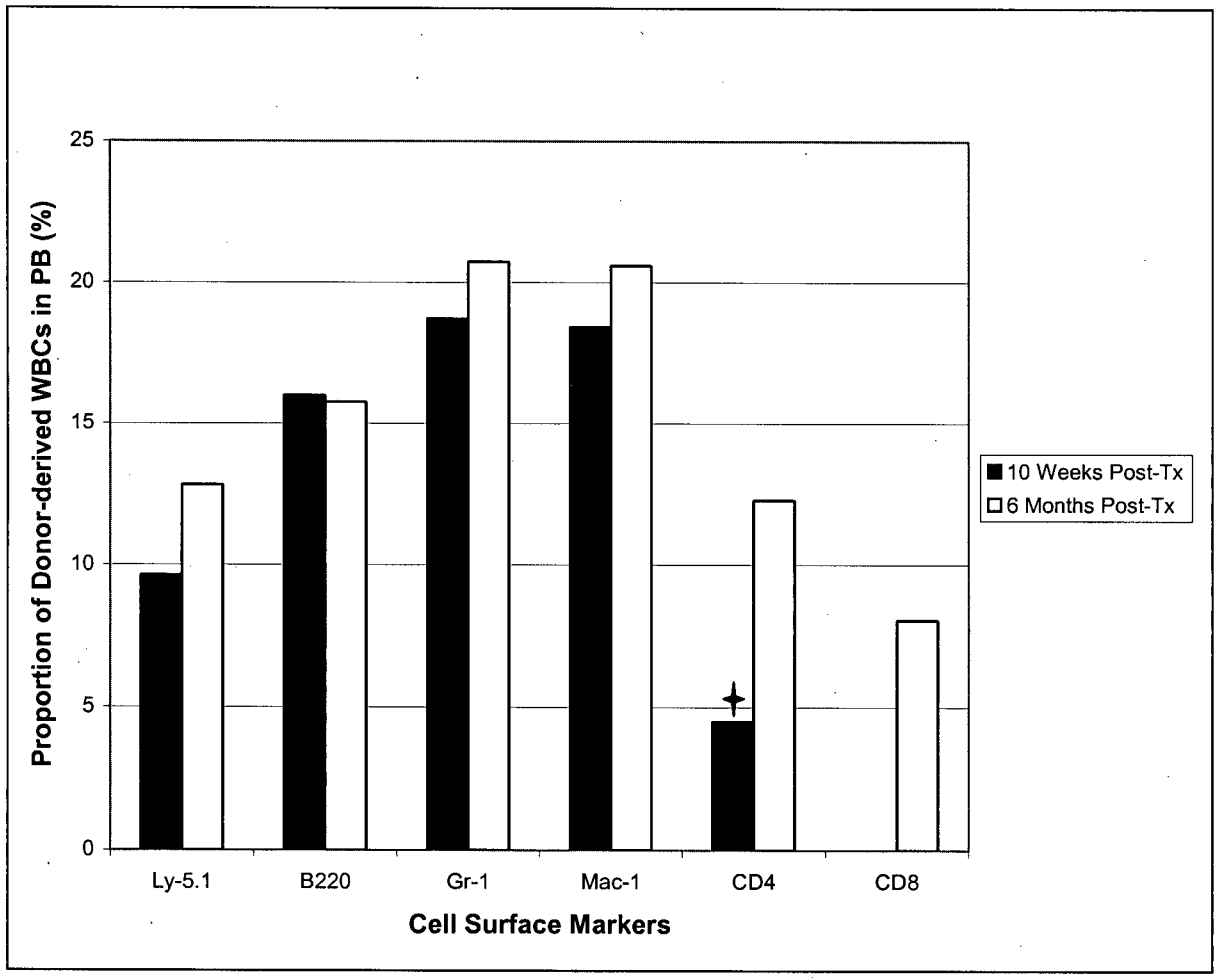

E
2 %
35 %
Ly 5.1 - APC

Figure 5 – Proportion of Donor-derived WBCs as a Whole or in Each of Five Blood Lineages in a Mouse Injected with 205 x 10^6 Unfractionated BM Cells at 10 Weeks and 6 Months Post-transplant.

Values for the proportion of donor-derived cells in each case were determined from the percentage of cells in each region of FACS profiles depicted in Figures 1A-1E. Proportions of donor-derived cells in each of the blood lineages were determined at 10 weeks (black) and 6 months (white) post-transplant (Post-Tx), by dividing the percentage of cells in the upper right quadrant by the sum of the percentage of cells in the upper and lower right quadrants. Each value was multiplied by 100 in order to report a percentage of donor-derived cells in each case. The data point marked with a star represents CD4 and CD8 positive cells at 10 weeks post-transplant.


25
20
15
10
5
0
Proportion of Donor-derived WBCs in PB (%)
Ly-5.1
B220
Gr-1
Mac-1
CD4
CD8
Cell Surface Markers
10 Weeks Post-Tx
6 Months Post-Tx

indicate that the presence of large numbers of unperturbed endogenous HSCs within the BM may reduce observed levels of engraftment by some form of competition. Given the findings of others who have tried to stimulate synchronized donor and host HSC differentiation by the repeated administration of cytotoxic drugs post-transplant (Quesenberry, unpublished data), it would seem that this competition is exerted at the level of HSC engraftment in the BM and not at the level of hematopoietic contribution once engrafted. It may be that there are a limited number of niches available for HSC engraftment in the unirradiated setting.

3.2 Hematopoietic Stem Cell Engraftment in the Sub-Lethally Irradiated Host

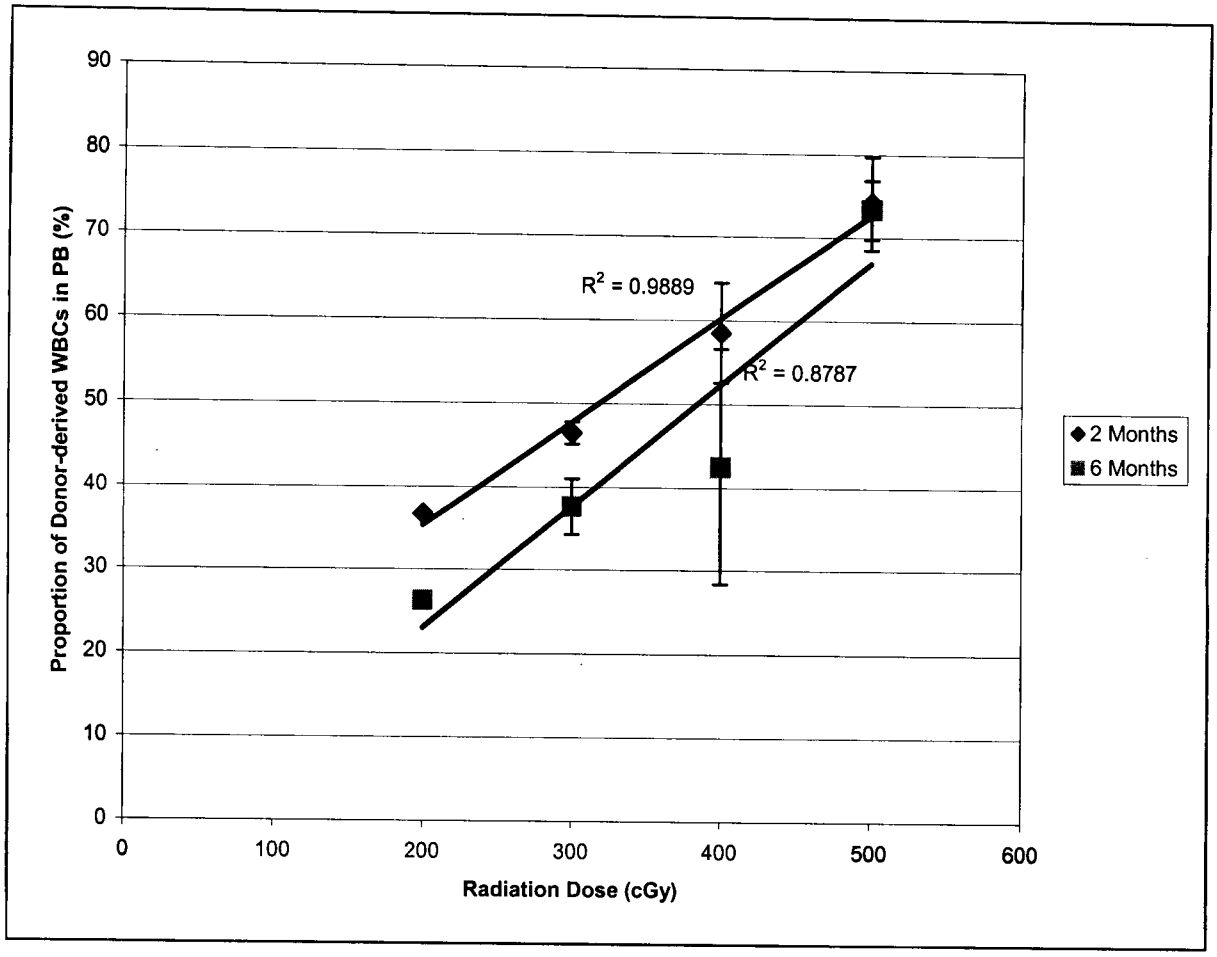
Given the large number of cells infused (20×10^6) and the modest level of engraftment obtained in the completely unconditioned setting, a means of reducing effective cell doses was deemed to be pertinent. Several previous studies had suggested that the ratio of donor HSCs to recipient HSCs likely determines observed levels of engraftment following BM transplantation (Stewart et al., 1993; Stewart et al., 1998; Stewart et al., 2001). If such was true, levels of engraftment could potentially be increased by either increasing the number of donor HSCs infused in the transplant dose, or by decreasing the number of endogenous HSCs within the recipient. While in theory it is feasible to increase the HSC content of the inoculum, previous studies had shown that this approach was generally unsuccessful (Stewart et al., 1993; Ramshaw et al., 1995; Nilsson et al., 1997; D'Hondt et al., 2001). On the other hand, the positive effect of host preparative conditioning on the observed levels of engraftment in transplant recipients was well known. Past studies had shown that cytoreductive drugs and ionizing radiation were both effective means of increasing the

observed levels of engraftment in transplant recipients (van Os, 1993; Mardiney & Malech, 1996; Stewart et al., 1998; Westerhof et al., 2000; Adams et al., 2001; Kean et al., 2002) and the use of either method would have been justified. However the effects of ionizing radiation were deemed to be more consistent between mice and humans, therefore it was decided that this approach to preparative conditioning would be taken. Previous studies defining the effects of radiation on post-transplant levels of chimerism, had established that long-term engraftment of infused donor cells is detectable and quite high following non-lethal, pre-transplant conditioning (van Os et al.,1993). While these studies demonstrated high levels of donor-derived cells in the PB, BM, spleen and thymus, after pre-transplant conditioning of either 200 cGy or 600 cGy, the lack of data between these extremes and the large number of cells (1×10^7) infused in all cases makes it difficult to decipher the relationship between pre-transplant radiation dose and observed levels of engraftment. In order to more clearly define the minimum radiation dose required to obtain therapeutic levels of engraftment in the context of β-thalassemic mice, the effects of different levels of preparative, sub-lethal irradiation on observed levels of engraftment at a constant cell dose, were to be examined. Since previous studies had defined ~100 cGy as the lower threshold of radiation efficacy (Stewart et al., 1998) and 570 cGy as the point at which the benefits of radiation dose escalation diminish (Mardiney & Malech, 1996), it was decided that mice would be irradiated over the radiation dose range of 200 cGy to 500 cGy. In order to reveal any subtleties in the radiation dose response of engraftment levels, radiation doses were to be separated by 100 cGy intervals between these extremes. Again, levels of engraftment were to be determined on the basis of multi-lineage donor-derived WBC content of the PB approximately 2 months and 4 months after transplantation.

In an attempt to make the results of the experiment more applicable in the context of gene therapy protocols, which are generally limited by viral titer and multiplicity of infection, a transplant dose of 5 x 10^6 cells was decided upon. While this is still a relatively large number of cells to infect with virus, it was chosen with precaution to guarantee success and a wide range of engraftment levels. PepC3 donors and B6C3 recipients were again chosen as the transplant model system for the reasons previously described. Bone marrow was harvested from two unmanipulated male PepC3 donors, was enumerated and was then injected into eight male B6C3 recipients that had been previously divided into groups of two and conditioned with varying doses of radiation over the range 200 cGy to 500 cGy. In addition, two unirradiated mice and two mice given lethal doses of radiation (i.e.- 900 cGy) were injected with 5 x 10^6 donor cells as negative and positive controls respectively. An assessment of donor engraftment on the basis of FACS analysis of WBCs, was made on all mice 10 weeks and 6 months after the transplant. Immuno-staining was carried out as previously described. Overall engraftment levels were found to be linearly related to pre-transplant, sub-lethal radiation doses at both time points, demonstrating correlational coefficients of 0.99 and 0.88 at 10 weeks and 6 months respectively (Fig. 6). Levels of donor engraftment increased from 36.7% to 74.1% over the radiation dose range 200 cGy to 500 cGy, when analyzed 10 weeks after transplantation. Control mice receiving no pre-transplant irradiation showed average engraftment levels of 1.7% and mice preconditioned with 900 cGy averaged 89.7% at the same time point. Evidence of donor engraftment in sub-lethally irradiated recipients persisted and was stable over time up to 6 months post-transplant, as no significant differences were found between linear regressions or measurements obtained at the two different time points (Student's t-test).

Figure 6 - Average Engraftment Levels of Mice Conditioned with Varying Doses of Sub-lethal Radiation Prior to the Transplantation of 5 x 10^6 Cells.

Mice were analyzed 10 weeks (diamonds) and 6 months (boxes) post-transplant. Each datum is the average of two mice and errors bars represent the standard deviation from the mean. Least squares linear regression for each data set is shown along with correlational coefficients. In cases where a datum is not visible, it is hidden by overlapping data points. In cases where error bars are not apparent, they are smaller than the size of the marker.


90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
Proportion of Donor-derived WBCs in PB (%)
Radiation Dose (cGy)
R2 = 0.9889
R2 = 0.8787
2 Months
6 Months

The results of the present experiment indicated that a simple direct relationship exists between the amount of preparative, sub-lethal radiation given to prospective transplant recipients and the levels of long-term engraftment observed at later time points. Despite this conclusion, it seemed likely that the engraftment levels were related to more factors than just preparative radiation dose. While the results of previous studies had already identified cell dose as one such factor, the multi-dimensional interplay of radiation dose, cell dose and engraftment levels, was unexplained.

3.3 Defining the Relationship Between Radiation Doses, Cell Doses and Observed Levels of Engraftment

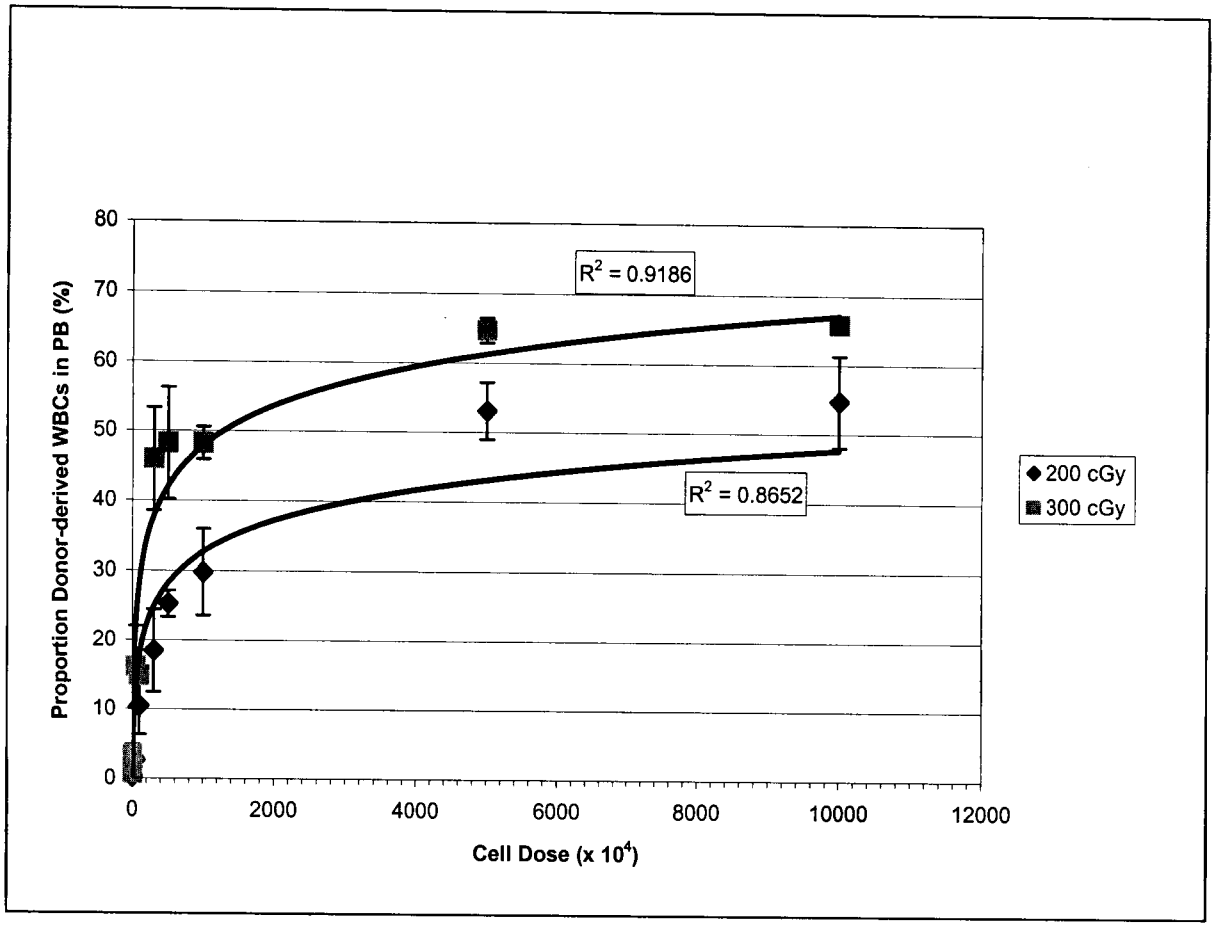
In addition to preparative radiation dose, the total number of nucleated cells (and thus the total number of HSCs) infused has been shown to be an important factor in determining the observed level of engraftment following BM transplantation (Stewart et al., 1998). The relationships between cell doses and engraftment levels and between pre-transplant radiation doses and engraftment levels are intuitive. Previous results detailed in this thesis have unequivocally shown that levels of true HSC engraftment increase linearly in direct proportion to preparative radiation doses up to some maximal level, after which the further escalation of radiation doses has a minimal effect. In addition, other studies have shown that increasing transplant doses in either unirradiated or minimally irradiated mice also has the effect of increasing engraftment levels up to some maximal level (Rao et al., 1997; Blomberg et al., 1998; Stewart et al., 1998). Despite the aforementioned studies, the three-way interplay between radiation doses, cell doses and engraftment levels has not been previously described. The elucidation of this interplay would likely shed light on the

mechanisms and factors that are important to HSC engraftment in the sub-lethally irradiated setting. Accordingly, two experiments were designed to explore the effect of increasing cell doses on engraftment levels, at varying levels of preparative radiation. Since the benefit of increasing cell dose on engraftment levels was known to be diminished at higher radiation doses (Malech & Mardiney, 1996), radiation doses of 200 cGy and 300 cGy were chosen for the exploration of cell dose effects. In planning these experiments, the intention was to evaluate cell dose effects over a range that would be limiting at one extreme and saturating at the other. As such, the cell dose range in each case was set to be 1×10^4 to 1×10^8 nucleated cells, representing 1 to 1×10^4 CRU (Szilvassy et al,1990).

In the first experiment, BM was harvested from 15 male donors and was then enumerated immediately prior to injection. Thirty-two prospective male transplant recipients that had been previously conditioned with 200 cGy of radiation, were then infused with the unfractionated BM over dose range 1×10^4 to 1×10^8 cells. Three mice were transplanted at each cell dose except 1×10^8, where there were only enough cells for two mice. The second experiment was essentially a reiteration of the first, except that all mice were given 300 cGy of preparative radiation instead of 200 cGy. Briefly, BM was harvested from 18 female donors, was enumerated and was then injected into 31 female recipients over the dose range 1×10^4 to 1×10^8 cells. Three mice were transplanted at each cell dose, with the exception of 1×10^8, where there were only enough cells to inject one mouse. In both experiments, mice were bled 2 months after transplant and engraftment levels were assessed by FACS analysis of WBCs as previously described (Fig. 7). Engraftment levels ranged from 0% to 59% for mice given 200 cGy and from 0% to 66% for mice given 300 cGy of preparative radiation and notable differences between the two groups were found at all

Figure 7 – Cell Dose Response of Donor Engraftment Levels in Mice Given Sub-lethal Doses of Preparative Radiation Prior to Transplantation.

Mice were given either 200 cGy (diamonds) or 300 cGy (boxes) of preparative radiation prior to transplantation. Engraftment levels were assessed on the basis WBC chimerism 2 months later. Each datum is the average of three mice except for those at cell doses of $10{,}000 \times 10^4$ where only two mice and one mouse were transplanted after 200 cGy and 300 cGy respectively. Error bars represent the standard deviation from the mean. In cases where a datum is not visible, it is hidden by overlapping data points. In cases where error bars are not apparent, they are smaller than the size of the marker. Logarithmic fits are shown.

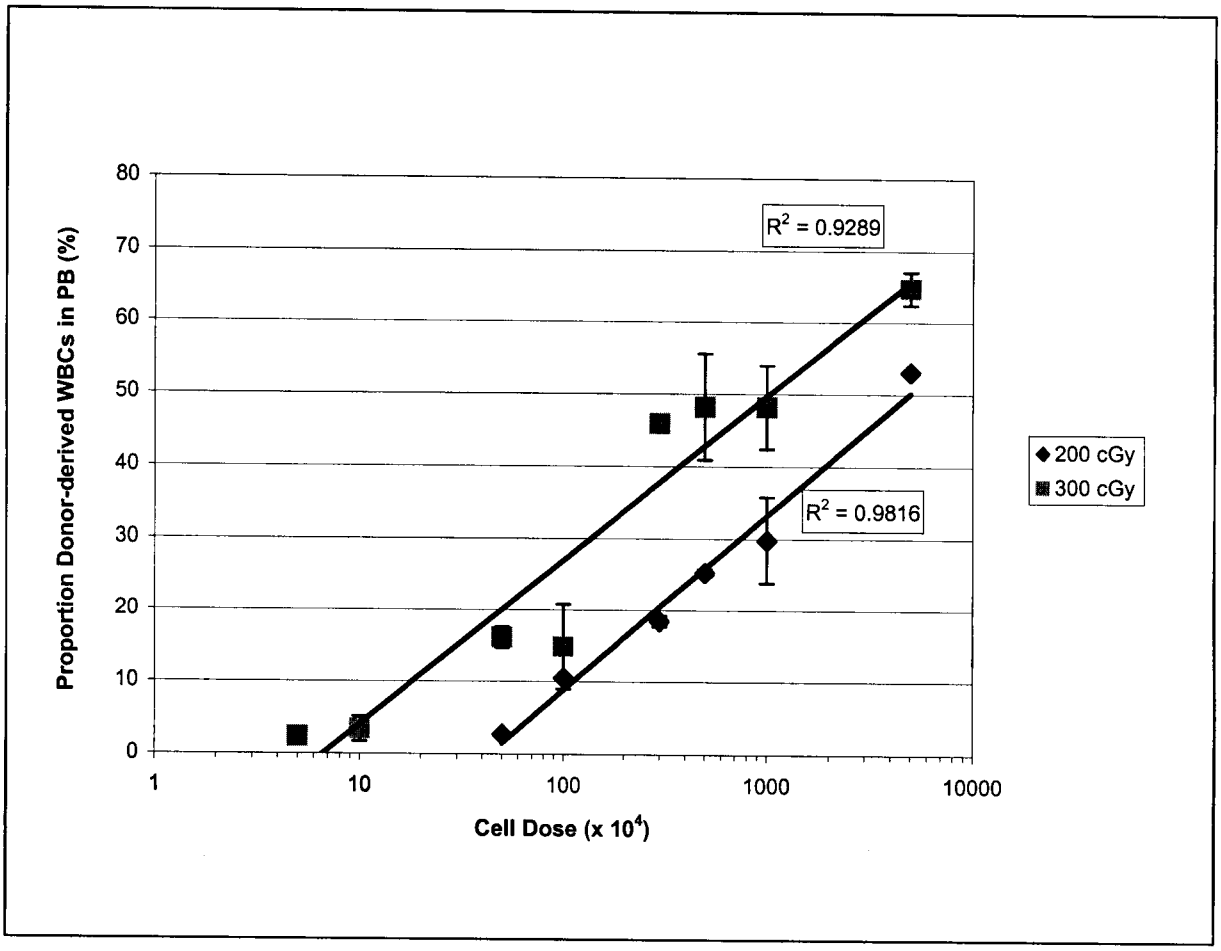

80
70
60
50
40
30
20
10
0
Proportion Donor-derived WBCs in PB (%)
R2 = 0.9186
R2 = 0.8652
200 cGy
300 cGy
0
2000
4000
6000
8000
10000
12000
Cell Dose (x 10^4)

cell doses above 300 x 10^4. From the shape of each curve, it is apparent that mice given 300 cGy of pre-transplant radiation achieved considerably higher levels of maximal engraftment, than mice given 200 cGy. In addition, mice that were given 300 cGy of preparative radiation demonstrated engraftment levels that were log-linearly related to cell doses that were 10-fold lower than that of mice given 200 cGy. Since the engraftment levels at 2 months post-transplant were found to be indicative of engraftment levels at 4 months post-transplant in the prior experiments, mice were only assessed at the earlier of the two time points.

Taken together, the results of the present study indicate that there exists an optimum cell dose for achieving high level engraftment in the sub-lethally irradiated setting, whose value is dependent upon the preparative radiation dose given. Increasing cell doses past this optimal level results in rapidly decreasing engraftment efficiency and in short, a waste of valuable stem cells. In addition, the results of the present study show that higher levels of engraftment may be achieved with the use of lower optimal cell doses, if the level of sub-lethal preparative radiation is increased. There also appears to be a preparative radiation-dependent threshold below which engraftment levels do not increase in proportion to transplanted cells doses. Therefore, in regards to the interplay of radiation doses, cell doses and engraftment levels, the conclusions drawn from the results of this experiment are that increasing pre-transplant radiation doses has the effect of increasing the maximal level at which engraftment levels plateau, decreasing the cell dose at which engraftment efficiencies

Figure 8 – Comparison of the Linearity of Cell Dose Response Curves of Donor Engraftment Levels Between Mice Given Either 200 cGy or 300 cGy of Preparative Radiation Prior to Transplantation.

Mice were given either 200 cGy (diamonds) or 300 cGy (boxes) of preparative radiation prior to transplantation. Engraftment levels were assessed 2 months later on the basis of WBC chimerism. Data points from mice transplanted with cell doses falling within the linear range of the curves in Figure 7 are shown plotted on a logarithmic abscissa. Each datum is the average of three mice and error bars represent the standard deviation from the mean. In cases where error bars are not apparent, they are smaller than the size of the marker.


80
70
60
50
40
30
20
10
0
Proportion Donor-derived WBCs in PB (%)
R^2 = 0.9289
R^2 = 0.9816
200 cGy
300 cGy
1
10
100
1000
10000
Cell Dose (x 10^4)

begin to taper-off and decreasing the threshold after which engraftment levels increase linearly with log cell doses.

3.4 An Evaluation of 5-Fluorouracil as a Means of Reducing Effective Cell Doses

The results of previous studies detailed in this thesis have shown that increasing preparative radiation doses is an effective way of reducing the cell doses needed to obtain potentially therapeutic levels of engraftment. However, the associated systemic toxicity and mutagenic nature of this treatment makes the unnecessary escalation of radiation-based conditioning regimens something to be avoided. Another approach that has been explored as a means of reducing effective cell doses in the unirradiated setting, is the use of the cytotoxic drug 5-FU (Stewart, 1993; Ramshaw, 1995; D'Hondt, 2001). The use of 5-FU as a means of enriching the BM for HSCs has become widely used, especially in the context of murine gene therapy protocols, where it is necessary to have a relatively large number of HSCs in a small total number of cells. There are two reasons for this requirement. First and foremost, is the relatively poor efficiency with which HSCs are infected by retroviruses. Despite improvements in viral pseudotyping which have allowed for the concentration of viral preparations to values in excess of 10^9 infectious units per milliliter, the requirement for high multiplicities of infection (i.e.- 100X) to transduce even the majority of HSCs necessitates that transplant doses remain small. The second reason for keeping transplant doses small, is the financial and logistical limitations of viral production. Even the production of viral concentrates on a small scale (<100 μL) consumes an enormous amount of material and human resources. Taking these two factors into account, simple arithmetic reveals that the efficient infection of more than 1 million to 2 million cells is prohibitive.

Therefore, it became obvious that a means of decreasing therapeutic cell doses in the absence of radiation dose escalation, would be advantageous. As such, an experiment was designed to evaluate the use of 5-FU in the context of sub-lethal BMTs.

Treatment of BM donors with 5-FU is known to decrease the cellularity of the BM by approximately 10-fold without affecting its repopulating potential in lethally irradiated mice (Lerner & Harrison, 1990). However, previous studies in unirradiated mice had shown that 5-FU treatment was detrimental to the engraftment potential of HSCs (Stewart et al, 1993; Ramshaw et al., 1995, D'Hondt et al., 2001). However, the magnitude of this defect had only been described on the basis of "whole femur equivalents", which fail to account for the HSC enriching effect of 5-FU treatment (Stewart et al., 1993). Therefore, an exploration of the use of 5-FU within the context of non-myeloablative transplants was deemed to be pertinent. As such, an experiment was designed to compare the engraftment potential of 5-FU and normal BM in sub-lethally irradiated recipients. Since previous studies had shown that the frequency of CRU within normal BM is 1 in 1 x 10^4 cells and that the frequency of CRU in 5-FU treated BM is about 1 in 3000 cells (Szilvassy & Cory, 1993), 5-FU treatment of donor mice was assumed to result in a 3.3-fold enrichment of engraftable HSC within the BM. Therefore, transplant doses of 1.5 x 10^6 and 5 x 10^6 nucleated cells were decided upon so that direct comparisons could be made between 5-FU treated and normal BM. It then follows that if 5-FU bone marrow was enriched 3.3-fold for engraftable HSCs, 1.5 x 10^6 5-FU treated BM cells would engraft equally as well as 5 x 10^6 normal BM cells. In addition, since it is also reasonable to predict that differences between 5-FU and normal BM might be obliterated at higher preparative radiation doses since fewer engraftable HSCs are needed to saturate available HSC niches, the experiment was reiterated at 200 cGy and at

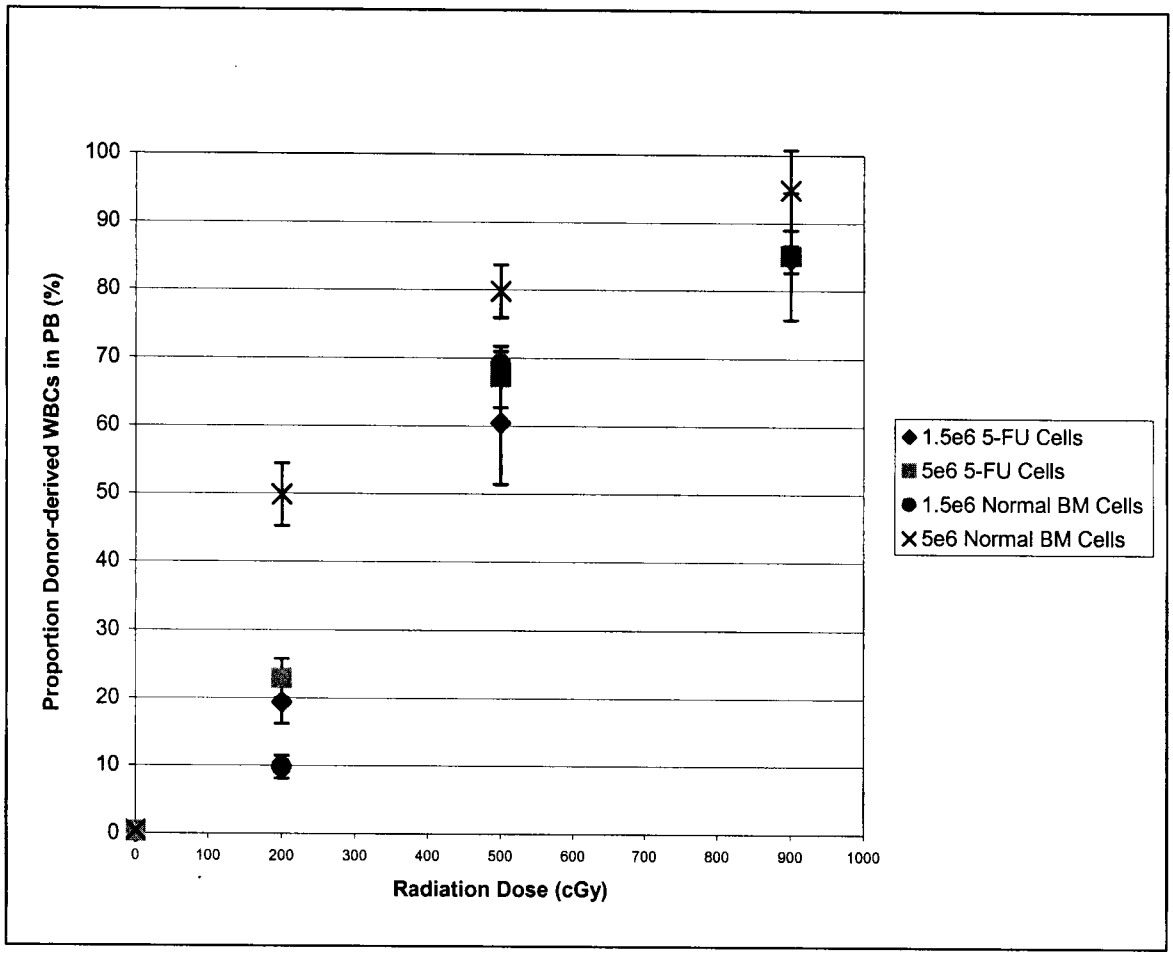
500 cGy. Engraftment levels were to be assessed on the basis of donor-derived WBC content in each of five lineages of the PB at 2 months and 4 months post-transplant.

Differences in the engraftability of transplanted HSCs may exist between strains of mice (Stewart, 1993; van Os, 1993; Ramshaw, 1995). In order to address this concern and at the same time make the results interpretable in the context of our transgenic mouse model of β-Thalassemia, the experiment was designed using Pep3b donors and B6 recipients instead of the previously used PepC3 and B6C3 strains. Like the PepC3/B6C3 model system, Pep3b and B6 strains of mice are congenic for the Ly5 WBC antigen. However, in this case both donors and recipients are homozygous. Nonetheless, since Pep3b WBCs carry only the Ly5.1 antigen and B6 carry only the Ly5.2 antigen, the presence of Ly-5.1 positive cells within recipients would be indicative of donor engraftment. In preparation for BM harvest, 16 Pep3b donor mice were treated with 5-FU. A second cohort of mice was injected with PBS. Four days later, 36 B6 mice that were to be infused with Pep3b BM were irradiated in preparation for transplants. Prospective recipient mice were divided into groups of 12 and were given 200 cGy, 500 cGy or 900 cGy of preparative radiation. Four hours later, BM was harvested from all donor mice and was pooled keeping 5-FU treated and normal BM separate. The BM suspensions from each cohort were then enumerated. An average of 50×10^6 nucleated cells were collected from each normal male mouse, while only 6.5×10^6 and 4.5×10^6 nucleated cells were collected from each 5-FU treated female and male mouse, respectively. Immediately following enumeration, the mice in each radiation group were divided into four sub-groups and were injected with 1.5×10^6 or 5×10^6 normal cells, or 1.5×10^6 or 5×10^6 5-FU treated cells. Three unirradiated controls in each of the four groups were also injected with donor cells. All mice were bled 2 months after

transplantation and donor engraftment was assessed on the basis of immuno-staining and FACS analysis of WBCs as previously described. While no significant differences in the levels of engraftment were evident between any unirradiated cohorts, sub-lethally irradiated cohorts of mice transplanted with 5 x 10^6 normal BM cells engrafted at significantly higher levels than those transplanted with 5 x 10^6 5-FU treated BM cells, whether given 200 cGy ($0.001<P<0.002$), or 500 cGy ($0.02<P<0.05$) of preparative radiation (Fig. 9). However, for mice given only 1.5 x 10^6 cells, 5-FU treated BM engrafted significantly better than normal BM in mice given 200 cGy ($0.01<P<0.02$) of preparative radiation and as well as normal BM in mice given either 500 cGy or 900 cGy of preparative radiation (Fig 9). For mice given sub-lethal doses of preparative radiation and transplanted with normal BM, those infused with 5 x 10^6 cells had significantly higher levels of engraftment than those infused with 1.5 x 10^6 cells whether given 200 cGy ($P<0.001$) or 500 cGy of preparative radiation ($0.01<P<0.02$). Mice given 900 cGy of preparative radiation prior to the infusion of normal BM showed no significant differences between mice given different cell doses. For mice given sub-lethal doses of preparative radiation, those given 500 cGy showed significantly higher levels of engraftment than those given 200 cGy, whether given 1.5 x 10^6 normal BM cells ($P<0.001$), 1.5 x 10^6 5-FU treated BM cells ($0.001<P<0.002$), 5 x 10^6 normal BM cells ($P<0.001$) or 5 x 10^6 5-FU treated BM cells ($P<0.001$). Differences between other cohorts that differed in more than one parameter were not assessed at this early time point. Despite these early findings, re-analysis of these mice 12 weeks later revealed that differences between mice infused with normal and 5-FU treated BM, were only apparent in the case of mice given 900 cGy and 5 x 10^6 cells ($0.02<P<0.05$), where mice given 5-FU treated BM cells engrafted at significantly lower levels than mice given normal cells (data not shown).

Figure 9 – Comparison of the Engraftability of BM Harvested From Normal Donor Mice or Mice that were Treated 4 Days Prior with 5-Fluorouracil.

Engraftment levels of mice were analyzed 2 months after preparative radiation and transplant of 1.5×10^6 normal cells (circles), 1.5×10^6 5-FU treated cells (diamonds), 5×10^6 normal cells (crosses) or 5×10^6 5-FU (boxes) treated cells. Unirradiated controls are also included. Each datum was obtained from the average engraftment level of three mice and error bars represent the standard deviation from the mean. In cases where a datum is not visible, it is hidden by overlapping data points. In cases where error bars are not apparent, they are smaller than the size of the marker.

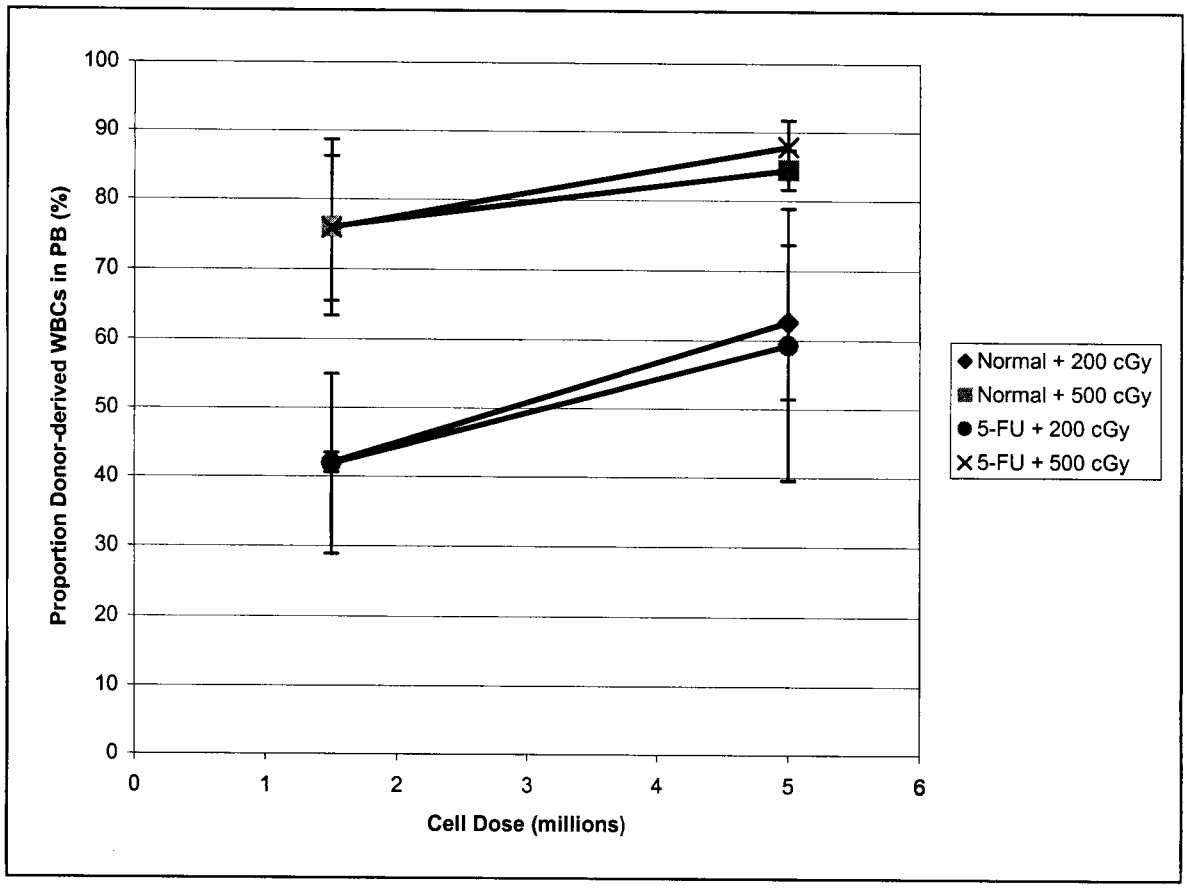

Proportion Donor-derived WBCs in PB (%)
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
700
800
900
1000
Radiation Dose (cGy)
1.5e6 5-FU Cells
5e6 5-FU Cells
1.5e6 Normal BM Cells
5e6 Normal BM Cells

For mice given sub-lethal doses of preparative radiation and a given cell dose, 500 cGy of preparative radiation resulted in notably higher levels of engraftment than 200 cGy (Fig. 10). Engraftment levels were found to be independent of cell doses at all preparative radiation doses for mice given either normal or 5-FU treated BM (data not shown for 900 cGy). In all cases, the overall level of engraftment was closely reflected by the proportion of donor-derived cells in each WBC lineage (data not shown).

The results of the present experiment suggest that the treatment of BM donors with 5-FU prior to BM harvest is not an effective means of decreasing potentially therapeutic cell doses, as the transplantation of equal numbers of 5-FU treated or normal BM cells results in near identical levels of engraftment at a given dose of preparative radiation. Apparently, the more than 3-fold enrichment of CRUs in 5-FU treated BM appears to be completely offset by an induced engraftment defect associated with the treatment. Despite these discouraging results, the finding that the transplantation of 1.5×10^6 cells resulted in relatively high level WBC chimerism, suggested that higher levels of engraftment may be obtained in the B6 recipients than in the previously used B6C3 recipients. The reason for this difference is uncertain, but could be related to differing degrees in the antigenicity of the Ly5 protein, leading to increased immune rejection of the BM grafts in B6C3 recipients as compared to B6 recipients. Alternately, HSCs in B6 mice may be more sensitive to the ionizing effects of preparative radiation.

Figure 10 – Effect of Cell Dose on Engraftment Levels in Mice Given Normal or 5-Fluorouracil Treated Cells.

Mice were given either 200 cGy (diamonds and circles) or 500 cGy (boxes and crosses) of preparative radiation prior to transplant of the indicated number of cells. Cells were harvested either from BM of normal mice or mice treated 4 days prior to harvest with 5-FU. Each datum was obtained from the average engraftment level of three mice 20 weeks after BM transplant and error bars represent the standard deviation from the mean. In cases where a datum is not visible, it is hidden by overlapping data points. In cases where error bars are not apparent, they are smaller than the size of the marker.


Proportion Donor-derived WBCs in PB (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Cell Dose (millions)
Normal + 200 cGy
Normal + 500 cGy
5-FU + 200 cGy
5-FU + 500 cGy

3.5 Curing beta-Thalassemia in Mice Using Sub-lethal Irradiation and Bone Marrow Transplant

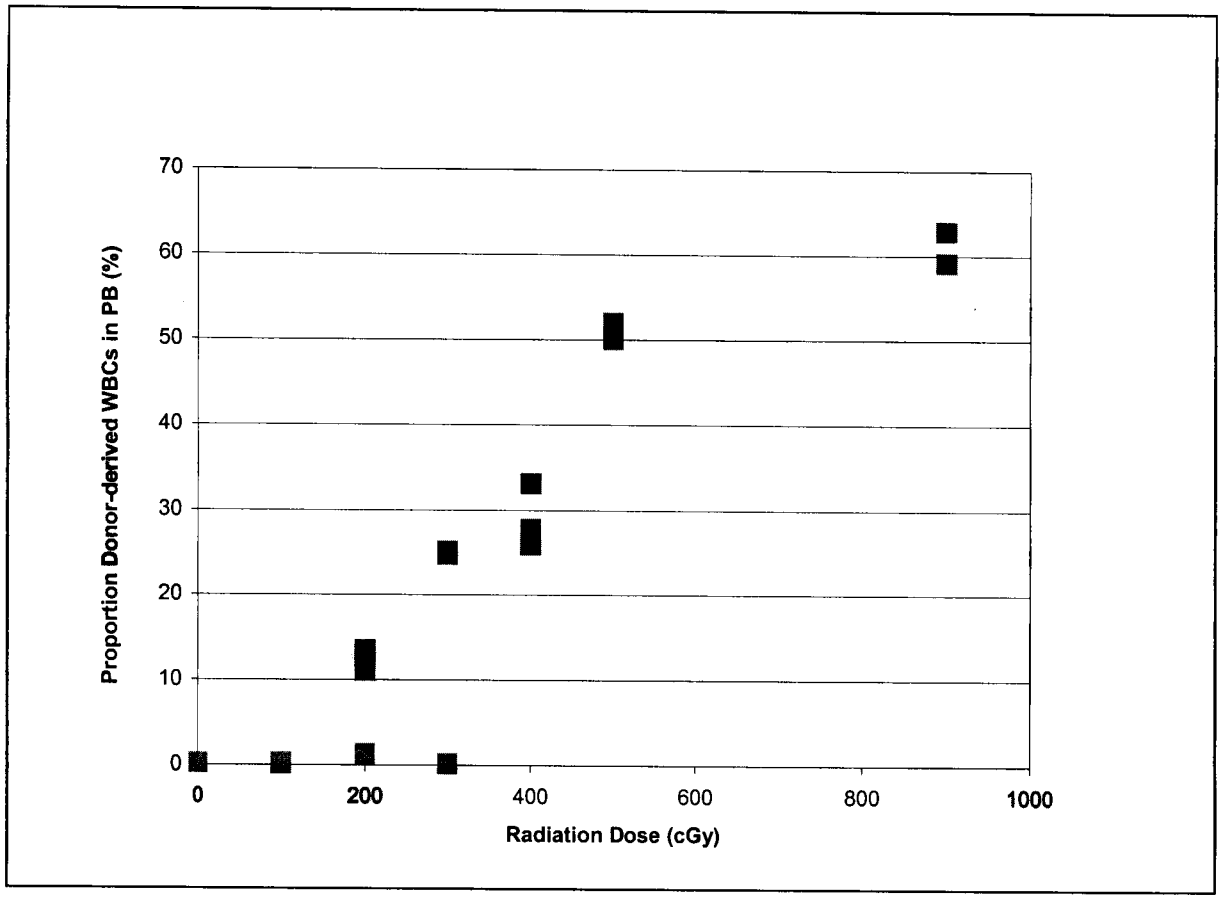
One of the main questions that exists in regards to the success of non-myeloablative BMTs in the context of β-Thalassemia concerns the level of chimerism that is necessary to alleviate the symptoms of the disease. Longitudinal studies of humans receiving BMTs for β-Thalassemia have shown that levels of donor chimerism as low as 25% can confer transfusion independency for up to 11 yrs, the longest time point analyzed (Andreani et al., 2000). These results have also been confirmed in mouse models of β-Thalassemia where indicators of β-Thalassemia were substantially improved with as little as 20% to 30% HSC chimerism (Persons et al., 2001). Since the previous experiments detailed in this thesis had already confirmed the attainability of these levels of engraftment under conditions of sub-lethal irradiation, it seemed reasonable to hypothesize that sub-lethal BMT would be curative in the context of our MDD mice. Mouse models of β-Thalassemia had recently been cured using gene therapy techniques and BMT, however this was achieved under conditions of lethal irradiation (May et al., 2000). Therefore, an experiment was planned in which MDD mice would be sub-lethally irradiated and transplanted with normal HSCs. Further, in order to make this approach applicable to gene therapy protocols, an attempt was made in planning the experiment to model this situation in a typical murine gene therapy trial. In recent years, retroviral vectors and in particular lentiviral vectors, have moved to the forefront of globin gene therapy research. In our hands, the transduction efficiency of retroviral vectors has been in the range of 60% to 100% (unpublished data). Therefore, 60% was taken as a conservative estimate of gene transfer efficiency in a typical experiment. In addition, it was assumed that gene transfer translated into therapeutic expression of the

globin transgene and that RBCs derived from transduced HSC would approximate the normal condition. As such, it was hoped that transplantation of a 60% to 40% mixture of genetically normal to thalassemic BM cells would model the situation encountered during a typical murine gene therapy trial.

In total, 22 thalassemic mice were selected for transplantation. Mice that looked unhealthy or that had visible sores were excluded from the study. In preparation for transplantation, mice were divided into groups of three and were irradiated over the dose range 0 cGy to 500 cGy, at intervals of 100 cGy. In addition, three mice were given 900 cGy of radiation as positive controls. In order to allow for the acquisition of WBC counts, the transplantation of irradiated mice was delayed for 3 days. WBC numbers in the PB were 1.2×10^7/mL, 3.6×10^6/mL and 0.9×10^6/mL for mice given 100 cGy, 200 cGy and 300 cGy of preparative radiation respectively, compared to 1.4×10^7/mL in control mice. WBC numbers for mice given 400 cGy, 500 cGy and 900 cGy were too low to count (i.e.- $< 0.9 \times 10^6$/mL). Immediately after obtaining WBC counts from perspective transplant recipients, BM was harvested from six congenic Pep3b and two syngeneic MDD donors. Bone marrow suspensions prepared from donor mice were then enumerated and Pep3b and MDD cells were mixed in a 60 to 40 ratio. Suspension mixtures were then homogenized by briefly vortexing and then 5×10^6 cells were injected into each MDD recipient. Two months following transplantation, the engraftment of donor cells into the transplanted MDD mice was assessed on the basis of WBC immuno-staining and FACS analysis (Fig. 11). The levels of engraftment observed in mice given 100 cGy to 500 cGy of preparative radiation ranged from 0% to 52%. Transplantation failed in two cases, with one mouse given

Figure 11 – Engraftment of Normal Donor Cells into MDD Thalassemic Recipients 2 Months After Transplantation.

Mice were given 100 cGy to 900 cGy of preparative radiation prior to the injection of 3×10^6 Pep3b and 2×10^6 MDD unfractionated BM cells in a single dose. Unirradiated controls are also included. Data points shown are for individual mice.


Proportion Donor-derived WBCs in PB (%)
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
Radiation Dose (cGy)

200 cGy of preparative radiation and one mouse given 300 cGy of preparative radiation showing no detectable engraftment. Unirradiated and lethally irradiated controls were engrafted at average levels of 0.2% and 61.6 % respectively. Since no selective advantage of normal over thalassemic cells is expected at the level of WBCs and since little or no endogenous competition is present in lethally irradiated recipients, the finding that the proportion of Ly-5.1$^+$ WBCs in the PB closely approximates the proportion of Ly-5.1$^+$ cells in the original inoculum is in keeping with what would be expected, and attests to the accuracy of the mixing procedure. However, the level of engraftment was found to be significantly, though minimally higher than the expected value 60% for two of the three transplant recipients ($P < 0.001$; Chi-Squared test).

3.5.1 Normalization of Blood Indices with Increasing Preparative Radiation Dose

One month later, at 3 months post-transplant, hematocrits for all sub-lethally irradiated MDD mice were determined. Values ranged from 29% in mice given 100 cGy of radiation, to 38% in mice given 500 cGy of radiation (Fig. 12). Unirradiated controls had average hematocrits of 32% and lethally irradiated controls had average hematocrits of 45%. Sex averaged hematocrit values for MDD and Pep3b controls were 31% and 48% respectively. Peripheral blood smears were also prepared from all recipients 3 months following transplantation. In order to determine the proportion of normal RBCs in each mouse, smears were scored by visual inspection (Table 3). For mice that received less than 200 cGy of preparative radiation prior to transplantation, blood smears were extremely pathologic with few if any normal cells present. For mice given more than 200 cGy of preparative radiation

Figure 12 – Hematocrits of MDD Mice 3 Months After the Transplantation of 3 x 10^6 Normal and 2 x 10^6 MDD BM Cells Following 100 cGy to 900 cGy of Preparative Radiation.

Unirradiated transplant recipients (0 cGy) and unmanipulated MDD and Normal controls are included. Each bar represents an individual mouse.

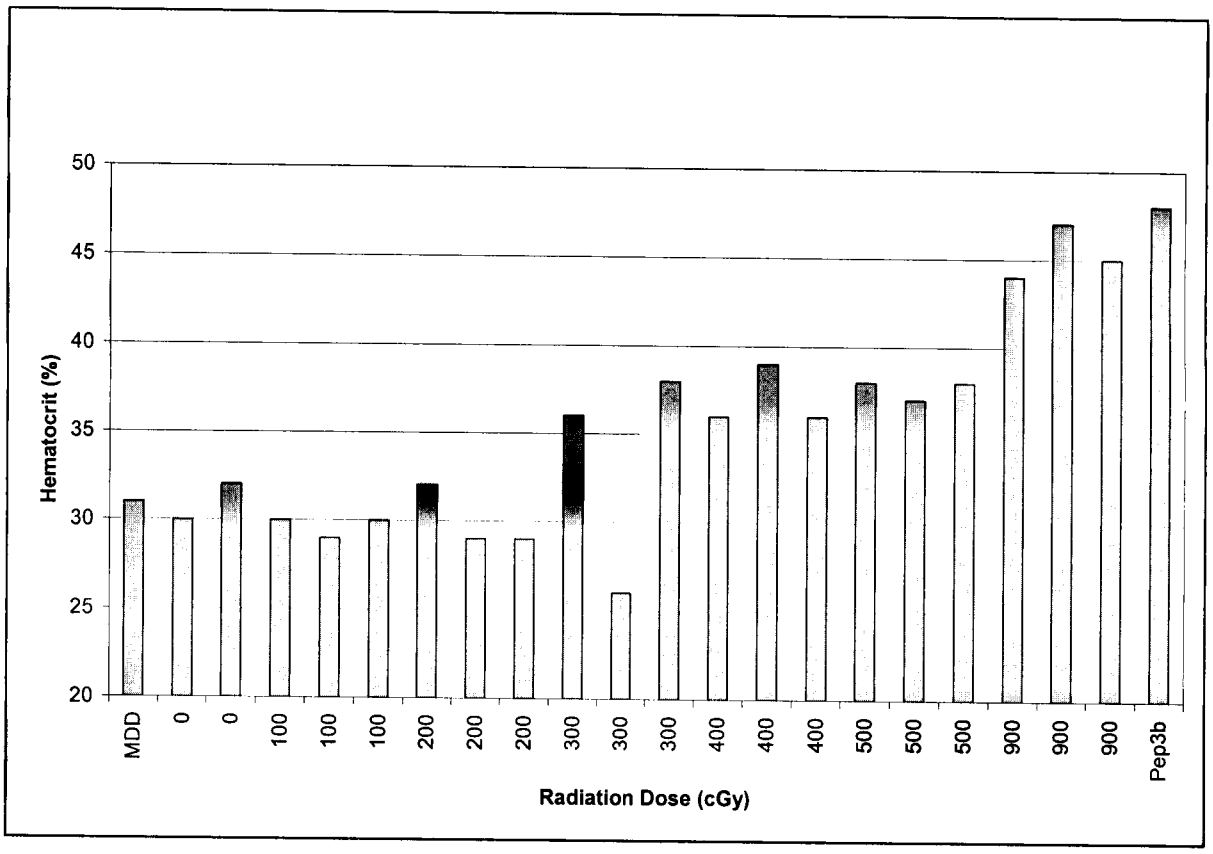

50
45
40
35
30
25
20
Hematocrit (%)
MDD
0
0
100
100
100
200
200
200
300
300
300
400
400
400
500
500
500
900
900
900
Pep3b
Radiation Dose (cGy)

Table 2 – Visual scoring results of blood smears prepared from MDD mice 3 months after the transplantation of 3 x 10^6 normal and 2 x 10^6 MDD BM cells following preparative radiation over the range 100 cGy to 900 cGy.

Unique ID	Proportion Normal RBCs (%)
MDD m#1	0
MDD m#2	0
100 cGy m#1	0
100 cGy m#2	0
100 cGy m#3	0
200 cGy m#1	15
200 cGy m#2	15
200 cGy m#3	0
300 cGy m#1	25
300 cGy m#2	0
300 cGy m#3	70
400 cGy m#1	60
400 cGy m#2	60
400 cGy m#3	70
500 cGy m#1	80
500 cGy m#2	60
500 cGy m#3	50
900 cGy m#1	75
900 cGy m#2	85
900 cGy m#3	95
Pep3b m#1	100
Pep 3b m#2	100

N.B. - Blood smears were scored by one individual on the basis of micrographs of modified Giemsa-Wright stained PB smears. All proportions are derived from a single count and are considered to be approximate.

prior to transplantation, the proportion of normal RBCs rapidly increased in going from 200 cGy to 300 cGy and then increased more slowly thereafter. Completely normal blood smears were not obtained even under conditions of lethal irradiation. Blood smears from unirradiated transplant recipients or recipients that were given 100 cGy of preparative radiation, did not look dissimilar to those of untreated MDD mice, with RBCs exhibiting marked disparity in size and shape, pallor, dark-staining inclusions and target cell morphology (Fig. 13). However, in transplant recipients given 200 cGy or more of preparative radiation, there is a progressive normalization in appearance, with the most apparent change between mice given 200 cGy and 300 cGy of preparative radiation. One blood smear from an MDD mouse given 300 cGy of radiation prior to transplantation, showed a large majority of RBCs that were uniform in size and shape, exhibited only a small central zone of pallor and were devoid of dark-staining inclusions. Blood indices for select MDD mice were also assessed 22 weeks after BMT (Table 4). In general there was a progressive normalization of indices for mice irradiated over the dose range 200 cGy to 500 cGy. However complete normality was approached only in those animals given lethal doses of radiation.

The present experiment definitively demonstrates the therapeutic potential of sub-lethal BMTs in thalassemic MDD mice. However, the magnitude of the therapeutic benefits seen, are directly related to the preparative dose of radiation given. A preparative radiation dose of 300 cGy appears to approach the lower limit of therapeutic potential as mice that are given 200 cGy or less show minimal improvement in disease pathology. Since mice given 200 cGy of radiation and BMT demonstrate significant levels of WBC chimerism, there

Figure 13 – Wright-Giemsa Stained Blood Smears from MDD Mice 3 Months After the Transplantation of 3 x 10^6 Normal and 2 x 10^6 MDD BM Cells Following Preparative Radiation Over the Range 100 cGy to 900 cGy.

Unirradiated and unmanipulated mice were included as controls. One blood smear from a representative mouse in each radiation group is shown. A) MDD; B) 0 cGy + BMT; C) 100 cGy + BMT; D) 200 cGy + BMT; E) 300 cGy + BMT; F) 400 cGy + BMT; G) 500 cGy + BMT; H) Normal.

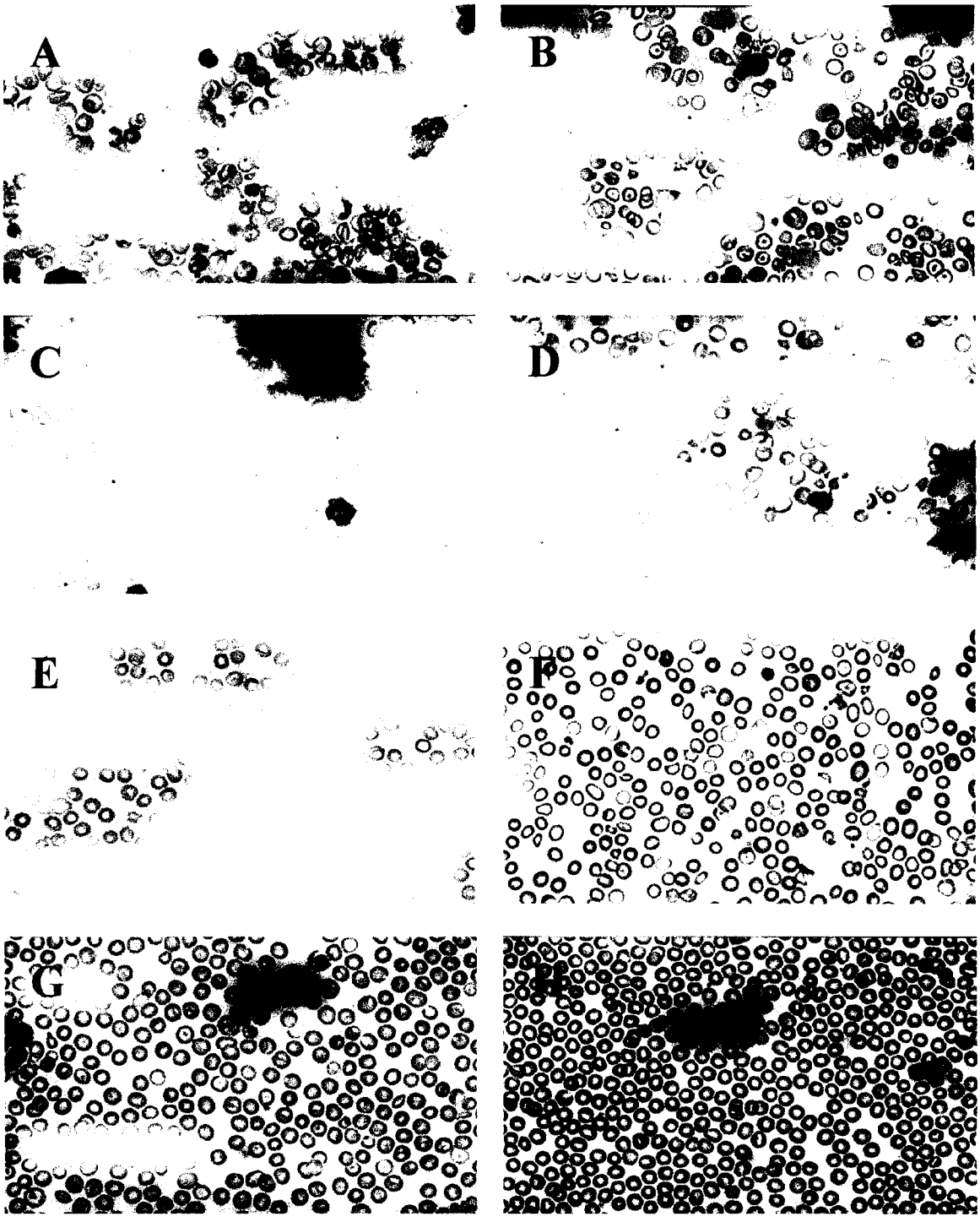

A
B
C
D
E
F
G

Table 3 – PB indices at 22 weeks post-transplant of MDD mice given sub-lethal preparative radiation and BMT.

Preparative Radiation Dose (cGy)	Unique ID	Blood Smear (% normal)	RBC (x 10^{12}/L)	HCt (%)	Hb (g/dL)	Reticulocytes (%)
None	m#1	too few to count	7.0	28.0	87	17
100	m#1	too few to count	6.0	25.0	71	23
200	m#1	too few to count	6.2	25.0	72	21
200	m#2	too few to count	7.0	29.0	86	22
200	m#3	too few to count	7.0	28.0	81	19
300	m#1	30	8.2	38.0	111	13
300	m#2	too few to count	5.4	22.0	62	16
500	m#1	90	9.3	44.3	134	6.1
500	m#2	60	8.6	43.0	128	5.3
500	m#3	50	8.0	39.0	122	6.1
900	m#2	95-99	10.6	49.0	152	2.5
900	m#3	95-99	9.7	44.5	138	2.5

N.B. - Mice were irradiated with 100 cGy - 500 cGy of radiation 3.5 days prior to receiving 3 x 10^6 normal and 2 x 10^6 MDD unfractionated BM cells by intravenous infusion. Mice were bled 22 weeks later and PB indices were determined. For logistical reasons only select mice were analyzed. In radiation groups with less than three samples, samples were unusable due to coagulation.

appears to be a threshold of RBC production below which the alleviation of disease symptoms does not occur.

CHAPTER 4 DISCUSSION

4.1 Summary of Findings

The results detailed previously demonstrate that BMT and true HSC engraftment is possible in a completely unconditioned murine host. About 10% of WBCs are donor-derived with demonstrable representation in all major lineages 6 months after the transplantation of two-thirds of a mouse equivalent of HSCs. These findings confirm the previous findings of others and solidify the notion that complete myeloablation is unnecessary for successful HSC engraftment. While the mechanism by which engraftment occurs in this context is unclear, it would appear that the creation of more available stem cells niches within the BM compartment prior to transplantation is not essential. This suggests that even under normal conditions, stem niches are available for engrafting HSC.

While preparative myeloablation is not an absolute requirement of HSC engraftment, the results described herein indicate that it significantly increases the observed levels of engraftment following BMT. Preparative radiation doses ranging from 200 cGy to 500 cGy were sufficient to obtain potentially therapeutic levels of long-term engraftment between 25% and 75%, despite transplant doses that were decreased by more than 40-fold. Irradiation of mice within this dose range was sub-lethal in all cases and caused only transient leukopenia. The overall effect was not strain specific as preparative radiation had an amplifying effect in both B6C3 and B6 murine recipients. However, B6 mice showed consistently higher levels of engraftment with levels between 49% and 87% for mice transplanted over the same radiation and transplant dose ranges. In addition, cell doses in this model system could be reduced by an additional factor of 3.3 without significantly

effecting observed engraftment levels at a given preparative radiation dose. This observation is difficult to explain as Pep3b cells that are homozygous for Ly-5.1 would be expected to elicit a stronger immune response in B6 Ly-5.2 homozygotes than heterozygous PepC3 cells in B6 Ly-5.2 homozygotes. Apparently there are other important factors at play in this situation.

In light of the previous results described here and by others, it is apparent that there is a relationship between increasing preparative radiation doses and the observed levels of engraftment. In addition, the previous studies of others have demonstrated a strong relationship between the transplant cell doses and the observed levels of engraftment. However, to my knowledge the three-way relationship between these factors has not been previously documented. The results of experiments detailed in this thesis demonstrate that there is a curvilinear relationship that exists between cell dose and observed levels of engraftment at a given level of preparative irradiation. This is to say that there is a minimum cell dose below which and a maximum cell dose above which engraftment levels do not change in linear proportion to log cell doses. The most probable explanation for the upper limit is a saturating effect of the transplanted cells that is likely spatial in nature. The lower limit is conceptually more difficult to explain and there are at least two explanations for this observation. The first pertains to the means by which the engraftment levels are determined and implies that there may be a lower limit of detection associated with immuno-staining techniques. However, given the single-cell basis of FACS analysis, this seems unlikely. The second possibility is that there exists a weak immunological barrier to the engraftment of congenic HSCs such as that alluded to previously in the PepC3/B6 model system. In this scenario, some minimal number of congenic HSCs is necessary to induce

immune tolerance of transplanted cells. Indeed, a recent study by van Os et al. has demonstrated that the Ly-5 markers used in these studies are mildly immunogenic under conditions where less than 200 HSCs are transplanted or where less than 400 cGy of preparative radiation is used (van Os et al., 2001). Lastly, increasing preparative radiation doses from 200 cGy to 300 cGy had a marked effect on both the position and limits of the curve. The overall result was an apparent decrease in the cell dose threshold and an increase in the maximal level of engraftment obtained, suggesting both a decrease in immunological barriers and endogenous HSC competition for stem cell niches. Indeed, there is evidence in the literature that supports the notion that immunological barriers may be overcome by increasing preparative conditioning (van Os et al., 1992).

In addition to preparative radiation of recipients, it is also theoretically possible to increase observed levels of engraftment by increasing the frequency of HSCs within the transplant inoculum. However, this approach has been unsuccessful in my hands and in the hands of others previously. The results described in the present thesis show that the use of 5-FU as means of enriching transplant inocula for HSCs is not beneficial. Apparently the approximate 3-fold enrichment of CRU frequency in BM treated 4 days previously with 5-FU is coincident with an approximate 3-fold decrease in the frequency of engraftable HSCs. The reasons for this observation are unclear, but previous studies have suggested that this phenomenon may be related to the induction of cell-cycle and a subsequent engraftment defect in HSCs treated with 5-FU. Nonetheless, this finding suggest that the use of 5-FU as a means of reducing transplant doses in the sub-lethally irradiated setting may be contraindicated. On the other hand, the use of 5-FU may still be beneficial in regards to retroviral infection protocols, which require active HSC cycling. However, based on the

previous observations of others, it seems likely that those HSCs that are induced to cycle are also those that demonstrate an engraftment defect.

Finally, the results of experiments described in this thesis demonstrate that therapeutic levels of engraftment can be obtained in MDD thalassemic mice with as little as 300 cGy of preparative radiation in combination with the transplantation of 3×10^6 normal cells. Under these conditions considerable improvements in blood smear morphology and indices were seen. Despite notable improvements, blood indices remained pathologic even in mice that received 500 cGy of preparative radiation and were not normalized until lethal doses of preparative radiation were administered. Similar findings were recently published by Bradley et al. using the $Hbb^{th-3/+}$ model system (Bradley et al, 2002). In addition to confirming the therapeutic potential of sub-lethal BMTs in the context of β-thalassemia that I am reporting, this study extends the findings presented herein, demonstrating that it is possible to obtain curative results with as little as 100 cGy of preparative radiation provided transplant doses are sufficiently large. Indeed, one-third of a mouse-equivalent of HSCs (i.e.- 100×10^6 cells) had to be transplanted to obtain normalization of blood profiles under these conditions. Similarly, it would appear that transplant doses approaching 20×10^6 and 10×10^6 cells are required for the normalization of blood profiles following 200 cGy and 300 cGy of preparative radiation respectively. It is therefore not surprising, that I achieved partial cures after transplanting 3×10^6 cells under these conditions.

4.2 The Enzymatic Theory of Hematopoietic Stem Cell Engraftment.

The relationship between preparative radiation doses, cell doses and observed levels of engraftment may be intuitively obvious and one would likely predict that engraftment levels would increase as either the cell dose or preparative radiation dose increased. Indeed this is

the case. However, predictions such as this do not give any indication as to what the mechanisms of engraftment might be. Here I have attempted to explain the results of my experiments exploring the interdependence of these three factors through a direct comparison to enzymatic catalysis.

The kinetics of enzymatic reactions are very well known and have been thoroughly described in the past. When a biological enzyme (E) comes into contact with its substrate (S) in an amenable environment, a reaction in which the substrate is converted to some product (P) takes place. Such reactions are typically described by the following equation:

$$E + S \leftrightarrow ES \rightarrow E + P \qquad \text{(Eq. 1)}$$

In this equation, ES represents the enzyme/substrate complex. In reactions such as these, the enzyme is neither a reactant nor product of the reaction, but is rather a catalyst that serves to lower the enthalpy of reaction. The rate at which such enzymatic catalysis reactions proceed depends on the concentration of substrate, the concentration of enzyme and the enzyme's affinity for the substrate. When the initial rate or velocity of a simple enzymatic catalysis is plotted as a function of substrate concentration, characteristic curvilinear plots are obtained. Such curves are described mathematically by the Michaelis-Menton Equation

$$\nu_o = V_{max}\,[S]/\,(K_m + [S]) \qquad \text{(Eq. 2)}$$

where ν_o is the initial reaction velocity, V_{max} is the empirically determined maximal reaction

velocity, [S] is the substrate concentration at time 0, and K_m is a constant that is dependent upon the affinity of an enzyme for a given substrate and which is determined empirically from the substrate concentration when the reaction velocity is ½ V_{max}. Such relationships are not uncommon in biological systems and can be found in the context of pharmacological studies of therapeutic efficacy as a function of plasma drug concentration and in the kinetics of membrane transport systems.

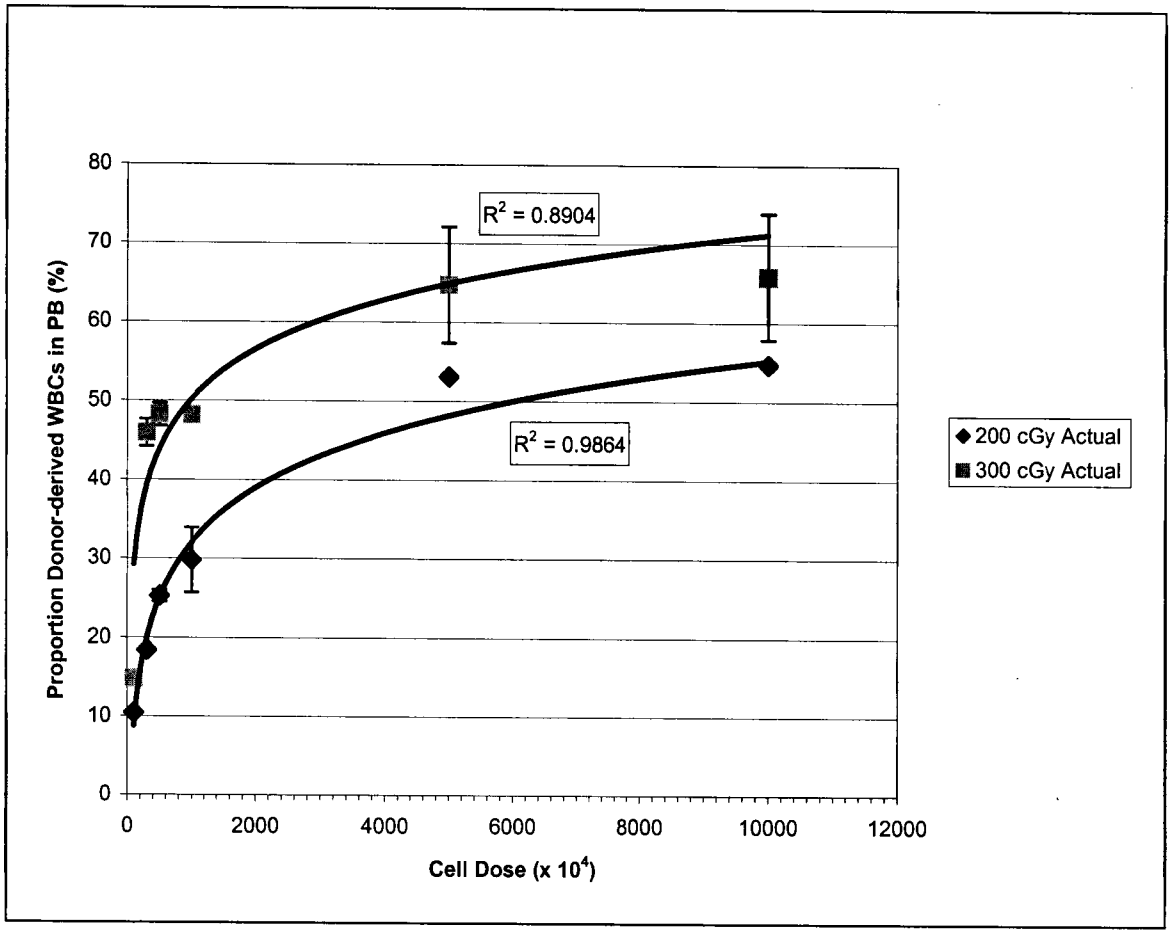
The results of the experiments assessing the relationship between preparative radiation doses, cell doses and observed levels of engraftment bare a striking resemblance to curves obtained in studies of enzymatic kinetics. As such, an attempt was made to model this system using the Michaelis-Menton Equation. Equations describing the observed level of engraftment as a function of cell dose have the form

$$E_{ob} = E_{max} \times D_{HSC} / (D_{50} + D_{HSC}) \qquad \text{(Eq. 3)}$$

where E_{ob} is the observed level of engraftment, E_{max} is the maximal level of engraftment in a given experiment, D_{HSC} is the number of HSC transplanted and D_{50} is the HSC dose at which ½ E_{max} is obtained. Actual data for these experiments are shown below, along with calculated curves based on the above equation (Fig.14). Values for D_{50} were 600 cells and 200 cells for mice given 200 cGy or 300 cGy of preparative radiation respectively. It is apparent from a comparison of empirical and calculated curves, that Equation 3 closely predicts the effects of increasing cell doses on observed levels of engraftment at a given level of preparative radiation. In light of the marked similarities between simple enzymatic kinetic theory and

Figure 14 – Comparison Between Actual Cell Dose Response Curves and Curves Predicted on the Basis of the Enzymatic Model of HSC Engraftment.

Mice were given either 200 cGy (diamonds) or 300 cGy (boxes) of preparative radiation prior to transplantation. Engraftment levels were assessed on the basis WBC chimerism 2 months later. Each datum is the average of three mice except for those at cell doses of $10{,}000 \times 10^4$ where only two mice and one mouse were transplanted after 200 cGy and 300 cGy respectively. Error bars represent the standard deviation from the mean. In cases where a datum is not visible, it is hidden by overlapping data points. In cases where error bars are not apparent, they are smaller than the size of the marker. Also shown are the curves calculated from Equation 3.


80
70
60
50
40
30
20
10
0
0
2000
4000
6000
8000
10000
12000
Proportion Donor-derived WBCs in PB (%)
Cell Dose (x 10^4)
R^2 = 0.8904
R^2 = 0.9864
200 cGy Actual
300 cGy Actual

the results of the present experiment, I propose here a model of HSC engraftment and subsequent hematopoiesis that is based on known mechanisms of enzyme catalysis. As such, I have called this model "The Enzymatic Model of HSC Engraftment".

The long-term engraftment of transplanted BM requires that HSCs "home" to the recipient BM and take up residence there. One long-standing theory is that there are "stem cell niches" within the BM that are capable of supporting transplanted HSC. It is from these niches, that lymphomyelo-reconstitution is thought to be expedited. One of the central dogmas of hematopoiesis is that it is facilitated by totipotent cells that reside within stem cell niches of the BM. These cells are considered to be capable of self-renewal, clonal expansion and multi-lineage differentiation and are thought to produce all of the types of cells normally found in the blood. A fitting analogy of HSC function, is that of a "blood cell factory". Such an analogy allows for an essentially inexhaustible source of all blood cells throughout life. However, in order to understand the "enzymatic model" proposed here, it is necessary to replace this conventional understanding of stem niches and HSC function with more abstract concepts. If instead of viewing the HSC as "blood cell factory", we view it as the *machinery employed within* a blood cell factory, it is possible to envision the *stem cell niche* as the blood cell factory in itself. Such a view allows us to impart several characteristics to the stem cell niche. Firstly, it becomes obvious that the stem cell niche is an essentially inexhaustible source of blood cells assuming that the necessary machinery (HSC) is present. Implicit in this characteristic is the assumption that neither the stem cell niche nor the HSC is consumed in the process. Secondly, it can be assumed that any given stem cell niche and associated HSC is capable of a finite rate of blood cell production and that the stem cell niche pool as a whole, is therefore also limited. This implies that the

utilization of more stem cell niches and associated HSCs would result in the production of a larger number of blood cells in a given period of time. Finally, given the above it becomes apparent that if there are more HSCs than stem cell niches, excess HSCs will be "unemployed" and the number of available niches will limit the rate of production of blood cells. Therefore, as illustrated by the foregoing characteristics it does not seem unreasonable to compare the function of a stem cell niche to that of an enzyme. Both facilitate the expeditious production of biological products, both are undiminished in the process, both have a finite rate at which products can be made and both can be saturated, enzymes by an excess of substrate and stem cell niches by an excess of HSCs. In addition, in keeping with this analogy it can then be inferred that preparative radiation has the effect of increasing the affinity of the stem cell niches for transplanted HSCs. We know from previous studies that stem niches are available even under normal conditions. However, as supported by the need for mega-doses of HSCs in this setting, there is likely a high level of competition for a limited number of niches and a subsequently low ability of transplanted HSC to engraft in the absence of conditioning. Therefore, just as a shortage of factory workers would drive up demand, the mechanism underlying radiation-induced increases in HSC engraftment could very well be a decrease in the endogenous competition for stem cell niches and the job of blood cell production. In any event, preparative radiation can be viewed as increasing the affinity of endogenous stem cell niches for transplanted HSCs. As predicted by Equation 3 and as evidenced in Figure 14, increasing preparative radiation would then cause a left- shift in the cell dose response curve as a result of a increased propensity of transplanted cells to engraft. In addition, a concomitant increase the maximal level of engraftment as a result of higher overall rates of blood cell production, would also be observed. Therefore, not only

does the present theory accurately describe the phenomenon of HSC engraftment in the sub-lethally irradiated setting, but it also predicts the effects of perturbations in one or more of the governing dynamics. While it may not be without flaws, I believe the above described Enzymatic Theory of HSC Engraftment to be a potentially useful tool in the planning of future sub-lethal BMT experiments.

4.3 Conclusions and Future Directions

One of the major limitations to sub-lethal BMTs in the context of globin gene therapy has been the relatively large number of HSCs required to obtain therapeutic levels of engraftment. As such, the work detailed in this thesis was focused on defining the conditions under which sub-lethal BMTs could be therapeutic in the context of hemoglobinopathies and in particular β-Thalassemia. It would appear that at least 300 cGy of preparative radiation in combination with the transplantation of at least 300 corrected HSCs will be necessary to obtain curative results. In theory, this number of HSCs could represent as few as 300 cells, however current enrichment procedures are not capable of producing this level of HSC purity. In addition, previous attempts to purify HSC to even a small extent have been plagued by apparent losses in the engraftability of recovered cells. More recently, advances have been made in HSC *in vitro* expansion techniques that may prove useful in the future (Antonchuk et al., 2002). Using retrovirally encoded HOXB4, Antonchuk et al., have demonstrated a 40-fold expansion in CRU. Assuming no loss in engraftment potential, such a 40-fold expansion of HSCs would translate into at therapeutic cell dose of 75,000 cells in the context of sub-lethal BMTs. This is a number well within the reach of current gene therapy protocols, even with multiplicities of infection approaching

100. However, until such time as these studies have been completed, the retention of engraftment potential in HOXB4 expanded HSCs remains in question. In the meantime, others have already begun to explore ways of selectively expanding HSCs *in vivo* using drug resistance genes. One particularly successful approach has employed the use of a retrovirally encoded mutant gene known as O6-Methylguaninine DNA-methyl transferase (ΔMGMT). In combination with the cytotoxic drugs 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and O_6-benzylguanine (BG), the group of Gerson has demonstrated *in vivo* expansion of as few as 40,000 transduced cells in non-myeloablated recipients, resulting in levels of donor-derived CFU chimerism as high as 47% (Davis et al., 2000). By increasing the transplant dose to 250,000 cells they were able to achieve levels of chimerism exceeding 85%. Based on the results presented in this thesis, such high levels of chimerism would be undoubtedly therapeutic in the context of β-thalassemia. The initiation of experiments using either of the foregoing approaches is the next logical step in the progression towards the goal of curative sub-lethal BMTs for β-thalassemia and hemoglobinopathies in general. It is my hope that the work described in this thesis has helped to bring this goal closer to fruition.

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