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Development of Cellular and Gene Therapies for β-Thalassemia and Sickle Cell Disease

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Development of Cellular and Gene Therapies for β-Thalassemia and Sickle Cell Disease

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RÉSUMÉ

La β-thalassémie et la drépanocytose sont deux maladies hématopoïétiques qui résultent d'anomalies affectant la chaine β-globine de l'hémoglobine (Hb). L' Hb est la molécule qui transporte l'oxygène et c'est normalement un tétramère formé de deux chaines d'α-globine et deux chaine de β-globine. La β-thalassémie est caractérisée par un débalancement entre les chaines de α- et β-globine dû à une absence ou diminution du taux de la β-globine résultant en une anémie. La drépanocytose est causée par une β-globine anormale qui entraine la formation de globules rouges anormaux allongés, appelés drépanocytes. Ceci résulte en une anémie, ainsi que des vaso-occlusions aboutissant à des complications ischémiques dans tous les organes. Les deux maladies sont associées à une morbidité et une mortalité élevée. La transplantation de moelle osseuse (m.o.) est un traitement potentiellement curatif. La transplantation allogénique est disponible pour une minorité de patients qui auront des donneurs histocompatibles, et laisse les receveurs vulnérables à la réaction de greffon contre l`hote. La transplantation autologue devrait en principe permettre d`éviter ces complications. D'autre part, des cas où des patients receveurs de transplantation de m.o. ne dépendaient plus de transfusions même avec seulement un chimérisme partiel, suggèrent qu`un remplacement total de la m.o. ne semble pas nécessaire pour avoir un effet thérapeutique. De plus, des études cellulaires chez des modèles murins suggèrent que les anomalies de la lignée érythroide thalassémique et drépanocytaire pourront conférer un avantage sélectif pour les cellules érythroides normales suite à une transplantation de m.o. Ce qui précède suggère qu'une correction de ces deux maladies pourrait être possible avec un remplacement partiel de la m.o., et indique qu' il y a un besoin important de développer des stratégies de transplantation de m.o. appropriées pour établir un chimérisme stable et efficace.

Cette thèse porte principalement sur le développement de conditions optimales de thérapies cellulaires pour la β-thalassémie et la drépanocytose par transplantation de m.o., avec des pré-requis minimals. En utilisant des modèles murins de la β-thalassémie *intermedia* et de la drépanocytose, j'ai d'abbord déterminé le pourcentage minimal de cellules de m.o. normales nécessaire pour avoir une correction du phénotype à long terme, en générant des séries de chimères hématologiques. Mes résultats ont établis une

intervalle de 19-24% (β-thalassémie) et 26-31% (drépanocytose) de cellules de m.o. normales comme étant le niveau thérapeutique corrigeant les différents aspects des deux maladies, et la durée de vie des souris est devenue normale. Ces résultats ont démontré qu'un remplacement partiel de m.o. malade peut corriger les phénotypes thalassémiques et drépanocytaires. Conséquemment, mon étape suivante a été de déterminer le niveau minimal de cellules et de myélosuppression, ainsi que les stratégies optimales de transplantation, qui permettront d'atteindre ce niveau thérapeutique chez des receveurs malades. Pour cela, j'ai effectué des transplantations de m.o. sur des souris thalassémiques et drépanocytaires après une myélosuppression partielle en modulant cinq paramètres. J'ai établi les conditions de transplantations qui permettent à 100% des souris transplantées d'avoir une prise de greffon et des niveaux thérapeutiques de chimérisme après une dose d'irradiation de 1Gy ou 2Gy, à condition que suffisamment de cellules soient injectées au temps approprié après l' irradiation. J'ai aussi déterminé qu'une transplantation de m.o. sans aucune myélosuppression peut permettre jusqu' à 100% des souris transplantées d'avoir une prise de greffon. Finalement, j'ai effectué une évaluation *in vivo* d'un nouvel vecteur lentiviral portant un gène de γ-globine humaine et démontré une expression efficace et long-terme du transgène après transduction des cellules souches hématopoïétiques avec une faible quantité de particules virales.

En conclusion, j'ai déterminé le pourcentage minimal de cellules de m.o. nécessaire pour corriger significativement le phénotype murin de la β-thalassémie *intermedia* et de la drépanocytose à long terme. J'ai aussi optimisé les stratégies de transplantation de m.o. et d'une manière reproductible achevé un remplacement partiel thérapeutique de la m.o. de souris malades après un niveau de myélosuppression modérée des receveurs. De plus, j'ai démontré l'efficacité *in vivo* d'un nouvel vecteur lentiviral portant le gène de γ-globine humaine. Mes études constituent une base pour developper des transplantations de m.o. chez l'humain afin de traiter la β-thalassémie et la drépanocytose avec des pré-requis minimals de cellules et de myelosuppression.

Mots clés: β-thalassémie, drépanocytose, érythropoïèse, hémoglobine, globule rouge, transplantation de moelle osseuse, chimère, thérapie cellulaire, thérapie génique.

SUMMARY

β-thalassemia and sickle cell disease (SCD) are two hematopoietic disorders that result from anomalies affecting the adult β-globin chain of hemoglobin. Hemoglobin is a tetramer that transports oxygen and is constituted of two α-globins and two β-globins chains. β-thalassemia is characterized by an imbalance between α- and β-globin chains due to decreased levels or absence of β-globin resulting in anemia. SCD is due to the production of an abnormal β-globin leading to the formation of abnormal elongated red blood cells (RBC) called sickled RBCs. This results in anemia as well as vaso-occlusions leading to ischemic damage of all organs. Both diseases are associated with high morbidity and mortality.

Bone marrow transplantation (BMT) (stem cell transfer) is a potentially curative treatment. However, allogenic BMT which leaves the recipients vulnerable to graft versus host disease is available to only a minority of patients with histocompatible donors. Autologous BMT with gene therapy could in principle prevent these complications and expand the range of patients who could benefit from a BMT. On the other hand, while complete bone marrow (BM) replacement was previously considered necessary to overcome the disease phenotype, cases of occasional transfusion-independent patients with mixed chimerism after BMT suggest otherwise. Furthermore, strong myeloablative pre-conditioning regimens by irradiation and chemotherapy would not be required for achieving mixed chimerism, thus sparring multiple side effects. In addition, cellular studies in mouse models have suggested that the anomalies of the erythroid cells in β-thalassemia and SCD would confer a selective advantage for normal erythroid cells after BMT. What preceded implies that a correction of both diseases would be possible by a partial replacement of the recipient's BM, and highlights the need to develop appropriate BMT strategies that allow establishing stable and efficient chimerism.

This thesis describes mainly the establishment of optimal conditions of cellular therapy for β-thalassemia and SCD by BMT, with minimal requirements of cells and

myelosuppression. Using mouse models of β-thalassemia *intermedia* and SCD, I first determined the minimal percentage of normal bone marrow (BM) cells necessary for long term phenotype correction of both diseases by generating series of hematopoietic chimeric mice. My results established the range of 19-24% (β-thalassemia) and 26-31% (SCD) normal BM cells as the therapeutic threshold for correction of these diseases, and mice lived to a normal lifespan. These results demonstrated that a partial replacement of the diseased BM is sufficient to correct the thalassemic and SCD phenotypes. Therefore, my second step was to determine the minimal level of myelosuppression and cellular requirements, as well as the optimal transplantation strategies, which could allow reaching that therapeutic threshold. To this end, I performed BMTs on sickle and thalassemic mice after minimal myelosuppression by modulating five transplantation-related parameters. I established transplantation conditions allowing 100% of recipients to engraft with therapeutic levels of chimerism after an irradiation dose of 1Gy or 2Gy, provided appropriate number of cells and timing of infusion after conditioning. I also determined that BMTs without prior myelosuppression allows up to 100% of recipient mice to engraft. Finally I performed an *in vivo* evaluation of a novel lentiviral vector carrying the human anti-sickling γ-globin gene and demonstrated long term efficient expression of the transgene after transduction of hematopoietic stem cells with low load of infectious units.

In conclusion, I determined the minimal percentage of normal BM cells that can significantly correct murine β-thalassemia *intermedia* and SCD for long term. I have also optimized transplantation strategies and reproducibly achieved therapeutic partial replacement of diseased murine BM after minimal myelosuppression of recipients. Furthermore, I demonstrated the *in vivo* efficiency of a novel globin lentiviral vector. My studies constitute the basis for transplantation therapies in human β-thalassemia and SCD with minimal conditioning and cellular requirements.

Keywords: β-thalassemia, sickle cell disease (SCD), erythropoiesis, hemoglobin, red blood cell, bone marrow transplantation, chimera, cellular therapy, gene therapy.

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Appendix VI. Production of chimeric mice by CRA

List of Abbreviations

- % Hypo : Percentage of hypochromic cells
-% Retic : Percentage of reticulocytes
-& : And
-± : More or less
-↑ : Increase
-↓ : Decrease
-AHSP: Alpha hemoglobin stabilizing protein
-BM : Bone marrow
-BMT : Bone marrow transplantation
-bp : Base pair
-CA : Capsid protein
-CFU-S : Colony forming unit in the spleen
-cHS4 : Chicken hypersensitive site 4
-CRA : Competitive Repopulation Assay
-dl : Deciliter
-DNA : Deoxyribonucleic acid
-ESC : Epithelial stem cell
-Elts : Elements
-Fe++ : Ferrous iron
-fl : femtoliter (10^{-15} liter)
-Fibn : Fibronectin
-g : Gram
-G-CSF : Granulocyte colony stimulating factor
-Gln : Glutamine
-Glu : Glutamic acid
-GVHD : Graft versus host disease
-Gy : Gray
-Hb : Hemoglobin
-HbA : Adult hemoglobin (major form)
-HbA2 : Adult hemoglobin (minor form)
-HbC : Hemoglobin C
-HbF : Fetal hemoglobin
-HbS : Hemoglobin S
-HCT : Hematocrit
-HDW : Hemoglobin distribution width
-Hemi-βthal : Hemizygous β-thalassemic
-HG : Human genomic
-HIV : Human immunodeficiency virus
-HLA : Human leukocyte antigen
-HS : Hypersensitive
-HSC : Hematopoietic stem cell
-HSCT: Hematopoietic stem cell transplantation
-HSPC: Hematopoietic stem and progenitor cell
-HTLV: Human T-cell leukemia virus

List of Abbreviations...continued

-Hyper % : Percentage of hyperchromic cells
-IBM : Intra-bone marrow
-IL : Interleukin
-IN : Integrase
-Inc : Incubation
-Ins : Insulin
-ISC : Irriversibly sickled cell
-IV : Intravenous / intravenously
-K+ : Potassium cation
-kb : Kilobase
-kd : Kilodalton
-LCR : Locus control region
-Lin- : Lineage negative / negative for lineage markers
-LTR : Long terminal repeat
-Lys : Lysine
-M:E : Myeloid-to-erythroid ratio
-MA : Matrix protein
-MCH : Mean corpuscular hemoglobin
-MCHC : Mean corpuscular hemoglobin concentration
-MCV : Mean corpuscular volume
-MHC-I : Major histo-compatibility antigen type I
-MLV : Murine leukemia virus
-MMTV : Moloney murine leukemia virus
-MOI : Multiplicity of infection
-MSC : Mesenchymal stem cell
-N:C : Nuclear-to-cytoplasmic ratio
-N/A : Not applicable
-Na+ : Sodium cation
-NaPy : Sodium pyruvate
-NC : Nucleocapsid
-ND : Not determined
-Nef : Negative regulator
-N.I. : Not indicated
-NK cells : Natural killer cells
-NLS : Nuclear localization signal
-PB : Peripheral blood
-PCR : Polymerase chain reaction
-PE : Phosphatidyl ethanolamine
-pg : Picogram
-Pr. : Protein
-PR : Protease
-Prot : Protamine
-Pre : Preculture
-PS : Phosphatidyl serine

List of Abbreviations...continued

-RBC : Red blood cell
-RDW : Red cell distribution width
-RES : Reticular endothelial system
-Ret : Retronectin
-Rev : Regulator of expression of viral proteins
-Ref : References
-RNA : Ribonucleic acid
-RRE : Rev response element
-RT : Reverse transcriptase
-SCA : Sickle cell anemia
-SCD : Sickle cell disease
-SCF : Stem cell factor
-SIN : Self-inactivating
-SpG : Splenomegaly
-T cells : T lymphocytes
-Transf : Transferrin
-Tat : Transcription activator
-Tg : Transgene
-USA : United States of America
-Vif : Viral infectivity
-Vpr : Viral protein R
-Vpu : Viral protein U
-WBC : White blood cell
-wt : Wild-type
-YS : Yolk sac
-α : Alpha
-β : Beta
-β^{maj} : β-globin major
-β^{min} : β-globin minor
-β-ME : β-mercapto-ethanol
-γ : Gamma
-δ : Delta
-ε : Epsilon
-ζ : Zetta
-μm : Micrometer

To my parents....

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CHAPTER I

INTRODUCTION

I.1 Hematopoiesis and the hematopoietic system

Hematopoiesis represents the dynamic processes of blood cell production and development (Baudurant & Khoury, 1998) through a complex network of tissues, organs, stem cells and regulatory factors. (Perkins, 1998). Hematopoiesis proceeds in a hierarchial fashion where an immature undifferentiated pluripotent cell called the hematopoietic stem cell (HSC), under the influence of various cytokines, leads to the progressive formation of hematopoietic cells of restricted potential known as precursors, each committed to give one type of blood lineage (Bell, 2002) (**Figure 1**). The normal hematopoietic system continuously maintains a cell population of erythrocytes or red blood cells (RBCs) that transport oxygen, leukocytes or white blood cells (WBCs) that perform immune functions, and platelets (or thrombocytes) that play a role in hemostasis. (Baudurant & Khoury, 1998). The hematopoietic system of vertebrates, derived from the mesodermal germ layer in early embryogenesis occurs in two waves. At early stages, the first wave, primitive hematopoiesis, takes place in visceral yolk sac (YS) or its analog (Dieterlen-Lievre, 1975; Lassila *et al*, 1978), and is characterized by the transient circulation of large, nucleated red cells (Maximow, 1909; Ingram, 1972) that originate in structures known as blood islands (Haar *et al*, 1971; Silver *et al*, 1997). The primitive hematopoiesis is followed by a second wave, known as definitive hematopoiesis which takes place in intraembryonic areas (Dieterlen-Lievre, 1975; Lassila *et al*, 1978), and is characterized by the continuous circulation of small enucleated red cells (Maximow, 1909; Ingram, 1972). The predominant anatomic site of definitive hematopoiesis changes several times during human and murine development. Definitive hematopoiesis occurs in the liver, spleen and bone marrow (BM) in the fetal life of humans (Baudurant & Khoury, 1998; Erslev & Gabuzda, 1995) and mice (Medvinsky, 1996; Cumano, 1996), and in the BM and spleen of adult mice (Keller *et al.*, 1999; Clapp *et al.*, 1995; McGrath *et al.*, 2003; Ji *et al.*, 2003) and BM of adult humans (Baudurant & Khoury, 1998; Williams *et al.*, 1995). Thymus and lymph nodes have only minor contribution to hematopoiesis, during the fetal stage (Papayannopoulo and Abkowitz, 1995).

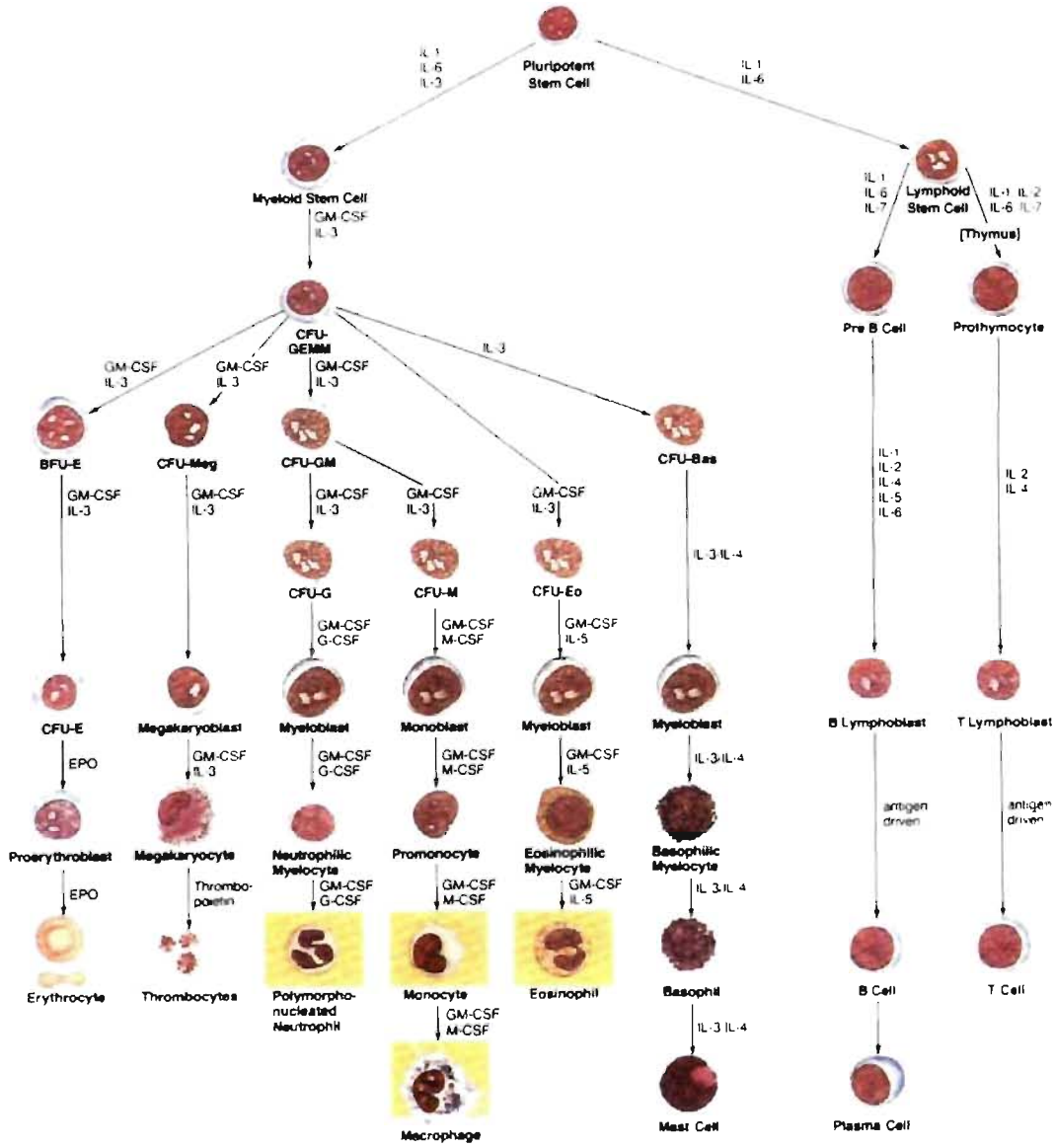

Figure 1. The hierarchial model of hematopoiesis and its regulation by cytokines. BFU-E = burst forming unit-erythroid; CFU-Bas = colony forming unit-basophil; CFU-E = colony forming unit-erythroid; CFU-Eo = colony forming unit-eosinophil; CFU-G = colony forming unit-granulocyte; CFU-GEMM = colony forming unit-granulocyte, erythroid, monocyte, macrophage, megakaryocye; CFU-M = colony forming unit-monocyte; CFU-Meg =colony forming unit-megakaryocyte; EPO = erythropoietin; G-CSF = granulocyte-colony stimulating factor; GM-CSF = granulocyte-monocyte-macrophage-colony stimulating factor; M-CSF, monocyte-colony stimulating factor; Meg-CSF = megakaryocyte colony stimulating factor. The name ``CFU`` reflects the ability of the designated cells to form a colony when cultured in a semi-solid medium (Adapted from Bell, 2002).

I.1.1 The hematopoietic tissue

I.1.1.1 Bone marrow

The bone marrow is one of the bodies largest organs, representing 3.4 to 6 percent of total body weight and averaging about 1500 grams in adult humans (Bloom & Fawcett, 1975). It consists of hematopoietic cells (erythroid, myeloid, lymphoid, and megakaryocyte), bone and its cells (osteoblasts, osteoclasts), and stroma and stromal cells (such as adipocytes and fibroblasts) (Bloom & Fawcett, 1975). The hematopoietic marrow is organized around the bone vasculature (Tavassoli & Jossey, 1978; Lichtman, 1981). An artery entering the bone branches out toward the periphery to specialized vascular spaces called "sinuses". Several sinuses combine in a collecting sinus, forming a central vein that returns into the systemic circulation. Hematopoiesis takes place outside of the sinuses in spaces called hematopoietic cords (**Figure 2**). Following maturation in the cords, the hematopoietic cells cross the walls of the sinuses and enter the blood (Tavassoli & Shaklai, 1979; Aoki & Tavassoli, 1981a, b).

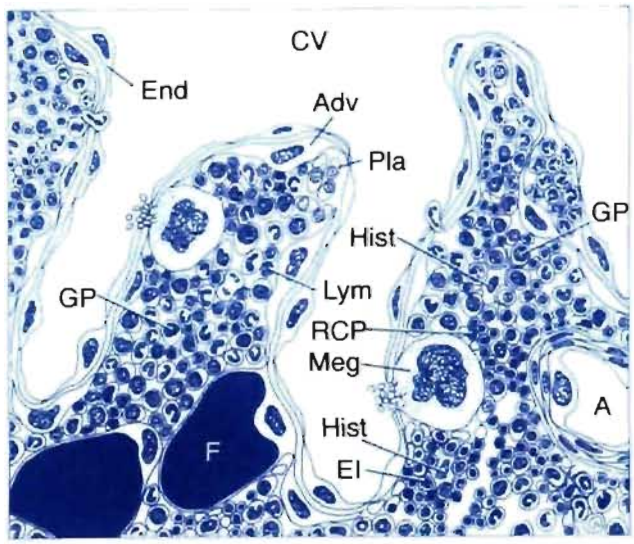

Figure 2 – Graphic representation of the bone marrow hematopoietic tissue.
The vascular compartment consists of arteriole (A) and central sinus (CV). The venous sinusoids are lined by endothelial cells (End), and their wall outside is supported by adventitial-reticulum cells (Adv). Fat tissue (F) is part of the marrow. The compartmentalization of the hematopoiesis is represented by areas of granulopoiesis (GP), areas of eryhropoiesis (RCP), and erythropoietic islands (EI) with their nutrient histiocyte (Hist). The megakaryocytes protrude with small cytoplasmic projections through the vascular wall (Meg). Lymphocytes (Lym) are randomly scattered among the hemopoietic cells, whereas plasma cells (Pla) are usually situated along the vascular wall. (Adapted from Ehsan 2002)

I.1.1.2 Spleen

The spleen is a dark red to blue-black organ located in the left cranial abdomen and surrounded by a fibrous capsule. It's an elongated organ, roughly triangular in cross section. Although the size of the spleen varies among species, the ratio of splenic weight to body weight remains fairly constant within the same individual, regardless of age (Losco, 1992).

The functions of the spleen are centered on the systemic circulation. It is comprised of two functionally and morphologically distinct compartments, the red pulp and the white pulp (**Figure 3**). The red pulp is a blood filter that removes foreign materials and damaged or aged erythrocytes.

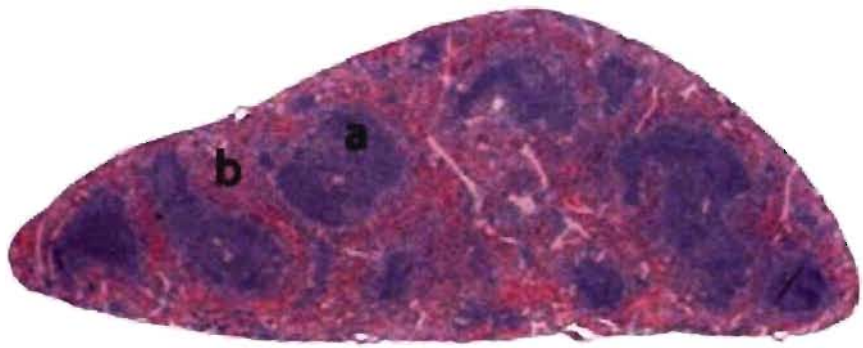

Figure 3. Histological section of a normal spleen. Note the while pulp (a) organized as follicles, and the red pulp (b) as diffuse formation. (Modified from Suttie, 2006)

It is also a storage site for iron, erythrocytes, and platelets, and plays a role hematopoiesis. The spleen is also the largest secondary lymphoid organ containing about one-fourth of the body's lymphocytes and initiates immune responses to blood-borne antigens (Kuper *et al.*, 2002; Nolte *et al.*, 2002; Balogh *et al.*, 2004). This function is performed by the white pulp which surrounds the central arterioles (Schmidt *et al.*, 1993).

I.1.1.3 Developmental hematopoietic organs

Embryonic blood islands are clusters of primitive erythroblasts surrounded by an endothelial covering and nestled between the outer visceral endoderm and inner mesothelial cell layers comprising the yolk sac, and involved in embryonic hematopoiesis (Moore, 2004). The thymus is a flat, bilobed lymphoid organ situated above the heart, surrounded by a capsule and divided in lobules, involved in the maturation of T-cells (Goldsby *et al.*, 2000). Lymph nodes are encapsulated bean-shaped structures containing a reticular network packed with lymphocytes, macrophages and dendritic cells, and constitutes a major site for antigen recognition and antibody production (Goldsby *et al.*, 2000). The liver is a complex organ of many functions including the production of numerous proteins and detoxification of nocive products. In fetal life it is involved in hematopoiesis for some duration, while in the adult this function could be resumed only in severe anemias (Papayannopoulo & Abkowitz, 1995).

I.1.1.4 Blood

Blood is a viscous substance that circulates in blood vessels. It varies in color depending on how much oxygen it carries. Arterial blood is well oxygenated and therefore, bright red, while venous blood is less well oxygenated and has a much darker color. Blood constitutes approximately 6-8% of total body weight (Hinchliff & Montague, 1988), and is made up of two components: fluid, known as plasma, and cells that float freely in the plasma. Plasma accounts for 55-60% of the blood volume and is 91% water, which helps to dissolve blood proteins. The blood proteins constitute 7% of plasma volume, and the electrolytes 2% (Huether & McCance, 1996). Plasma proteins, such as albumin, globulin and fibrinogen, are the most abundant solutes in the plasma. Most plasma proteins are formed in the liver and have a variety of functions. The remaining 40-45% of blood consists mainly of red blood cells (Kapit, 1987). The volume of red cells in 100 ml of blood is known as the hematocrit. White blood cells and platelets constitute only a small fraction of the blood.

I.1.2 Stem cells of the hematopoietic system

I.1.2.1 Definition and types

Stem cells are immature non-differentiated cells, characterized by three properties: proliferation, self-renewal, and capacity to differentiate (Hillman & Finch, 1996). There are at least three different types of stem cells that exist in the hematopoeitic tissue: hematopoietic stem cell (HSC), epithelial stem cell (ESC), and mesenchymal stem cell (MSC). HSC and ESC are believed to arise from a common precursor stem cell known as hemangioblast (Choi *et al.*, 1998; Keller *et al.*, 2001; Robertson *et al.*, 2000; Sabin, 1920). These cells can be distinguished by various morphological and functional characteristics.

- Hematopoietic stem cells (HSCs)

Hematopoietic stem cells are pluripotent stem cells that give rise to blood cellular elements (RBCs, WBCs, and platelets) and they can repopulate a recipient host after lethal irradiation. Two major subpopulations of HSCs could be distinguished: long-term repopulating HSCs and short-term repopulating HSCs, based on their ability to repopulate irradiated hosts for long-term and short term, respectively (Lanzkron *et al.*, 1999; Zhao *et al.*, 2000).

The purification of HSCs usually leads to a cell suspension enriched in HSCs. HSCs are usually defined by their ability to regenerate all of the hematopoietic lineages in vivo. Purification of cell populations capable of such repopulation is usually done on the basis of particular cell surface markers. Yet, the results obtained suggest that even such populations are not 100% pure HSCs (Osawa *et al,* 1996; Wagers *et al.*, 2002). Therefore, depending on the particular combination of markers used, subpopulation with various purities of HSCs could be obtained. The majority of human hematopoietic cells capable of producing multlineage hematopoietic engraftment in myeloablated recipients express the CD34 ($CD34^+$) (Zanjani *et al.*, 1997; *Larochelle et a*l., 1996; Andrews *et al.*, 1992), and human HSCs are usually considered negative for lineage markers (Lin^-), $CD34^+$, $CD38^-$ (Hogan *et al.*, 2002; Verstegen *et al.*, 1998; Mazurier *et al.*, 2003). Murine HSCs are usually considered Lin^-, $cKit^+$, $Sca1^+$ (Spangrude *et al.*, 1998; Osawa *et al.*, 1996), but mouse HSCs also express the CD34 although its regulation is different from humans (Okuno *et al,* 2002; Morel *et al.*, 1998). However, studies both in mouse and humans indicate that CD34 is a marker of activated and/or cycling stem cells, and that $CD34^+$ stem cells can revert to quiescent $CD34^-$ state (Sato *et al.*, 1998; Dao *et al.*, 2003; Zanjani *et al.*, 2003). Some reports have indicated that the most primitive murine HSCs are $CD34^-$, $cKit^+$, $Sca1^+$, and Lin^- (Osawa *et al.*, 1996a; Smith *et al.*, 1991; Spangrude *et al.*, 1995; Uchisda *et al.*, 1996; Matsuzaki *et al.*, 2004), while reports on whether human $CD34^-$ $CD38^-$ population is more primitive than the $CD34^+$ $CD38^-$ population are still controversial (Verfaillie *et al.*, 2000; Uchida *et al.*, 2001). A subpopulation of stem cells was identified based on its ability to extrude the Rhodamine 123 or the Hoechst 33341, and called side population (SP), both in mice (Goodell *et al.*, 1996) and humans (Goodell *et al.*,

1997). A high-purity HSCs is positive for side-population (SP^+), $CD34^+$, $CD38^-$ in humans (Uchida *et al.*, 2001), while the cells that have the strongest efflux activity (``Tip`` SP cells) have the phenotype $CD34^-$, $cKit^+$, $Sca1^+$, Lin^- in the mouse (Matsuzaki *et al.*, 2004).

• Epithelial stem cells (ESCs)

Also called endothelial progenitor cells or angioblasts, these cells can give rise to mature endothelial cells in vessels. (Asahara *et al.*, 1999; Lin *et al.*, 2000; Shi *et al.*, 1998; Kocher *et al.*, 2001).

• Mesenchymal stem cells (MSCs)

Also called stromal cells (Phinney *et al.*, 1999; Prockop, 1997; Pittenger *et al.*, 1999), MSCs can differentiate into many cell types including adipocytes, chondrocytes, osteocytes and muscle cells (Prockop, 1997; Pittenger *et al.*, 1999).

I.1.2.2 Hematopoietic Stem cell niche

The control of stem cell properties is affected by the localization of HSCs themselves (Croizat *et al.*, 1970; Gidali & Lajtha, 1972). The niche hypothesis, formulated by Schofield (Schofield, 1978) suggested that true HSCs exist in association with one or more other supporting cells and would therefore, in essence, be fixed tissue cells. These microenvironmental cells were postulated to form a specific niche that, when in close association with the HSC, confer on it the attribute of indefinite self-renewal capacity, while effectively inhibiting differentiation and maturation of the cell. HSCs exhibit selective lodgment in the hypoxic area at the endosteal region (Nilsson *et al.*, 1997 & 2001; Cipolleschi *et al.*, 1993; Lord 1992), as opposed to the proliferating and maturing progenitors which tend to be closer to blood vessels in areas with more oxygen (Cipolleschi *et al.*, 1993; Lord 1992).

I.1.2.3 Hematopoietic stem cell homing

Homing is the process by which stem cells reach the bone marrow after intra-venous injection into recipient hosts (**Figure 4**). It involves many complex processes such as

cell adhesion, rolling, passage through the bone marrow endothelium (Mazo *et al.*, 1999; Zanjani *et al.*, 1999; Kollet *et al.*, 2001; Peled *et al.*, 2000;Lapidot *et al.*, 2005; Quesenberry *et al.*, 2005) and entering the BM extravascular tissue (Calvi *et al.*, 2003; Avecilla *et al.*, 2004; Sorrentino, 2004; Zhang *et al.*, 2003; Kopp *et al.*, 2005). Following the entry into the BM other processes take place, including interaction with BM stromal cells (Quesenberry & Becker, 1998), endolgement in one of the extravascular bone marrow niches (Calvi *et al.*, 2003; Avecilla *et al.*, 2004) where they interact with the endosteal niche and vascular niche (Calvi *et al.*, 2003; Avecilla *et al.*, 2004; Zhang *et al.*, 2003; Kopp *et al.*, 2005) and proliferate in the bone marrow microenvironment. All of the different classes of cell adhesion receptors appear to play a role in anchoring hematopoietic cells within the marrow or in promoting hematopoietic differentiation (Carlos & Harlan, 1994; Long, 1992; Lewishon *et al.*, 1990; Yong *et al.*, 1998; Long *et al.*, 1992; Bruno *et al.*, 1995; Quesenberry *et al.*, 1998; Aizawa & Tavassoli, 1987)

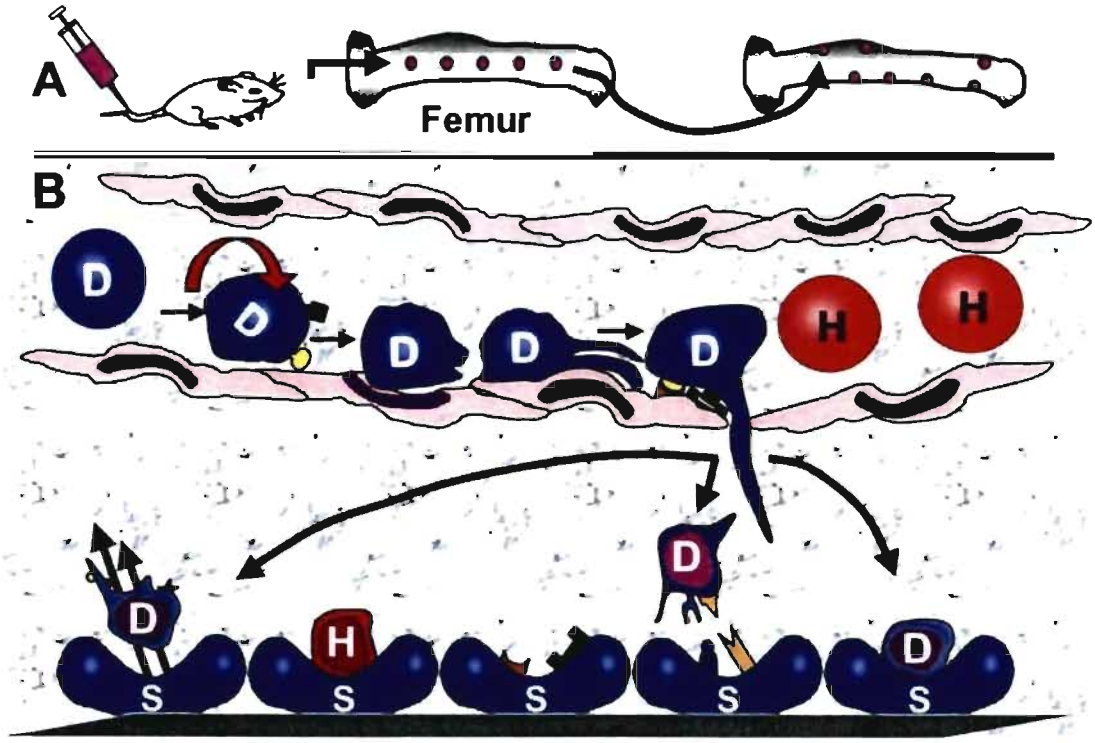

Figure 4. Stem cell homing after intravenous injection. After injection (A), the stem cells leave the vascular space and enter the trabecular marrow compartments from central marrow vessels. Once in this space, there is subsequent localization within the bone marrow according to the phenotype of the cells. Stem cells demonstrate selective redistribution to the endosteal region. Taking a closer look at the vascular space within marrow is depicted in (B). The donor (D) and host (H) cells travel through marrow vessels and sinusoids. Adhesion receptors such as VCAM-1, P- and E-selectins and others on the stem cells are responsible for the initial process of endothelial migration. This occurs by the "stickiness" of the cell, by rolling, crowling, and then migrating into the extravascular marrow stromal space. The cells find their respective niche in stromal (S) cells by additional cell receptors. Note the open spaces within the marrow cavity. Stem cells are not static, they leave the marrow stroma and go out into the circulation. Proteopodal (pseudopod-like) extensions may be the mechanism of how the cells home to their respective marrow niche. (Adapted from Quesenberry *et al.*, 2005)

I.1.3 Erythropoiesis

The term erythropoiesis refers to the entire process by which RBCs (erythrocytes) are produced (Dessypris, 1998; Bull *et al.*, 1995). In response to erythropoietin, a growth factor that stimulates the erythroid precursors, erythropoiesis occurs in the central sinus beds of medullary marrow over a period of about 5-7 days through at least 3 successive reduction divisions from proerythroblast to basophilic erythroblast, then to

polychromatophilic erythroblast, and finally to orthochromatophilic erythroblast. With successive developmental stages, the following changes occur (**Table I**): reduction in cell volume, condensation of chromatin, decrease in N:C ratio, loss of nucleoli, decrease in ribonucleic acid (RNA) in the cytoplasm, decrease in mitochondria, and gradual increase in synthesis of hemoglobin (Dessypris, 1998; Bull *et al.*, 1995). The nucleus of the orthochromatophilic erythroblast is eventually extruded, leaving a non-nucleated polychromatophilic erythrocyte (reticulocyte) which is released into the circulating blood to mature in 1 to 2 days. Progressive cellular divisions of one proerythroblast results in the production of 14 to 16 RBCs (Dessypris, 1998). Erythropoiesis takes place in distinct anatomic units called erythropoietic islands (Gulati *et al.*, 1988). Each island consists of a macrophage surrounded by a cluster of maturing erythroblasts. Hemoglobin synthesis occurs as early as the proerythroblastic stage, but most hemoglobin synthesis occurs in the polychromatophilic stage. The average life span of circulating red cells is ~120 days in humans (Ehsan, 2002), and ~60 days in mice (Singer, 1986)

Table I. Morphological characteristics of erythrocyte precursor subpopulations

Morph \ Type	Proerythroblast*	Basophilic Erythroblast	Polychromatophilic Erythroblast	Orthochromatophilic Erythroblast	Reticulocyte	Mature Erythrocyte
Cell size, μm	14-19	12-17	12-15	8-12	7-10	7-8
N:C ratio	4:1	4:1	4:1	1:1	n/a	n/a
Nuclear shape	Round	Round	Round	Round	n/a: nucleus has been extruded	n/a
Nuclear position	Central	Central	Central	Central	n/a	n/a
Nuclear color/chromatin	Reddish-blue finely stippled, granulate chromatin	Increased, larger granularity of nuclear chromatin	Dark blue, smaller nucleus with parachromatin, increased clumped chromatin	Blue-purple, small nucleus with pyknotic degeneration/condensed chromatin	n/a	n/a
Nucleoli	0-2 μm	Usually none, occasional indistinct nucleolus	None	None	None	None
Color/amount of cytoplasm	Dark or royal blue/slight	Basophilic/slight	Bluish-pink/moderate	Pink/moderate	Clear gray-blue, polychromatophilic to pink	Pink
Cytoplasmic granules	None	None	None	None	None	None

Morph, morphology of the cells; type, the type of erythroid subpopulation; n/a, not applicable; N:C, nucleus-to-cytoplasm ratio (Bell, 2002)

*Proerythroblast is the earliest recognizable erythroid precursor by light microscopy. Its can be very large in size (up to 20-25μm in diameter). The nuclear –cytoplasmic ratio is high, the chromatin pattern is immature (high proportion of euchromatin), nucleoli are present, and a characteristic pale, small golgi zone abuts the nucleus. The Golgi contains small acid phosphatase-positive granules containing ferritin and the cytoplasm is filled with polyribosomes, which results in its characteristic basophilia (intensely blue color) (Lichtman *et al.*, 2007).

I.1.4 The red blood cells (RBCs)

I.1.4.1 General properties of RBCs

The red blood cells (RBCs) or erythrocytes are 6- to 8-μm biconcave discs (Harmening, 2002) representing the blood cells that transport oxygen to the different organs of the body via its major component, hemoglobin (**Figure 5**) (Bunn, 1994). The RBC membrane contains numerous proteins (Cabantchik, 1999), including glycophorin, a major transmembrane protein (Mohandas & Evans, 1994). Glycophorin is heavily glycosylated and accounts for most of the membrane sialic acid, which gives the erythrocytes their negative charge as a result of which they repel each other as they move through the circulation. Anomalies in the RBC membrane components can lead to loss of its deformability and pliability (Harmening, 2002). Affected cells are at a marked disadvantage when they pass through the small (3- to 5-μm diameter) sinusoidal orifices of the spleen, one of the functions of which is extravascular sequestration and removal of aged, damaged or less deformable RBCs or fragment of their membranes. (Telen & Kaufman, 1999). The normal RBC membrane is freely permeable to water and anions and relatively impermeable to cations. It is primarily through the control of the sodium and potassium intracellular concentrations that the RBC maintains its volume and water homeostasis (Harmening, 2002).

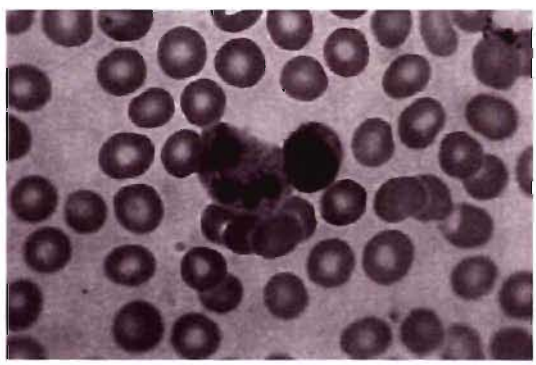

Figure 5. Normal red blood cells (RBCs) on peripheral blood smear. Note the roughly round appearance and a central pallor due to the biconcave shape of RBCs (Adapted from Schrier, 1997)

I.1.4.2 RBC senescence

RBC senescence represents the process of RBC aging during its life in the circulation (Harmening, 2002). Each day ~1% of the old RBCs in the circulation are taken out by a system of fixed macrophages in the body known as the reticuloendothelial system (RES). These RBCs are replaced by the daily release of ~1% of the younger RBCs reticulocytes from the bone marrow storage pool. As erythrocytes become older, certain glycolytic enzymes decrease in activity, resulting in a decrease in the production of energy and loss of deformability. At a certain critical point, the RBCs are no longer able to traverse the microvasculature and are phagocytized by the RES cells. Although RES cells are located in various organs and throughout the body, those of the spleen are the most sensitive detectors of RBC abnormalities (Cotran *et al.*, 1994). Various metabolic and physical changes associated with the aging of RBCs are listed in **Table II**

Table II. Changes occurring during aging of red blood cells

Increases	Decreases
Membrane-bound IgG	Several enzyme activity
Density	Sialic acid
Spheroidal shape	Deformability
MCHC	MCV
Internal viscosity	Phospholipid
Agglutinability	Cholesterol
Na^+	K^+
Methemoglobin	Protoporphyrin
Oxygen affinity	

Abbreviations: MCHC = mean cell hemoglobin concentration; MCV = mean cell volume; Na^+, sodium cation; K^+, potassium cation (Garratty, 1981)

Ninety percent of the destruction of senescent RBCs occurs by the process of extravascular hemolysis. During this process, old or damaged RBCs are phagocytized by the RES cells, digested by their lysosomes, and their hemoglobin components recycled for the major part, in a process involving the liver (Deiss, 1999). Only 5% to 10% of normal RBC destruction occurs through intravascular hemolysis. During this process, RBC breakdown occurs within the lumen of blood vessels. The RBC ruptures, releasing hemoglobin directly into the bloodstream. The hemoglobin molecule dissociates into αβ dimers and is picked up by the protein carrier,

haptoglobin (Deiss, 1999). The haptoglobin-hemoglobin complex prevents renal excretion of the hemoglobin and carries the dimers to the liver cell for further catabolism

I.1.5 Hemoglobin

The most important protein of red blood cells is hemoglobin, the molecule involved in the delivery of oxygen from the lungs to the tissues and facilitation of carbon dioxide exchange from the tissues to the lungs (Rodwell, 2000; Garratty, 1981). It constitutes 95% of the RBC dry weight or 33% of the RBC weight by volume (Bunn, 1994). Approximately 65% of the hemoglobin synthesis occurs during the nucleated stages of RBC maturation, and 35% occurs during the reticulocyte stage.

Hemoglobin is a conjugated globular protein with a molecular weight of approximately 64.4 Kilodaltons (Kd). Normal hemoglobin is a tetramer of two pairs of unlike globin polypeptide chains, two alpha-like (141 amino-acids) and two beta-like (146 amino acids) (Harmening, 2002) each folded around a heme molecule (**Figure 6**). Each heme consists of a protoporphyrin ring plus ferrous iron (Fe^{2+}) (Bunn, 1994). The net synthesis of α- and β-type globin polypeptide chains is normally balanced at 1:1 (Whitney, 1977). Anomalies affecting globin chains lead to disorders of various severities.

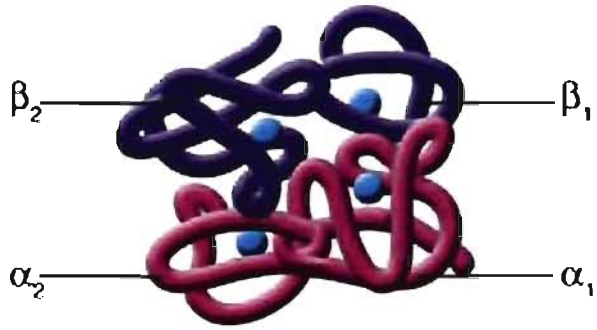

Figure 6. Schematic representation of normal hemoglobin molecule. Normal hemoglobin is a tetramer of two α-like and two β-like globin chains. The light blue spheres in the center of each globin represents the heme (Adapted from Bank, 2005)

I.1.5.1 Normal globins in human

Human β-globin is encoded by a structural gene found in a cluster with the other β-like genes on chromosome 11. The cluster contains five functional genes, ε, $^{G}\gamma$, $^{A}\gamma$, δ, β, which are arranged in the order of their developmental expression (Bulger *et al.*, 2000). ε is an embryonic globin gene expressed primarily in yolk sac-derived cells from 3 to 8 weeks of gestation; Gγ and Aγ are fetal globin genes expressed primarily in fetal liver-derived cells from 6 weeks of gestation to ~ 3 months after birth; and δ and β are adult globin genes expressed primarily in bone marrow-derived cells starting shortly before birth and persisting throughout adult life. The β gene is responsible for 97-98% of adult β-globin, and the δ gene is responsible for ~2-3% (Whitelaw *et al.*, 1990). Upstream of the entire β-globin complex is the locus control region (LCR), a regulatory element essential for the expression of all the genes in the complex (Bulger *et al.*, 2000). α-globin genes are found on chromosome 16, including ζ chain expressed in the embryonic stage and α chain expressed in the fetal and post-natal stages. β-like chains pair with the α-like globin ζ chain in the embryonic life, and with α-chain in fetal and post-natal life, to form the various hemoglobin molecules (**Table III**) (Harmening, 2002). The change in globin production from embryonic to fetal and from fetal to adult is called hemoglobin switching (Peterson, 2003).

Table III. Composition of the various normal human hemoglobin molecules

Stage of Development	Globin Chains	Hemoglobin
Embryo	$\zeta_2\varepsilon_2$	Gower 1
	$\alpha_2\varepsilon_2$	Gower 2
	$\zeta_2\gamma_2$	Portland
Fetus	$\alpha_2{}^{A}\gamma_2$	F
	$\alpha_2{}^{G}\gamma_2$	F
Adult	$\alpha_2\beta_2$	A
	$\alpha_2\delta_2$	A_2

(Harmening, 2002)

I.1.5.2 Normal globins in the mouse

Genes for mouse β-globin are encoded in a complex of β-like loci on chromosomes 7 (Hutton, 1969; Popp, 1969). There are four functional genes in the mouse β-globin gene cluster: bh1 (or βh1), an early embryonic globin gene expressed primarily in yolk sac-derived cells from 9.5 to 12.5 days of gestation; γ, (or $\varepsilon\gamma^2$) a late embryonic globin gene expressed primarily in fetal liver-derived cells from 11.5 to 16.5 days of gestation. Two β-globin genes located toward the 3' end of the β-globin gene complex are normally expressed in adult mice (Jahn & Hatchison, 1980; Edgell *et al.*, 1981), first at 9.5 days of gestation in yolk sac-derived cells, then in fetal liver-derived cells, then in spleen, and finally in bone marrow-derived cells throughout adult life (Whitelaw *et al.*, 1990). These genes occur in polymorphic forms (Ranney *et al.*, 1960; Russel & McFarland, 1974) that encode four types of β-globin polypeptides (Popp, 1973; Popp *et al.*, 1973; Gilman, 1976). Mice of the Hbb^s haplotype possess two genes that make an identical β-globin subunit, *β-single*. Mice of the Hbb^d haplotype produce two distinct globins, *β-major* and *β-minor*, while mice of the Hbb^p haplotype make β-major and a variant form of β-minor. The *β-major* gene is responsible for ~80% of adult β-globin, and *β-minor* is responsible for ~20% (Whitney, 1977), while in the Hbb^s, both β-globin genes contribute equally (Ranney and Gluecksohn, 1955; Morton, 1962). The organization of the mouse β-globin gene complex is similar to that of humans (Lawn *et al.*, 1978; Fritsch *et al.*, 1979), goats (Haynes *et al.*, 1980), sheep (Kretshmer *et al.*, 1981) and rabbits (Lacy *et al.*, 1979) and appears to be representative of mammals in general. Mouse β-globin genes are

also regulated by a locus control region (LCR). Murine α-globins are encoded in a complex of α-like locus on chromosome 11 (Russel & McFarland, 1974) and include ζ chain in the embryonic life and α-chain in the fetal and adult stages (Trimborn, 1999); they pair in a 1:1 ratio with β-like globins. The hemoglobin switching in mice involves the switch from the production of embryonic globins to fetal-adult globins (Trimborn, 1999).

I.1.5.3 Hemoglobin variants

Several hemoglobin variants exist. Most are accompanied by normal phenotype. Some, however, result in anomalies including altered affinity to oxygen ($Hb_{Chesapeake}$, $Hb_{Bethesda}$, Hb_{kansas}, $Hb_{Seattle}$, $Hb_{Antille}$), slight anemia and morphological changes of RBCs (HbC, HbM), or severe anemia (HbS) (Harmening *et al.*, 2002).

I.2. Anemias and hemoglobinopathies

Anemia is defined as the decrease in the amount of hemoglobin per unit of blood volume, below normal range (Bernard *et al.*, 1998, DeRossi & Raghavendra, 2003). Hemoglobinopathies are defined in the broadest sense as conditions in which there are either qualitative or quantitative abnormalities in the synthesis of hemoglobin (Huisman *et al.*, 1998). More than 625 hemoglobin variants have been described (Huisman *et al.*, 1998) and most of them have no clinical significance. Only about one third (200) of these variants represent hemoglobinopathies with clinically significant hemolytic anemia.

I.2.1 General symptoms of anemias

The symptoms of anemia are related to its severity, the rapidity of its onset and the overall condition of the organism. A rapid onset of anemia (acute anemia) is accompanied by more severe symptomatology than a progressively established anemia (chronic anemia), for the same degree of anemia, since adaptation to hypoxia

takes place progressively in the latter. On the other hand, the patient age and overall conditions play an important role in the possibilities of adaptation. (Bernard *et al.*, 1998). The common symtpoms of anemia are listed in **Table IV**.

Table IV. Clinical symptoms of anemia

-Pallor
-Weakness
-Fatigue
-Lethargy or malaise
-Exercise dyspnea
-Palpitation
-Pica (consumption of substance such as ice, starch or clay)
-Syncope (particularly following exercise)
-Dizziness
-Headache
-Tinnitus or vertigo
-Irritability
-Difficulty sleeping or concentrating
-Gastrointestinal symptoms

(Sonakul *et al.*, 1988)

I.2.2 Pathophysiology and classification of anemias

The decrease in the amount of hemoglobin can result from one of two mechanisms: increased loss where compensation by the bone marrow is not enough to correct the situation, and a decreased medullary production. In the first case, there is an increase in the reticulocyte count, accounting for the medullary regeneration, and which appears slightly after the onset of anemia, whereas in the latter, there is a decrease in reticulocyte count preceeding the onset of anemia, and this is the *primum movens* of the anemia. The first group is usually referred to as regenerative anemias and the second as non-regenerative anemias (Bernard *et al.*, 1998). Anemias could be classified according to their etiology into hemolytic, hemorrhagic, deficiencies, and aplastic anemia (Ravel, 1994), as shown in **Table V**

Table V. Classification of anemias by etiology

I- Hemorrhagic anemia: due to acute or chronic hemorrhage
II- Hemolytic anemia: Hyperhemolysis
  II-1 Intra-corpuscular origin
    a-Enzyme abnormalities
    b-Hemoglobin abnormalities (sickle cell disease, thalassemias)
    c-Membrane abnormalities
    d-Marchiafava-Micheli
  II-2 Extra-corpuscular origin
    a-Toxins and toxic substances
    b-Parasitism
    c-Mechanic aggressiveness
    d-Immunological aggressiveness
III- Aplastic Anemia
  III-1Pure: anerythroblastic anemia or erythroblastopenia
  III-2 Global medullary failure:
    a-Invasion
    b-Pure medullary aplasia:
      -Destruction of stem cells
      -Destruction of the scaffolding tissue
IV- Deficiencies Anemia:
  IV-1 Affecting DNA:
    a-Deficiency in folic acid or Vit. B12
    b-Primitive
  IV-2 Affecting hemoglobinogenesis:
    a-Iron deficiency or retention in macrophages
    b-Disorders in iron utilization
V- Diverse causes: renal failure, endocrine disorders, inflammation

(Bernard *et al.*, 1998; DeRossi & Raghavendra, 2003)

I.2.3 The thalassemias

The thalassemias were first described by Thomas B. Cooley and Pearl Lee in 1925 (Roitt *et al.*, 1998) and they refer to a diverse group of hemoglobin disorders characterized by a reduced synthesis of the globin chains, thus resulting in anemias of varying degrees (Weatherall & Clegg, 2001). The major subgroups are α-thalassemia (α-globin deficiency) and β-thalassemia (β-globin deficiency) (Harrison, 2002).

I.2.3.1 Epidemiology of the thalassemias

The thalassemias are the most common monogenic disorders in the world and globally it is estimated that there are 250 million to 270 million carriers, of which 80 million are carriers of β-thalassemia. (Choudhry, 2000; Filon *et al.*, 1995). The most common mutations are those that are prevalent in the malarial tropical and sub-

tropical regions where a few mutations have reached high gene frequencies because of the protection they provide against malaria. (Flint *et al.*, 1998). The estimated prevalence includes 16% in Cyprus, 3-14% in Thailand, 3-8% in india, Pakistan, Bengladesh and China. There are also prevalences in descendants in Latin America, the Carribean, and Mediterranean countries. In some regions in Africa, the frequency is around 0.9%, while in African Europeans, it is almost 0.1% (Broodie, 2005).

I.2.3.2 Molecular basis of β-thalassemia

More than 200 β-thalassemia alleles have been characterized, however about 40 account for 90% or more of the β-thalassemia worldwide (Flint *et al.*, 1998). The β-thalassemias are rarely caused by large deletions (Borget, 2001; Thein, 1998). In such cases, expression of the β-gobin genes can also be silenced by deletions of the β-globin complex (Weatherall & Clegg, 2001; Rooks *et al.*, 2005) as part of $(\varepsilon\gamma\delta\beta)^0$ thalassemia. At a molecular level, the deletions causing $(\varepsilon\gamma\delta\beta)^0$ thalassemia fall into two subgroups: one removes all or a greater part of the β-globin gene complex including the β-gene itself; the other removes extensive upstream regions leaving the β-globin gene intact, although its expression is silenced because of inactivation of the β-globin LCR.

The vast majority of β-thalassemias are caused by point mutations within the gene or its immediate flanking sequences. These single base substitutions, minor insertions or deletions of a few bases are classified according to the mechanism by which they affect gene regulation: transcription, RNA processing or RNA translation (Thein, 1998). Approximately half of the β-thalassemia alleles affect different stages of RNA translation and no β-globin is produced resulting in β^0-thalassemia (Maquat, 1995).

- β^0 Thalassemia

This gene results in complete absence of β chains production (Harrison, 2002). It could be due to absence of expression, large deletions, aberrant splicing and degradation of the product.

- β^+ Thalassemia.

The β^+ thalassemia genes produce a reduced amount of β chains. There is a heterogeneity in β^+ thalassemia, and at least three different groups of genes have been described. The type 1 β^+ thalassemia gene produces the least amount of β chains (about 10% of normal production). The type 2 β^+ thalassemia gene produces a greater amount of β chains (about 50% of normal production). The type 3 β^+ thalassemia gene produces an even greater amount of β chains, and causes a much milder form of β thalassemia (Harrison, 2002).

I.2.3.3 Pathophysiology of β-thalassemia

β-thalassemia occurs when there is a deficiency of β-globin; typically, it is caused by a direct down-regulation in the synthesis of structurally normal β-chains. However, a thalassemia phenotype can also arise from structural β-chain variants if they are synthesized at a reduced rate, e.g. HbE. Alternatively, the variants are produced at a normal rate but are so unstable that they are rapidly destroyed giving rise to a functional deficiency. The former group of variants is also referred to as β-thalassemic hemoglobinopathies. The hyper-unstable β-chain variants act in a dominant negative fashion, causing a disease phenotype even when present in a single copy, and hence, have been referred to as dominantly inherited β-thalassemia (Thein, 1999). In contrast, typical β-thalassemia is inherited as a haplo-insufficient Mendelian recessive disease (Thein, 1999).

In β-thalassemia the disease does not manifest itself until the switch from γ-chain to β-chain synthesis has been completed. This usually occurs several months after birth. Thus, the clinical presentation of a patient with this disease usually occurs during the first year of life. There often is a compensatory increased production of γ chains and δ chains resulting in an increased level of hemoglobin F and hemoglobin A_2 (Harrison, 2002).

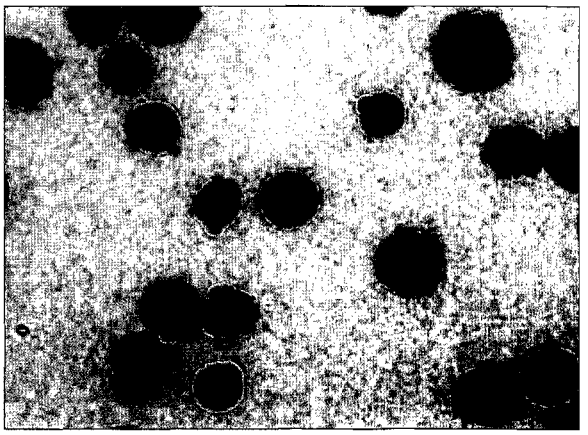

Figure 7. Heinz Bodies. With crystal violet staining of RBCs, Heinz bodies appear as spheroid formation at the level of the RBC membrane (Adapted from Harmening, 2002)

The deficiency in β-globin leads to decreased hemoglobin content per RBC (mean corpuscular hemoglobin, MCH), resulting in hypochromic cells (Harrison, 2002). The RBC volume (mean cell volume, MCV) decreases, resulting in microcytosis (Harrison, 2002). The excess unmatched α-globin chains accumulate at the membrane and its skeleton, forming rounded precipitates called Heinz Bodies (**Figure 7**) where they lead to membrane alterations consisting of reduced incorporation, misincorporation or clustering of some membrane protein components in the plasma membrane (Shinar *et al.*, 1987, 1989; Aljurf *et al.*, 1996; Advani *et al.*, 1992; Yuan *et al.*, 1992), affecting RBC deformability, stability and hydration (Schrier, 1989). This results in many abnormalities in RBC morphology and physiology, including fragmented cells (**Figure 8**) (Bunyaratvej *et al.*, 1985; Bessis, 1973; Schreir, 1989), and abnormal osmotic properties (Wyrick-Glatzel & Hugues, 2002; Harrison, 2002). Abnormal RBCs have a reduced half-life, and will be sequestered more rapidly than normal in the spleen resulting in splenomegaly. A highly expressed protein called alpha hemoglobin stabilizing protein (AHSP) acts as a chaperone for some free alpha chains and prevent their precipitation (Kihm *et al.*, 2002; Gell *et al.*, 2002) and the absence of which results in more severe β-thalassemic phenotype. The bone marrow shows intense erythroid hyperplasia, and 60-80% of the thalassemic erythroid precursors die in the marrow (normal value: 10-20%) or in sites of extramedullary erythropoiesis (Finch *et al.*, 1970) due to increase in both apoptosis and phagocytosis (Centis *et al*, 2000; Weiner *et al.*, 1999; Wanachlwanawin *et al.*, 1993; Angelucci *et*

al., 2002), commonly known as ineffective erythropoiesis. The decreased RBC half-life and the ineffective erythropoiesis result in decreased number of RBCs in the peripheral blood and subsequent aggravation of anemia.

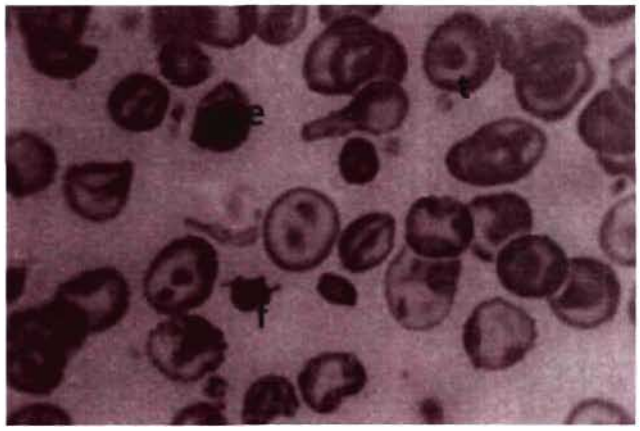

Figure 8. Red blood cells in β-thalassemia *major*. The cells are pale or hypochromic, with different shapes, including the target cells (t) or "bull's eye". Note the presence of fragmented cells (f), and some nucleated RBCs or erythroblasts (e). (Modified from Schrier, 1997)

Iron overload frequently develops in thalassemic patients as a consequence of blood transfusions (Sonakul *et al.*, 1998; Bothwell *et al.*, 1979; Link *et al.*, 1985) and increased iron absorption (Harrison, 2002). As iron loading progresses, the capacity of the its main transport protein, serum transferrin, to bind and detoxify it may be exceeded and a non-transferrin bound fraction of plasma iron may promote the generation of free hydroxyl radicals, resulting in oxygen related damage (Hershko & Weatherall, 1988; Hershko *et al.*, 1998). In the absence of chelating therapy, the accumulation of iron results in progressive dysfunction of many organs including the heart, liver, endocrine glands (Oliveri & Brittenham, 1997; Du *et al.*, 1997; Gullo *et al.*, 1993; Skalr *et al.*, 1987) and pancreas (Cavallo-Perin, 1995).

I.2.3.4 Clinical manifestations of β-thalassemia

The clinical presentation of β-thalassemia is very heterogeneous, and is broadly divided in three groups according to the severity: β-thalassemia *major*, β-thalassemia *intermedia*, and β-thalassemia *minor*.

Thalassemia *major* is the most severe clinical expression of β-thalassemia and results from homozygosity for a β^0- or β^+-thalassemia type 1 gene, or compound heterozygozity for these genes. Infants with thalassemia major usually present within the first year of life with failure to thrive, pallor, a variable degree of jaundice, and abdominal enlargement with hemoglobin levels from 4 to 8 g/dl (Harrison, 2002). Patients present characteristic deformities of the skull and face, as well as osteopenia and local defects in bone mineralization (Rioja *et al.*, 1990; Orvieto *et al.*, 1992). High blood transfusion program (hypertransfusion) is required to keep the patient alive. Iron accumulation results in liver cirrhosis, growth retardation and diabetes.

Thalassemia *intermedia* results from homozygous type 2 or type 3 β^+-thalassemia gene or compound heterozygous states, or heterozygous state for β^0- or β^+-thalassemia type 1 gene. It could be defined as a form of thalassemia in which patients have variable degrees of symptomatic anemia, jaundice, splenomegaly, and many of the complications of thalassemia major, but survive into adulthood without a large blood transfusion requirement. It covers a broad spectrum of clinical expression, and usually presents at a somewhat older age, generally after age of 2, and with a slightly higher level of hemoglobin (between 6 and 10 g/dl). Children usually have an acceptable level of growth and development, and they reach adulthood if infections are controlled and a good nutrition is administered (Harrison, 2002). Iron overload develops slower, and its complications occur much later at life (Chen *et al.*, 2000).

Thalassemia *minor* is a mild form that results either from heterozygozity for the type 2 or type 3 β^+-thalassemia genes, or heterozygozity for the β^0- or type 1 β^+-thalassemia genes with concomitant elevation in the levels of fetal hemoglobin (HbF) (Harrison, 2002; Bunn & Forget, 1986). It is a clinical entity in which the genetic defects of thalassemia are expressed as a mild microcytic, hypochromic anemia usually in the 9 to 11 g/dl range. Patients are asymptomatic except during periods of stress, and usually require no therapy if they maintain good nutrition (Harrison, 2002).

I.2.3.5 Treatment options for β-thalassemia

Patients with severe anemia receive regular blood transfusions (Cazzola *et al.*, 1995; Piomelli, 1995); with time, however, iron accumulates, and could cause intoxication or hemochromatosis, necessitating iron chelation (Olivieri & Brittenham, 1997; Kontoghiorghes *et al.*, 1990; Grady *et al.*, 1998; Wonke *et al.*, 1998), and reducing iron absorption from the gut (Urbinati, 2006). The therapy of β-thalassemia also includes HbF induction with agents like butyrate, 5-azacytidine, hydroxyurea, and erythropoietin, which are administered for long periods, with potential toxicities and variable outcome (Zeng *et al.*, 1995; Alberto *et al.*, 1998; Arruda *et al.*, 1997; Olivieri *et al.*, 1992; Dover, 1998; Collins *et al.*, 1995; Cappelini *et al.*, 1998; Ley *et al.*, 1982). β-thalassemia therapy also includes the use of anti-oxidants: protecting the cell membrane (Grinberg *et al.*, 1994, 1997), as well as other supportive and symptomatic therapies (Urbinati, 2006). Hematopoietic stem cell transplantations (HSCT) provide the means to cure β-thalassemia. Few HSCT are being done in human clinics with variable degrees of success (Lucarelli *et al.*, 2005; Vellodi *et al.*, 1994; Boulad *et al.*, 1998; Walters *et al.*, 1994).

I.2.3.6 Spectrum of the thalassemias

The most frequent forms of thalassemia are α- and β-thalassemias. **Table VI** summarizes the main characteristics of α-thalassemia, and **Table VII** gives a comparative view of the other forms of thalassemia.

Table VI. Overview of α-thalassemias

Type	Genotype[a]	Phenotype	Hemoglobin[b]
α-Thalassemia silent carrier (α^+-thalassemia)	Single α-gene deletion (-α/αα)	Asymptomatic; no hematological abnormailities	Normal
α-Thalassemia minor (α^0-Thalassemia)	Two α-gene deletions Trans (-α/-α) or cis (--/αα) deletions	Asymptomatic; erythrocytosis, microcytosis, hypochromia	Normal
HbH disease	Three α-gene deletions	Mild to moderate microcytic hypochromic anemia, chronic hemolysis, episodic severe anemia	Hb Bart's (γ_4) HbH (β_4)
Hb Bart's hydrops fetalis	Four α-gene deletion	Hydrops fetalis	HbH, Hb Bart's

[a] α^+-Thalassemia refers to deletion of one of the four α-globin genes; α^0-thalassemia refers to two α-globin gene deletions, either in trans (deletion of one α-globin on each of the two different alleles), or in cis (deletion of two α-globin on the same allele). The symptomatic forms of α-thalassemia result from three or four α-globin gene deletions.

[b] Types of hemoglobin (Hb) found on electrophoresis. When there is a paucity of total lack of α-globin chains, the complementary unpaired γ-globin or β-globin chains form tetramers and precipitate as Hb Bart's (γ_4) or HbH (β_4) respectively, both detectable as distinct entities (Urbinati *et al.*, 2006)

Table VII. Spectrum of the thalassemia and the thalassemia-like syndromes

α-Thalassemia syndromes

1-Heterozygous α-thalassemia 2 or "silent carrier" state
2-Heterozygous α-thalassemia 1 or α-thalassemia trait
3-HbH disease: double heterozygozity for α-thalassemia 1 + α-thalassemia 2
4-Hydrops fetalis with Hb Bart's: homozygous α-thalassemia 1
5-Hb constant spring syndromes
6-α + β thalassemia
7-HbS or HbSS/α-thalassemia

β-Thalassemia syndromes

1-Heterozygous β-thalassemia, β-thalassemia trait, or β-thalassemia minor
  a-With elevated HbA_2 ± elevated HbF
  b-With normal HbA_2, and elevated HbF; δβ-thalassemia, or F-thalassemia
    i-$^{G}\gamma^{A}\gamma(\delta\beta)^{0}$ thalassemia
    ii-$^{G}\gamma(^{A}\gamma\delta\beta)^{0}$ thalassemia
  c-With normal HbA_2 and HbF
    i-"Silent carrier", including Hb Knossos
    ii-Concomitant δ + β thalassemia, *in cis* or *in trans*
    iii-γδβ-thalassemia
    iv-Other: atypical δβ-thalassemia; concomitant iron deficiency
  d-Hb Lepore trait
2-Homozygous β-thalassemia, Cooley's anemia, or β-thalassemia major
  a-True homozygosity for one or another β-thalassemia major
  b-Double heterozygosity for any two different β-thalassemia genes
3-β-thalassemia intermedia

Rare forms of thalassemia

1-γ-thalassemia
2-δ-thalassemia
3-γδβ-thalassemia

Interacting thalassemia

1-α-thalassemia + α-chain variant
  a-HbQ/α thalassemia
  b-HbG/ α thalassemia
2-β-thalassemia + β-chain variant
  a-Sickle/ β thalassemia
  b-HbC/ β thalassemia
  c-HbE/ β thalassemia

Hereditary persistence of fetal hemoglobin (HPFH)

1-Pancellular
  a-$^{G}\gamma^{A}\gamma(\delta\beta)^{0}$ HPFH
  b-Hb Kenya ($^{G}\gamma$ HPFH)
  c-Black $^{G}\gamma\beta^{+}$ HPFH with high HbF
  d-Greek $^{A}\gamma$ HPFH
  e-Chinese $^{A}\gamma$ HPFH
2-Heterocellular
  a-Swiss-type $^{G}\gamma^{A}\gamma$ HPFH
  b-British-type $^{A}\gamma$ HPFH
  c-Other- Seattle-type $^{G}\gamma^{A}\gamma$ HPFH; Atlanta type-black $^{G}\gamma\beta^{+}$ HPFH with low HbF; Saudi high HbF determinant

(Bunn & Forget, 1986)

I.2.4-Sickle cell disease (SCD)

First described by Herrick in 1910 (Herrick, 1910), SCD (also known as sickle cell anemia, SCA) encompasses a group of conditions characterized by a hemolytic anemia and tissue damage, resulting from the production of an abnormal β-globin chain and therefore abnormal hemoglobin that polymerizes upon deoxygenation, distorting the RBCs into elongated "sickled" cells, hence the name (**Figure 9**) (Wang 2004; Lukens, 2004).

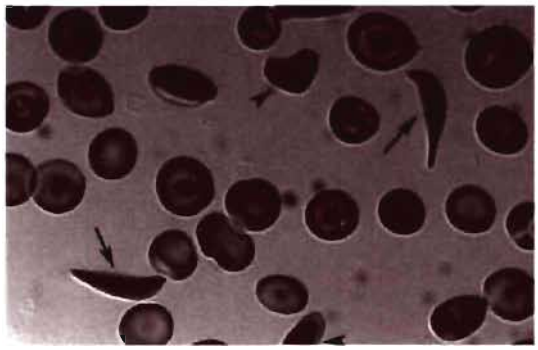

Figure 9. Sickled red blood cells
Peripheral blood smear from patient with SCD. Sickled cells appear elongated, with pointed or blunted ends, and many are crescent shaped. The smear shows classical sickle-shaped (arrows) and various other misshaped RBCs (arrowheads) (Adapted from Frenette & Atweh, 2007)

I.2.4.1 Epidemiology of SCD

The largest proportion of SCD occurs in Africa where it is estimated that between 200000 and 230000 infants are born each year with SCD (Diallo & Tchernia, 2002). In parts of Africa, as much as 45% of the populations are carriers of the mutant HbS allele. Countries, such as the USA, that have a significant population of African descent also have a significant incidence of SCD. The carrier state is seen in about 8% of populations of African descent in Latin America, the USA, and the Caribbean (Wang 2004). In several areas in Africa and in Asia the β^S gene has arisen independently as a new mutation. Recognized by differences in the β globin gene cluster, these haplotypes are named after the areas where they were first described (Senegal, Bantu, Benin, Cameroon, Arab, Indian, and Saudi Arabia haplotypes) (Embury & Stenberg, 1994).

Although homozygous SCD can be fatal if not treated or not associated with HPFH, the heterozygous state confers survival advantage with regards to infections with *Plasmodium falciparum* which may explain what epidemiologic studies have shown as association of malaria distribution and the distribution of sickle cell gene (Aidoo *et al.*, 2002; Eaton, 1994).

I.2.4.2 Molecular basis of SCD

A single point mutation in the 6th codon of the β-globin gene leads to substitution of glutamic acid by valine, resulting in an abnormal globin: β^S. This leads to the formation of "sickle hemoglobin", or HbS ($\alpha_2\beta^S_2$). The conformational change that HbS undergoes when deoxygenated exposes the substituted valine on the molecular surface of β^S globin, instead of the polar glutamic acid. This allows intermolecular hydrophobic interactions between β^S of adjacent hemoglobin molecules (in contrast to normal hemoglobins where no such interactions occur), ultimately resulting in the polymerization of HbS (Wang, 2004; Lukens, 2004). This is the molecular hallmark of sickle cell disease (SCD) (Bunn, 1997). As a consequence, the normally pliable RBC assumes a rigid non-deformable sickled shape, with ensuing erythrocyte membrane damage and hemolysis. The rigidity of these cells contributes to the overall process of microvascular occlusion that leads to tissue ischemia and organ dysfunction

I.2.4.3 Spectrum of sickle cell disorders

Although HbSS is the most severe form of SCD, many other forms exist, as shown in **Table VIII**. In particular, in HbS-Antille, the $\beta^{Antilles}$ mutation (β^{23}Ile) that occurs in cis to the β^S mutation (β^6Val β^{23}Ile), increases the clinical severity of the β^S mutation so that HbA/HbS-Antilles heterozygotes display a major sickle cell syndrome, in contrast to the asymptomatic A/S genotype (Montplaisir *et al.*, 1986). This is due, first, to the decreased oxygen affinity of HbS Antilles that favors HbS polymerization, and second, new contact sites are created in the polymer, as suggested by the low solubility of fully deoxygenated HbS Antilles. The β^{121}Gln mutation of HbD Punjab gives a very severe sickle cell syndrome in the compound

heterozygous state, HbS/HbD Punjab (Milner *et al.*, 1970). This is due to the greatly lowered solubility of the deoxygenated hybrid, Hb $\alpha\beta^S/\alpha\beta^D$, which results from the creation of new contact sites that stabilize the polymer (Padlan & Love, 1985).

Table VIII. Spectrum of sickle cell disorders

Disease	Genetic defect	Hb produced	Clinical consequences[a]
HbSS (Sickle cell anemia)	Homozygous for substitution of valine for glutamic acid at sixth codon of β-globin gene	HbS Variable HbF	Moderate to severe anemia, vaso-occlusive episodes, strokes, acute chest syndrome, priapism, hepatobilliary and liver disease, splenic infarction, life-threatening infections, renal disease, retinopathy shortened lifespan
HbAS (Sickle trait)	Heterozygous for sickle mutation and normal β-globin	HbA>HbS	No anemia and generally no other symptoms, rarely, hematuria, urinary tract infection and splenic infarcts; very rarely (with extreme conditions, e.g. high altitude, extreme physical exercise, pneumonia) symptoms of SCA. Normal life expectancy
HbSC (Sickle, HbC disease)	Heterozygous for sickle mutation and C mutation (lysine at sixth position of β-globin chain). HbS can polymerize with HbC, but does so less avidly than with another HbS	HbS = HbC Normal to slightly elevated HbF	Same constellation of symptoms as SCA, but significantly milder. Life expectancy 60-68 years[b]
HbS-β-Thal (Sickle, β-Thalassemia)	Heterozygous for sickle mutation and one of β-thalassemia mutations that causes decreased (β^+-thalassemia) or absent (β^0-thalassemia) normal β-globin	HbS with (1) no HbA (β^0) or (2) HbS > HbA (β^+) Variable HbF and HbA2	Severity of clinical symptoms are inversely related to the amount of HbA and HbF made. $S\beta^0$-thalassemia is clinically manifested like SCA. $S\beta^+$-thalassemia is milder and can be as mild as sickle trait
HbS-HPFH (SCA-Hereditary persistence of fetal hemoglobin)	Homozygous for sickle mutation with deletional or non-deletional mutations that increase HbF	HbS HbF: 20-30%	Milder symptoms because HbF prevents HbS polymerization
HbSE (Sickle, HbE disease)	Heterozygous for sickle mutation and E mutation (lysine for glutamic acid at 26th position of β-globin chain). HbS can polymerize with HbE, but does so less avidly than with another HbS	HbS HbE: 30%	Mild anemia, mild symptoms. HbE does not polymerize

Table VIII...continued

Disease	Genetic defect	Hb produced	Clinical consequences[a]
HbSD (Sickle, HbD)	Heterozygous for sickle mutation and D mutation (glutamine for glutamic acid at position 121 of β-globin chain). HbS can polymerize with HbD but does so less avidly than with another HbS	HbS HbD	Mild anemia, mild SCA symptoms; the mildest of the symptomatic SCD syndromes[b] unless HbS and HbD mutation on the same allele
$HbSO^{Arab}$ (Sickle, HbO^{Arab})	Heterozygous for sickle mutation and O mutation (lysine for glutamic acid at position 121 of β-globin chain)[c]. HbS can polymerize with HbO but does so less avidly than with another HbS	HbS HbD	Anemia and SCA symptoms; indistinguishable from SCA
HbS-α-Thal (SCA, α-Thalassemia)	Homozygous for sickle mutation with mutations causing decreased (trait or α^+-thalassemia) or absent (α^0-thalassemia) α-globin chain	HbS	SCA symptoms, but milder. Symptoms are ameliorated because less HbS is formed, as a result of low α-globin availability; HbS concentration is thus decreased, reducing sickling[d]

[a] Except where indicated otherwise, the information in this table was obtained from Lukens, 2004 and Wang, 2004
[b] Beutler, 2001
[c] Baglioni & Lehman, 1962; Wick *et al.*, 1987
[d] Embury, 1989

I.2.4.4 Pathophysiology of SCD

The pathophysiological hallmark of SCD is RBC sickling caused by HbS polymerization (Bunn, 1997). Factors which favor hemoglobin polymerization include reduction in the hemoglobin affinity to oxygen such as decrements in pH (Kaul *et al.*, 1996). Dehydration also favors polymerization (Bertles *et al.*, 1970; Noguchi & Schechter, 1985; Mozarelli *et al.*, 1987; Bookchin & Nagel, 1973). If sickled erythrocytes escape the microcirculation, the HbS polymer becomes soluble again after reoxygenation and the RBC can reassume its biconcave shape (Glader, 1994). Overtime, however, cycles of polymerization and depolymerization lead to membrane lesions and dehydration of RBCs (Lew *et al.*, 1991), resulting in cells that are permanently sickled even in the absence of polymers (irreversibly sickled cells,

ISCs) (Hofrichter *et al.*, 1947). ISCs have significantly reduced lifespan as compared with non-ISCs (Glader, 1994).

RBCs in SCD are characterized by an increase in the concentration of hemoglobin per RBC (mean corpuscular hemoglobin concentration, MCHC), an increase in the percentage of dense hyperchromic cells, due to dehydration (Harmening *et al.*, 2002) a decrease in RBC volume (mean cell volume, MCV), and change in their osmotic properties (Wyrick-Glatzel & Hugues, 2002). The RBC membrane lesions **(Figure 10)** lead to increased intravascular hemolysis and hemolytic anemia, and decreased RBC pliability resulting in their sequestration in the spleen and splenomegaly.

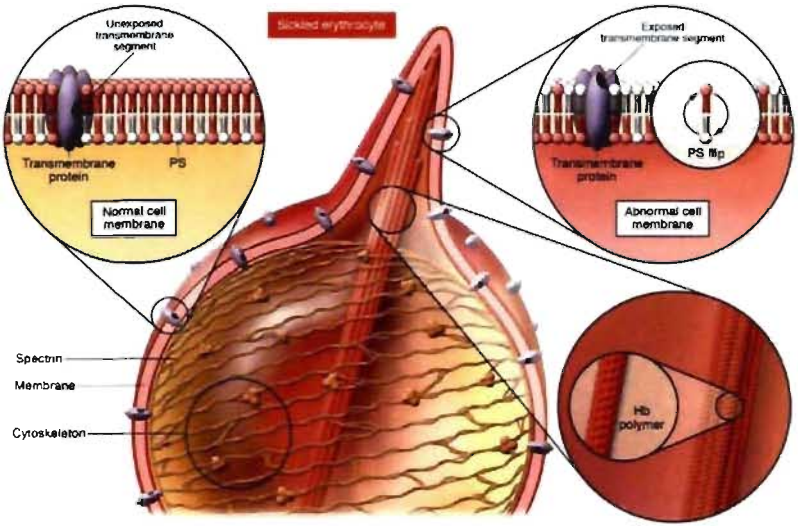

Figure 10. Alterations of the RBC membrane by polymers of sickle hemoglobin. Deoxygenation of HbS leads to the formation of hemoglobin polymer (Hb polymer, lower right, inset). The hemoglobin polymers disrupt the RBC membrane cytoskeleton and form protrusions, giving rise to the characteristic sickle or holly leaf appearance. Interruption of the attachment of the membrane to the protein cytoskeleton results in exposure of transmembrane protein epitopes and lipid exchanges, notably of phosphatidylserine (PS), between the inside and the outside of the cell (upper right, inset). Exposure of negatively charged glycolipids contributes to the proinflammatory and prothrombotic state of sickle cell blood (Adapted from Statius van Eps, 1999).

These RBCs will also occlude blood vessels resulting in ischemia and tissue damage. A combination of adhesive interactions between activated vascular endothelial cells, RBCs, leukocytes and platelets, and a proinflammatory state, hypercoagulability and

endothelial dysfunction contribute to the vaso-occlusive process **(Figure 11)** (Frenette, 2002; Ataga & Orringer, 2003; Reiter & Gladwin, 2003; Marks *et al.*, 2000). Iron deposits in tissues are observed in many but not all cases of SCD, in particular in the spleen (Rao *et al.*, 1983). However, an overt iron deficiency may occur in non-transfused patients, which could be attributed in part to excessive urinary losses of iron (Rao *et al.*, 1984; Washington & Boggs, 1975)

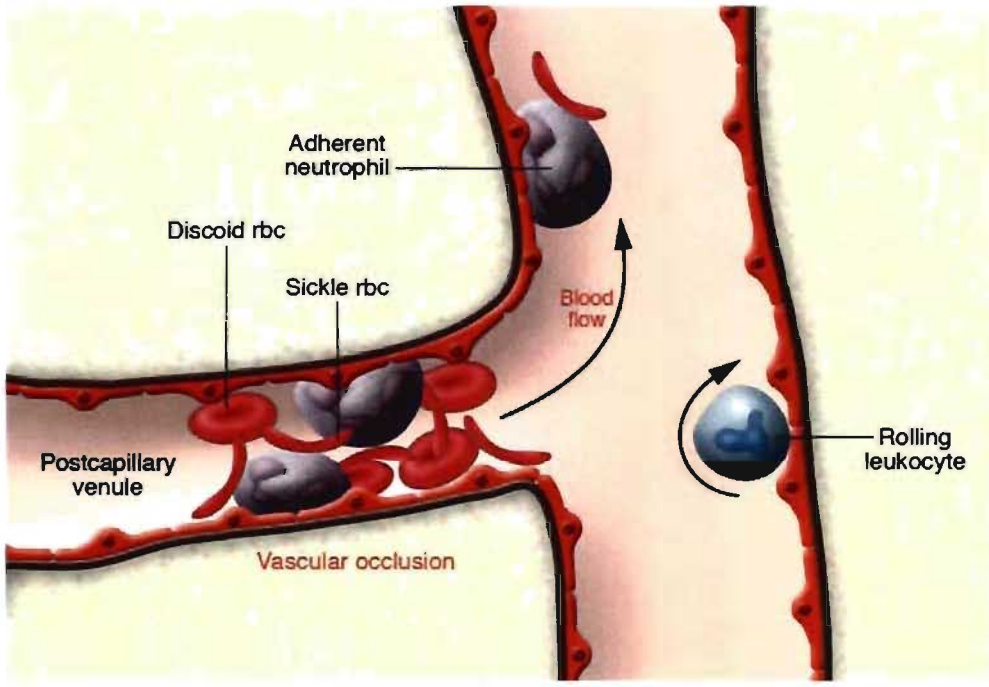

Figure 11. Simplified view of a vaso-occlusive process in sickle cell disease. Abnormal, sickle RBCs (rbc) induce the expression of inflammatory and coagulation mediators, leading to the activation of the vascular endothelium. Sickle RBC themselves may also stimulate endothelial cells directly by adhesion. The stimulated endothelial cells are poised to recruit rolling and adherent leukocytes in venules by expressing chemokines and cell adhesion molecules such as the selectins and immunoglobulin family members. Activated, firmly adherent neutrophils capture circulating discoid and sickle-shaped RBCs, leading to transient episodes of vascular occlusions that are initiated in the smallest post-capillary venules. Interactions between RBCs and leukocytes tend to occur at vessel junctions, where leukocyte recruitment is the most active. The large arrow indicates the direction of blood flow (Adapted from Frenette & Atweh, 2007).

Higher concentrations of HbS lead to more rapid polymerization. The presence of other hemoglobins like HbF and HbA_2, hinder HbS polymerization, while HbC and HbA integrate in HbS polymers limiting its formation to variable extent

(Stamatoyannopoulos *et al.*, 1994; Bunn & Forget, 1986; Embury & Stenberg, 1994; Kinney & Ware, 1994). HbS polymerizes effectively with other less common hemoglobin variants such as $HbD^{Los\ Angeles/D\text{-}Punjab}$ and HbO^{Arab} leading to less frequent double heterozygous form of SCD but not as severe (Kinney & Ware, 1994). Sickle cell disease is a relatively benign disorder in the first few months of life because of the potent anti-sickling properties of human fetal hemoglobin (HbF). HbF, which constitutes 70 to 90% of total hemoglobin at birth, is gradually replaced by HbS during the first few months of life. Rising HbS concentrations result in the onset of disease between 3 and 6 months of age (Stamatoyannopoulos *et al.*, 1994; Bunn & Forget, 1986). Combinations of SCD with β-thalassemias or α-thalassemias give rise to SCD with varying severity depending on the number and type of gene deletions (Kinney & Ware, 1994). The homozygous SCD (HbSS) is the most severe where most Hb is HbS, while in the heterozygous form (HbAS) or sickle cell trait, HbS represents approximately 40% of the total hemoglobin, and patients are generally asymptomatic (Serjeant, 1997).

I.2.4.5 Clinical manifestations of SCD

Clinical manifestations can be roughly attributed to two phenomena: hemolysis and vaso-occlusion, resulting in ischemia and tissue injury (Eaton & Hofrichter, 1990). All tissues and organs within the body are at risk for damage as a result of the vascular obstruction produced by the sickled red cells. HbSS typically presents as a severe chronic anemia with hemoglobin levels in the range of 6 to 8g/dl. Characteristically the patient demonstrates an asthenic physique and is mildly jaundiced. Many complications are associated with the disease, with the major manifestations being "sickling crises". There are three types of crises: aplastic, hemolytic, and painful (vaso-occlusive) (Steinberg, 1999; Embury *et al.*, 1994). An aplastic crisis is usually associated with infections, particularly to parvoviruses, which cause a temporary suppression of erythropoiesis. Aplastic crises usually spontaneously resolve within 5 to 10 days. A hemolytic crisis reflects an acute exacerbation of the anemia with a resulting fall in hemoglobin and hematocrit, an increased reticulocyte count, and jaundice. The usual clinical features of hemolytic

crisis include sudden weakness, rapid pulse, faintness, pallor of the lips and mucous membranes, and abdominal fullness caused by the enlarged spleen. Vaso-occlusive or painful crisis is the hallmark of sickle cell disease (Steinberg, 1999; Embury *et al.*, 1994). The crisis is usually associated with severe pain, caused by occlusion of small blood vessels mediated by the adhesion of sickled cells to endothelium, resulting in tissue damage and necrosis (Hebbel, 1991). A painful crisis usually lasts 4 to 6 days but sometimes persists for weeks (Steinberg, 1999; Embury *et al.*, 1994). It can be precipitated by infections, fever, acidosis, dehydration and exposure to extreme cold. **Table IX** summerizes the various clinical manifestations in SCD.

Table IX. Clinical features of sickle cell disease

Type of Complication	Feature
Vaso-occlusive complications	
Painful episodes	In more than 70% of patients; very frequent in some, rare in others
Stroke	In about 10% of patients in childhood; "silent" central nervous system damage with cognitive impairment in 5 to 9 times as many patients
Acute chest syndrome	In 40% of all patients; more common in children; more severe in adults
Priapism	In 10 to 40% of men; severe cases cause erectile dysfunction
Liver disease	In <2% of patients; many causes (e.g., iron overload, hepatitis B or C)
Splenic sequestration	In children <6 year old; often preceded by infection
Spontaneous abortion	In about 6% of pregnant women with sickle cell anemia; much less frequent in sickle cell-hemoglobin C disease
Leg ulcers	In about 20% of adults with sickle cell anemia; rare in sickle cell-hemoglobin C disease
Osteonecrosis	In 10 to 50% of adults with sickle cell anemia and sickle cell-hemoglobin C disease
Proliferative retinopathy	Rare in sickle cell anemia; in 50% of adults with sickle cell-hemoglobin C disease
Renal Insufficiency	In 5 to 20% of adults; severe anemia often present
Complications of hemolysis	
Anemia	Hematocrit values of 15 to 30% in sickle cell anemia; higher values in sickle cell-hemoglobin C disease
Cholelithiasis	Present in most adults; often asymptomatic
Acute aplastic episodes	Due to parvovirus B19 infection; appears with rapidly occurring, severe anemia
Infectious complications	
Streptococcus pneumoniae sepsis	In 10% of children <5 years old with sickle cell anemia
Osteomyelitis	Due to *Salmonella* and *Staphylococcus aureus*
Escherichia coli sepsis	In adults, initiated by urinary tract infection

(Steinberg, 2004)

1.2.4.6 Hereditary persistence of fetal hemoglobin (HPFH)

Hereditary persistence of fetal hemoglobin (HPFH) is a condition characterized by continuation of fetal hemoglobin (HbF) synthesis in the adult stage of erythropoiesis. Three types of abnormality are responsible for this phenotype. In deletional HPFH, the mutations delete sequences from the 3' end of the β-globin locus including the δ-

and β-globin genes (Stamatoyannopoulos and Grosveld, 2001). Levels of HbF in heterozygous carriers usually range from 20 to 30%, and HbF is distributed pancellularly among red blood cells. The mechanism of activation of HbF in deletional HPFH has been explained by various hypotheses, including the activation of the γ-globin gene by enhancer elements that are normally located in the 3' end of the locus but are juxtaposed to the γ genes as a result of the 3' deletions (Feingold and Forget, 1989; Anagnou *et al.*, 1995; Arcasoy *et al.*, 1997). The non-deletional HPFH is due to mutations that are either linked or non-linked to the β locus. The molecular basis of non-linked to the β locus HPFH is unknown. Non deletional HPFH linked to the β locus is due to mutations characterized by synthesis of either the Aγ globin chain (Aγ HPFH) or the Gγ chain (Gγ HPFH). Structural studies have shown that the Gγ or the Aγ HPFHs are due to mutations in the Gγ or Aγ gene promoters (Stamatoyannopoulos and Grosveld, 2001). The last group is characterized by modest elevations of HbF levels (1% to 4%) distributed in an uneven fashion among the F cells (subsets of erythroicytes containing HbF). In the non-linked group of HPFH cases (heretocellular HPFH), no mutations are identifiable within the β-globin cluster, and in many cases, the determinant is not linked to the β-complex, implicating the presence of transacting factors (Gianni *et al.*, 1983; Martinez *et al.*, 1989; Thein *et al.*, 1994; Giampaolo *et al.*, 1984). Surveys show that the distribution of F-cells values is skewed to the right and that approximately 10% of the normal population have at least 4.5% F cells (Sampietro *et al.*, 1992; Zago *et al.*, 1979; Miyoshi *et al.*, 1988). The importance of this condition is clearly demonstrated by the striking amelioration of the phenotype of individuals homozygous for β-thalassemia or sickle cell disease who also coinherit a HPFH determinant (Thein and Weatherall, 1989; Cappellini *et al.*, 1989).

I.2.4.7 Treatment options for SCD

The therapy for SCD includes blood transfusions (Steinberg, 1999; Embury *et al.*, 1994; Adams *et al.*, 1998; Wang *et al.*, 1991), and iron chelation for the treatment of

hemochromatosis (Vichinsky *et al.*, 2007). It also includes the use of agents that induce HbF such as hydroxyurea, erythropoietin, butyrate, and 5-azacytidine with variable outcome and toxicities (Steinberg, 2003; Saleh & Hillen, 1997; Blau *et al.*, 1993; Keefer *et al*, 2006; Atweh *et al.*, 1999).

SCD treatment also includes agents that decrease RBC dehydration like clotrimazole and magnesium (Brugnara, 2003; Stocker 2003; Muzyamba *et al.*, 2006; DeFranceschi *et al.*, 2000; Rust *et al.*, 2007), or relief from vaso-occlusion using vaso-dilators such as nitric oxide (Machado, 2007; Dasgupta *et al.*, 2006; Montero-Huerta *et al.*, 2006). The prevention and treatment of infections is also performed by chronic antibiotic administration (Steinberg, 1999; Gaston *et al.*, 1986).

As with β-thalassemia, hematopoietic stem cell transplantation (HSCT) represents the curative option. Variable degree of success is observed in the few HSCT done in human clinics. (Bernaudin *et al.*, 1993; Abboud *et al.*, 1994; Locatelli *et al.*, 2003; Kalinyak *et al.*, 1995; Ferster *et al.*, 1993; Vermylen & Cornu, 1994).

I.3 Bone marrow transplantation (BMT)

Bone marrow transplantation (BMT) belongs to the group of hematopoietic stem cell transplantation (HSCT). The objective in HSCT is to seed a damaged or destroyed host BM with pluripotent hematopoietic stem and progenitor cells (HSPC) to reestablish the host immune response and myeloid and erythroid cell lineages. HSCT is a bone marrow transplantation (BMT), peripheral blood HSCT, or cord blood HSCT, if the source of stem cells is the bone marrow (Kim *et al.*, 2007b; Chim *et al.*, 2007), peripheral blood (Gratwohl *et al.*, 2002a, b, c; 2003, 2004, 2005), or umbilical cord blood (Gratwohl *et al.*, 2005; Holyoake *et al.*, 1999; Kim *et al.*, 1999), respectively.

Under normal physiologic conditions, HSPC can enter into the blood circulation from the bone marrow. This process can be expanded by stem cell mobilization in

which cytokines such as granulocyte colony-stimulating factor (G-CSF) that increase the passage of HSPC into the bloodstream (Thomas & Ferrebee, 1962; Cavins *et al.*, 1962). Marrow grafts could be obtained from the recipient himself (autologous transplant), from a genetically identical person, i.e. identical twin (isogenic transplant) or from a histocompatible non-identical donor (allogenic transplant) (Saitos *et al.*, 2007; Yuza *et al.*, 2005). The cells responsible for recovery could be frozen and kept for long periods of time (Thomas & Ferrebee, 1962; Cavins *et al.*, 1962).

I.3.1 History of BMT

BMT trials started in the early 1900s, mostly by intravenous (IV) injection of bone marrow cells into recipient's blood, mainly in mice (Jackobson *et al.*, 1949; Lorem *et al.*, 1951), dogs (Reckers *et al.*, 1950), and humans (Osgood *et al.*, 1939; Thomas *et al.*, 1957, 1959). In the 1950s, some limited success was observed in trials with canines (Alpen & Baum, 1958; Mannick *et al.*, 1960), and humans (Mannick *et al.*, 1960). However, in these studies, allogenic bone marrow transplantation almost always resulted either in failure to engraft or in successful engraftment followed by lethal graft-versus-host disease.

It became clear that a successful allogenic BMT depended upon close histocompatibility matching between donor and recipient, and techniques were developed for histocompatibility typing (Epstein *et al.*, 1968; Schwartz & Dameshek, 1959). Only about one-fourth to one-third of patients could have a human leukocyte antigen (HLA)-identical sibling (Beatty *et al.*, 1985). The many failures in allogenic bone marrow grafting in human patients caused most researchers to abandon such studies in the 1960's. However, under the impetus of kidney grafting, the knowledge of human histocompatibility antigens progressed rapidly, and successful transplantation of HLA-matched allogenic bone marrow cells from healthy siblings into patients followed over the next two decades confirming that intravenously

administered human bone marrow stem cells were capable of homing to the marrow and providing long-term hematopoietic and immune reconstitution (Buckner *et al.*, 1970; Thomas *et al.*, 1975) and marked the beginning of the "modern" era of human allogenic BMTs (Thomas *et al.*, 1975). As follow-up times increased for patients transplanted for end-stage leukemia, it became apparent that a plateau was developing on a Kaplan-Meier plot of survival so that it became possible to use the term "cure" for these patients (Thomas *et al.*, 1977). Bone marrow transplantation is now carried out in hundreds of centers world-wide, and the number of diseases for which marrow graft might be successful continues to increase.

I.3.2 Immunological difficulties with allogenic BMTs

Allogenic BMT could be associated with complications that fall within two groups: infectious and non-infectious.

• In the non-infectious complications, the most important is the immunologic reactivity of both graft-versus-host disease (GVHD) and graft rejection. GVHD is the outcome of donor's T-lymphocytes reacting against recipient's antigens, depending on type and number of histocompatibility mismatchings (Armitage, 1994; Giliam, 2004). Acute GVHD is characterized by dermatitis, enteritis and hepatitis, appears during the first 100 days after BMT, and is a high mortality complication (Ratanathanathorn *et al.*, 2001; Barret & Bilous, 1984). Chronic GVHD develops more than 100 days after BMT and is a systemic autoimmune disease (Armitage, 1994; Giliam, 2004; Ratanathanathorn *et al.*, 2001). Graft rejection is a destructive process the recipient's residual T cells and NK cells do versus the graft cells (Murphy *et al.*, 1990).

• Infectious complications result especially when patients are greatly immuno-suppressed because of the conditioning regimens and graft-versus-host disease and its treatment, and therefore at a great risk for all kinds of opportunistic infections

(Witherspoon *et al.*, 1978). That includes bacterial, fungal and viral diseases (Meyers, 1985; Meyers *et al.*, 1982). Infectious complications could also occur after autologous/isogenic BMT following myeloablation.

I.3.3 Modalities of BMT

I.3.3.1 Route of cell transfer

BMT is classically done by infusing cells in the blood stream. Following intravenous infusion of donor cells in recipient mice, localization of stem cells to their niches is quite rapid, occurring within 5 to 15 hours and homing appears to have plateau by 1 hour (Quesenberry *et al.*, 2005). However, only a small fraction of donor stem cells will seed the bone marrow. This was demonstrated by studies showing that homing efficiency to the bone marrow after intra-venous injection of the cells in non-irradiated mice is ~17% (Jisong *et al.*, 1999), whereas in irradiated mice it is much lower, and was reported to be 1-2% (Jisong *et al.*, 1999), 6% (Stephen *et al.*, 1999), 9.3% (Cerny *et al.*, 2002), and 10% (Van der Loo & Ploemacher, 1995;Van Hennik *et al.*, 1999; Cashman & Eaves, 2000). The cells not reaching marrow niches are probably sequestrated by other organs with large capillary beds like the liver and lungs or are lost (Vos *et al.*, 1972). To overcome this problem, another strategy of transplantation has been developed: the intra-bone marrow (IBM) injection of hematopoietic stem cells. The IBM injection of allogeneic bone marrow was first used in rats (Van Bekkum *et al.*, 1967), then in mice (Kushida *et al.*, 2001), and monkeys (Ikehara, 2003). In clinical setting IBM infusion was first used in humans in 1934 (Josefson, 1934), and many trials followed (Tocantins & O'Neill, 1940; Spivey, 1987; Morrison & Samwick, 1940; Hagglund *et al.*, 1998). IBM BMTs allowed a significant reduction in the number of days of parenteral nutrition and a tendency to a reduction in the number of days on antibiotics. However, although allogeneic BMT can safely be performed by IBM infusion, hematopoieic recovery was not improved (Hagglund *et al.*, 1998). Recently, several investigators have tested and successfully used the IBM BMT to study the distribution and physiology of stem cells after IBM

injection in mice (Guillermo *et al.*, 2001; Taketoshi *et al.*, 2001; Zhong *et al.*, 2002; Tahata *et al.*, 2003; Mazurier *et al.*, 2003).

I.3.3.2 Number of cell doses

Most clinically performed BMTs consist on injecting donor cells all at once in one dose. However, some have investigated BMTs with repeated injections both in mice (Hofer *et al.*, 1989a, b; Xiang *et al.*, 2004) and humans (Basara *et al.*, 1998; Moreau *et al.*, 1996; Banerjee *et al.*, 2005; Byrne *et al.*, 2001). These studies reported that BMTs by fractionated cell infusion were efficient.

I.3.3.3 Timing of cell transfer after myeloablative conditioning

The timing of cell transfer after irradiation might have an impact on level of chimerism, as BMT after long intervals following irradiation led to lower level of donor chimerism (Hinchliff & Montague, 1988).

I.3.4 Conditioning regimens

Bone marrow transplantations performed around the world use a lethal/total or partial myeloablative regimens to eliminate recipient hematopoietic cells, whether by irradiation or by the use of chemotherapeutic drugs, or both. Such treatments that precede a BMT are called conditioning regimens. When low doses of myeloablation are used, the transplantation is referred to as reduced-intensity transplants or mini-transplants (Schanz, 2001; Kim *et al.*, 2007a; Giralt *et al.*, 2007; Worel *et al.*, 2007; Nakagawa *et al.*, 2007).

It is generally accepted that spaces have to be cleared for stem cell to engraft. Cytotoxic therapy damages marrow and actually appears to impair homing (Hendrikx *et al.*, 1996). Lethal myeloablative regimens are very toxic and limit the age of recipients to around 55 years. However, several studies showing high rates of engraftment in non-myeloablated mice have challenged the concept that myeloablation is a pre-requisite to open spaces for bone marrow stem cell

engraftment (Stewart *et al.*, 1993; Quesenberry *et al.*, 1994; Brecher *et al.*, 1979; Saxe *et al.*, 1984; Wu & Keating, 1993). It later seemed that "hemopoietic space", although useful, was not essential for engraftment of donor marrow (Reisner & Martelli, 2000).

Quantitative engraftment at the stem cell level in non-myeloablated mice indicated that the final engraftment phenotype is determined simply by the ratio of host-to-donor stem cells (Stewart *et al.*, 1993; Ramshaw *et al.*, 1995a, b; Rao *et al.*, 1997; Blomberg *et al.*, 1998). However, in most non-myeloablative regimens, low-level donor chimerism was observed after the infusion of large or very large amounts of cells (Qin *et al.*, 1989; Takada *et al.*, 1970, 1971; Micklem *et al.*, 1968; Brecher *et al.*, 1982; Saxe *et al.*, 1984), although engraftment efficiency, i.e. the percentage of recipient mice that showed donor cells in their peripheral blood, was high. Further studies showed that an irradiation dose of 1Gy with infusion of relatively low numbers of cells resulted in high levels of donor chimerism in engrafted mice (Stewart *et al.*, 1998). According to the authors, this treatment probably increased donor phenotype in syngeneic transplants, and also appeared to be stem cell toxic, but not myelotoxic since the total number of bone marrow cells was almost unchanged compared to non irradiated controls.

Irradiation is a common myeloablative conditioning (Peters *et al.*, 1979; Freedman *et al.*, 1996; Freedman *et al.*, 1996; Bierman *et al.*, 1997; Rohatiner *et al.*, 1994a, b). Radiation results in many side effects, including fatigue (king *et al.*, 1985), cataract (Hand *et al.*, 2004), acute skin reaction such as erythema and dry or moist desquamation, dermatitis (Chao *et al.*, 2001), depression, functional disabilities, changes in quality of sleep (Andersen and Tewfik, 1985; Smets *et al.*, 1998), as well as a variety of cancers (Hall and Cox, 2003; March, 1950; Myrden and Hiltz, 1969). Several chemotherapeutic agents are also used in humans clinics and animal studies. The particular types and combinations of agents used depend on many factors including the disease type and severity, the age of recipient, and the degree of myeloablation required, and therefore varies widely, and could be coupled to

irradiation. (Sandmaier *et al.*, 2007; Jantunen, 2006; Papageorgiou *et al.*, 2006). Chemotherapeutic agents also result in a variety of side effects. Among the most commonly used in clinics, busulfan and cyclophosphamide could result in hemorrhagic cystitis and pneumonitis (Morgan, 1991) among other things.

I.4. Gene Therapy

Gene therapy is defined as the administration of genetic material in order to modify or manipulate the expression of a gene product or to alter the biological properties of living cells for therapeutic use. Gene technology has become one of the most intensively developing strategies for current clinical research. It offers new treatment possibilities for many common acquired and inherited human diseases where conventional clinical procedures are less effective. These diseases include monogenic disorders, such as cystic fibrosis (Alton *et al.*, 1999; Konstan *et al.*, 2003), but also more complex disorders, such as diseases of the nervous system (Kaplitt *et al.*, 2007), and cancer (Sterman *et al.*, 1998; Holt *et al.*, 1996).

In the case of genetic diseases caused by a mutation in a specific gene, gene therapy usually involves the delivery of a functional copy of this gene into a target cell or tissue, to achieve a therapeutic benefit (Orkin, 1986). However, it can also be a tool for the treatment of non-genetic and polygenic disorders by delivering genes that stimulate immune response, suicidal genes inducing cell death, genes modifying cellular information or developmental program, or genes producing a therapeutic protein with specific function (Rubanyi, 2001).

The generic aim of gene therapy strategies is to achieve a stable expression of transgenes in the target tissue for as long as required, in an appropriately regulated form, without side effects, for example toxicity, or cellular carcinogenic transformation. According to the mode of gene delivery to the target cells, there are two major categories of somatic cell gene therapy. In the "e*x vivo*" gene therapy, cells are removed from the body, genetically modified, and returned to the body. This

procedure is generally limited to a few cell types such as blood cells, which are easy to remove and return (Hauser *et al.*, 2000). The second is the "*in vivo*" approach where the vector is administered directly to the tissue of patients (Chan *et al.*, 2003).

I.4.1 Gene delivery systems

A gene delivery system or vector is a system that enables delivery of genes into the target cells and their nucleus, provides protection from gene degradation, and ensures gene transcription in the cell (Landstrom, 2003).

Two kinds of vectors have been employed as vehicles for gene transfer. Viral vectors for gene transduction, such as retroviral, adenoviral, and adeno-associated viral vectors, and non-viral vectors for gene transfection, such as plasmids and liposomes. Each vector has its own advantages and disadvantages.

I.4.1.1 Viral delivery systems

Unlike wild-type viruses, these vectors are used to transfer therapeutic genes into target cells and thus are engineered by deleting the essential genes which allow replication and assembly of virions. These genes can be replaced by a therapeutic gene to make the genome of a gene therapy vector. Such vectors lose their ability to reproduce in target cells and can be replicated only in a cell line which supplies the deleted function. Replication deficiency ensures the safety of viral vectors per se, but on the other hand, vectors need to be produced in large amounts of virus particles. For this purpose, there are specialized cell lines called packaging cell lines engineered to replace a function of a deleted viral gene and for the production of recombinant viruses (Dando *et al.*, 2001). Required viral products could also be provided by transient transfection of producing cell lines with plasmids carrying the appropriate genes (May *et al.*, 2000, 2002).

I.4.1.2 Non-viral gene delivery systems

Non-viral techniques of gene transfer represent a simple and, more importantly, safer alternative to viral vectors. Various methods exist, including the use of lipid carriers for DNA (cationic and anionic liposomes) in the form of lipoplex (Dass, 2002; Gaucheron *et al.*, 2001; Zhdanov *et al.*, 2002), or complex DNA to polymers (polyplex) (Forrest and Pack, 2002; Merlin *et al.*, 2002) The simplest technique of non-viral gene transfer is the use of the so-called naked DNA, such as the ones used in pre-clinical and clinical trials (Nishitani *et al.*, 2000). An alternative method of naked plasmid method is the application of polymerase chain reaction (PCR) products that encode the therapeutic gene instead of plasmid DNA (Ogris, 2003).

Delivery of naked DNA to cells elicits minimal immune response as compared to DNA encapsulated in lipids or cationic polymers. The lack of immunogenicity of naked DNA makes it a good prospect for gene therapy. The limitations of this approach arise in that naked DNA is unprotected against nuclease degradation and the DNA does not have target specificity. Thus, the actual physical delivery of naked DNA must be directed towards the tissues of interest since no target ligands are attached to the DNA. Advances have been made in the development of methodologies to improve targeted delivery of naked DNA (Conweil & Huang, 2005).

I.4.2 Lentivirus and lentiviral vectors

I.4.2.1 Life cycle of Human Immunodeficiency Virus type 1 (HIV-1)

The life cycle of HIV-1 is similar to that of other retroviruses, and begins when an infectious particle encounters a cell bearing the appropriate receptor (CD4) and coreceptor (CCR5 or CXCR4) (Berger *et al.*, 1999). Following recognition between viral envelope glycoproteins and cell receptors, membrane fusion is triggered and the viral core carrying the dimeric RNA genome enters into the cytoplasm of the cell. The single-stranded genomic RNA is then converted into double-stranded proviral DNA by reverse transcriptase (RT) with the aid of nucleocapsid protein (NC). As

their name suggests, reverse transcription of RNA into DNA is a characteristic and distinctive feature of retroviruses. Subsequently, proviral DNA is transported into the nucleus and integrated into the host genome, via the viral enzyme integrase (IN). Once proviral DNA is integrated, transcription of viral mRNA occurs from the viral long terminal repeat (LTR) via the host RNA polymerase II (Coffin *et al.*, 1997). Provirus integration is a hallmark of successful replication of a retrovirus. Its life cycle can be thus divided into events that precede or follow integration, defined as early and late steps of the viral life cycle, respectively.

I.4.2.2 Genome of HIV-1

The HIV-1 proviral genome is flanked by two long terminal repeats (LTRs) and encodes the structural genes (*gag*, *pro*, *pol*, and *env*), and the non-structural genes *tat*, *rev*, *nef*, *vif* , *vpr* and *vpu*. The presence of such a large number of non-structural proteins sets lentiviruses apart from other classes of retroviruses (Coffin *et al.*, 1997).

I.4.2.3 Structural proteins of HIV-1

Gag, Pro, Pol, and Env are translated in the form of polyprotein precursors that, with the exception of Env, are processed by viral-encoded protease. Thus, Gag upon processing yields the following proteins: matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7), and p6. (Krausslich & Welker, 1996; Freed, 1998). These proteins as part of the Gag precursor carry out necessary and specific functions during viral assembly and are often referred to as domains of Gag.

In HIV-1, the gene encoding the viral protease, *pro*, and the gene expressing the polymerase and integrase, *pol*, is translated as part of a single Gag-Pro-Pol polyprotein precursor of 160 kDa (p160). Translation of the Pro-Pol requires a -1 frameshift that allows reading through the gag stop codon. This event is imposed in cis by a slippery sequence present on the genomic RNA and occurs at a frequency of about 1 in 20 translation events, which accounts for the relative ratio between Gag and Gag-Pro-Pol (Coffin *et al.*, 1997). As a result of viral protease processing, the protease itself (PR), the reverse transcriptase heterodimer (RT) and the integrase (IN) are released from the precursor.

I.4.2.4 Accessory proteins of HIV-1

There are 6 accessory proteins in HIV-1 and their major characteristics are summarized in **Table X**.

I.4.2.5 Lentiviral and γ-retroviral vectors

For the treatment of hemoglobinopathies, persistence of the transgene is required and therefore, integration in the genome of the HSC is a necessity. In the absence of stable episomes and highly efficient homologous recombination, the use of integrating viral systems has dominated this field of investigations. Recombinant retroviruses have established themselves as the tool of choice, eventually soliciting three different generations: γ-retroviruses (or C-type retroviruses, or oncoretroviruses, e.g. MLV, MMTV, HTLV-1, HTLV-2), lentiviruses (HIV-1, HIV-2), and spumaviruses. Other vector systems such as adeno-associated virus have so far met with limited success in the context of HSCs (Hargrove *et al*, 1997; Srivastava, 2005).

γ-Retroviral vector systems have been extensively used in small- and large-animal model transplant and approved for use in over 40 clinical trials based on the ex-vivo transduction of autologous stem cells (Kohn, 2003). Lentiviral vectors entered the stage a decade ago (Naldini *et al.*, 1996) and rapidly elicited broad interest for their ability to transduce non-dividing cells (Sutton *et al.*, 1998; Uchida *et al.*, 1998; Miyoshi *et al.*, 1999; Case *et al.*, 1999) and accommodate large genomic sequences needed for effective transgene regulation (May *et al.*, 2000). Spumaviral vectors are the most recent addition to this cast (Mergia & Heinkelein, 2003) and are only starting to be evaluated in HSCs (Vassilopoulos *et al.*, 2001; Trobridge *et al.*, 2006)

Retroviral vectors have been tested for many diseases, including blood disorders, and hemoglobinopathies in particular. During the long history of trials, several factors were target for optimization, including vector backbone, vector tropism (surface glycoprotein), and transgene expression (promoters, additional regulatory elements).

Table X. Accessory proteins of HIV-1

Protein	Role	References
Tat Transcription activator	Molecular adapter between transcription machinery & viral RNA[1,3,5,9,10] Does not bind DNA[9,10] Binds viral RNA on TAR (transactivation response region) [6,7,8] ↓MHC-I [2,4] NLS-like domain [9,10]	1-Benkirane *et al.*., 1998; 2-Kerkau *et al.*., 1997; 3-Kessler & Mathews, 1992; 4-Howcoft *et al.*, 1993; 5-Kamine *et al.*, 1996 ; 6-Ratnasabapathy *et al.*, 1990 ; 7-Zhou & Sharp, 1995 ; 8-Marcello *et al.*, 2001 ; 9-Marshall & Price, 1999 ; 10-Marzio *et* al., 1998
Rev Regulator of expression of virion protein	Possesses an NLS and NES [12, 13, 14] Binds viral RNA on RRE, as multimeric complexes[11,12] Exports full-length viral RNA from nucleus to cytosol[12,13] Binds RRE, located in the *env* gene [13,14,15]	11-Fankhauser *et al.*., 1991; 12-Malim *et al.*, 1989; 13-Meyer *et al.*., 1996; 14-Truent & Cullen, 1999; 15-Wen *et al.*, 1995
Nef Negative factor	Membrane associated [16] ↓CD4, ↓MHC-I [16] Stimulates virus infectivity [16,17,18] ↑FasL on infected cells, causing apoptosis of FasR harboring cells [17] ↑CD40 on macrophages, ↑ inflammation [17] N-terminal myristoylation site [16]	16-Aiken *et al.*, 1994; 17-Collins *et al.*., 1998; 18-Howcoft *et al.*, 1993
Vpr Viral protein R	Transports PIC into nucleus [21,22,25] Incorporated in virion [23,24] Induces cell cycle arrest in G2 phase [19,20] Anti-inflammatory [26] Interacts with importins and nucleoporins [21,22] NLS [21,22]	19-Emerman & Malim, 1998 ; 20-Sherman *et al.*, 2002; 21-Subbramanian *et al.*, 1998; 22-Eckstein *et al.*, 2001; 23,24-Tristen *et al.*, 1998, 1992; 25-Heinzinger *et al.*, 1994; 26-Muthumani *et al.*, 2004
Vpu Viral protein U	Transmembrane protein [27,28,34,40] Phosphorylated [32] Not incorporated in virion [39] ↓CD4, ↓MHC-I [35,36,37,38] Enhance virion release [33,36] May form ion channels [27,28,29,30,31]	27-Gonzalez & Carrasco, 1998; 28-Grice *et al.*., 1997; 29-Coady *et al.*, 1998;; 30-Cordes *et al.*, 2001; 31-Ewart et al., 1996 ; 32-Friborg *et al.*, 1995 ; 33-Gottlinger *et al.*, 1993 ; 34-Maldarelli *et al.*, 1993 ; 35-Margottin, 1998 ; 36-Paul *et al.*, 1997, 1998 ; 37-Piguet *et al.*, 1999 ; 38-Speth *et al.*, 1999 ; 39-Strebel *et al.*, 1988, 1989 ; 40-Tigamos *et al.*, 1998
Vif Virion infectivity	Enhances reverse transcription [43] Binds reverse transcriptase and NC [43] Enhances infectivity [42,47,48] Phosphorylated [44] Soluble [41] Incorporated in virion [41,44,45,46]	41- Camaur & Trono, 1996; 42-Courcoul *et al.*., 1995; 43-Gabuzda *et al.*, 1992; 44-Karczeweski *et al.*, 1996; 45-Khan *et al.*, 2001; 46-Liu *et al.*, 1995; 47-Ma *et al.*, 1994; 48-Madani *et al.*, 1998;

MHC-1, major histocompatibility complex type 1; NLS, nuclear localization signal; NES, nuclear export signal; PIC, pre-integration complex; NC, nucleocapsid; LTR, long terminal repeat; FasR, Fas receptor; FasL, Fas ligand.

Retroviral LTRs were frequently used to drive transgene expression because of the robust and ubiquitous expression they provide (Suzuki *et al.*, 2002; Uren *et al.*, 2005). However, cases of insertional oncogenesis occurred with LTR-driven vectors (Hacein-Bey-Abina *et al.*, 2003), calling into question the use of LTR in stem cell therapy. The deletion of the promoter region within the LTR was subsequently used to minimize the occurrence unwanted side effects, which led to the so-called self-inactivating (SIN) vectors (Emerman and Temin, 1986).

In the settings of gene therapy for globin diseases, the LCR was too large to be all incorporated in γ-retroviral vectors which could not be stably packaged owing to frequent rearrangements. Therefore, small LCR fragments consisiting of 200-300bp fragments from the hypersensitive sites (HS), containing only the core sequences, have been used. However, the inclusion of the core LCR elements into Moloney Leukemia Virus (MLV)-based γ-retroviral vectors led to low levels of expression that was prone to position effect (Karlsson *et al.*, 1988; Bender *et al.*, 1989; Sadelain *et al.*, 1995). Inclusion of larger LCR elements in transgenic mice resulted in high level transgene expression (Ellis *et al.*, 1997). However, due to the size constraints of MLV vectors, individual HS site cores or a few combinations of these have been tested in MLV vectors with variable expression (Bender *et al.*, 1989; Novak *et al.*, 1990; Raftapoulos *et al.*, 1997). In addition, globin gene expression is intron-dependent and the use of cDNA led met many difficulties in expression. Investigators therefore turned to the use of genomic sequences of globin genes, as depicted in **Table XII**. However, intron-2 of β-globin leads to low titers by destabilizing the vector. Deletion of a 372bp fragment of the intron-2 of the β-globin gene (Miller *et al.*, 1988; Leboulch *et al.*, 1994), which contains most of the "instability elements", helps stabilizing the viral genomic RNA.

Furthermore, γ-retroviral long terminal repeats (LTRs) transcriptionally interferes with LCR elements within MLV vectors, resulting in unstable proviral transmission and/or poor transgene expression (Sadelain *et al.*, 1995; Walsh *et al.*, 1993). Transcriptional interference from the LTR (Emerman and Temin, 1986) was

overcome by self-inactivating (SIN) vectors, the LTR promoter/enhancers were deleted upon integration of the provirus. However, SIN MLV vectors have a loss in titers resulting from the LTR deletion. Replacement of the viral LTR with enhancers from other erythroid genes (the GATA-1 (Grande et al., 1999) or HS-40 (Ren et al., 1996; Emery et al., 1999), the distal control element from the α-globin locus) has met reasonable success.

In order to increase the retroviral-mediated gene expression, many modifications to vectors have been done. 1-The delection of silencing elements in the U3 region of the LTR (Dalle *et al.*, 2005; Zufferrey *et al.*, 1998; Dull *et al.*, 1998); 2-the use of robust promoters (promoters less likely to be silenced), such as PGK (Woods *et al.*, 2000; Salmon *et al.*, 2000; Ramezani *et al.*, 2000); 3-the introduction of introns in LTR-driven retroviral vectors (Riviere *et al.*, 1995; Armentano *et al.*, 1987); 4-the introduction of the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (Salmon *et al.*, 2000; Ramezani *et al.*, 2000; Zufferey *et al.*, 1999; Schambach *et al.*, 2000); 5-the use of insulators or boundary elements (West *et al.*, 2002, 2004; Recillas-Targa *et al.*, 2002); 6-the use of scaffold or matrix attachment regions (S/MARs) (Agarwal *et al.*, 1998; Kurr *et al*, 2003).

Large regulatory elements were finally stably transduced in the context of lentiviral vectors, which possess the Rev protein to regulate RNA stability (May *et al.*, 2000). The use of lentiviral versus γ-retroviral vectors also spared the need for the target cells to be dividing, and promised several advantages for gene transfer (**Table X).**

An important element that remains a hindrance in the current gene transfer trials is the transduction efficiency, which appears to depend both on the nature of the target cells, and the vector surface glycoprotein (GP). In this regards, various GP were utilized over the time. Ecotropic proteins initially gave promising results in rodents but disappointing in higher mammals. Amphotropic proteins target receptors poorly expressed on the surface of higher mammalian cells, and did not yield the desired outcome. Various heterologous GP were tested, and the Vesicular Stomatitis Virus G-protein (VSV-G) became the most popular. VSV-G was suggested to interact with a ubiquitous receptor giving the vector a broad host-cell range (Schlegel, 1983). It also confers high vector particle stability (Burns, 1993; Bartz, 1996). One drawback for the VSV-G is that it is inactivated by human serum complement (DePolo, 2000) However, the most significant shortcoming for VSV-G is that it is toxic to cells if expressed constitutively (Ory, 1996).

Retroviral-mediated gene transfer may be used not only to express transgenes but also to delete undesirable transcripts using RNA interference (RNAi) (Tahara *et al.*, 2004). Recently, the use of RNAi for globin diseases was investigated in vitro in the context of SCD (Samakoglu *et al.*, 2006).

The difference in the ability of γ-retroviral and lentiviral vectors to transduce HSC of various species seems related to HSC properties. HSC are a minor population of the adult bone marrow (Boggs *et al.*, 1984; Szilvassy *et al.*, 1990; Benveniste *et al.*, 2003). This pool of essential cells remains in a relatively quiescent state (Bradford *et al.*, 1997; Mahmud *et al.*, 2001). Under steady-state hematopoiesis, most murine HSCs enter into cycle over a period of 1-2 months (Bradford *et al.*, 1997; Cheshier *et al.*, 1999). The HSC turnover rate in cats, rhesus macaques, baboons, and humans is even slower (1-2 years) (Nash *et al.*, 1988; Abkowitz *et al.*, 1995; Rufer *et al.*, 1999; Kim *et al.*, 2000), which may in part explain the least efficient γ-retroviral-mediated gene transfer in large animals. Some recent studies challenge this view, suggesting that viral particle uncoating, rather than nuclear import, is responsible for the cell cycle dependence of retroviral infection (Yamashita & Emerman, 2005).

However, although lentiviral vectors compare favorably to γ-retroviral vectors in overall G0/G1 stem cell populations (**Table XI**) (Uchida *et al.*, 1998), it has been clearly established that cells in G1 are more readily transduced than cells in G0 and it has been inferred that integration in G0/G1 stem is confined to cells that exit G0 and entering G1 (Sutton *et al.*, 1999; Korin & Zack, 1999). It's indeed generally agreed that cells must progress into a G1b state for reverse transcription to occur during lentivirus infection (Uchida *et al.*, 1998; Korin & Zack, 1998; Zack *et al.*, 1990; 1992).

Table XI. Major properties of lentiviral and γ-retroviral vectors

γ-Retroviral vectors	Lentiviral vectors	References
Integrate in genome of dividing cells only	Integrate in genome of both dividing and non-dividing cells	Miyoshi *et al.*., 1999; Lewis & Emerman, 1994; Korin & Zack, 1998
Integration restricted to S-phase	Integration not restricted to S-phase	Sutton *et al.*., 1998; Uchida *et al.*., 1998, Evans *et al*,., 1999, Guenechea *et al.*, 2000; Case *et al.*, 1999
No recognizable NLS in viral components	HIV-1 has NLS in gag matrix protein (MA), Vpr, integrase, Rev and tat cppt enhances nuclear import therefore increase transduction efficiency in unstimulated HSCs	Bukrinsky *et al.*, 1993; Gallag *et al.*, 1997 , 1995 ; Goff, 2000 ; Heinzinger *et al.*, 1994
Preferential integration near promoters as with MLV	Preferential integration of HIV-1 into transcribed regions of expressing genes	Wu *et al.*., 2003; Schroder *et al.*., 2002; Mitchell *et al*,. 2004
MLV integrates at 48.8% into or near genes	HIV-1 integrates at 65.2% into or near genes	Wu *et al.*, 2003; Ellis, 2005
MLV integrates into CpG islands at 16.8%	HIV-1 integrates into CpG islands at 2.1% 35% of HIV-1 integrations are greater than 5kb from any gene and therefore, candidate for integration in heterochromatin HIV-1 preferentially integrates into repetitive Alu elements (15%)	Wu *et al.*, 2003; Ellis, 2005
Wild type γ-retroviral vector expresses less efficiently than SIN lentiviral vector	SIN lentiviral vector expresses more efficiently than wild-type γ-retroviral vector	Lois *et al.*., 2002; Pfeifer *et al.*, 2002; Ikawa *et al.*., 2003
SIN γ-retroviral vector integrates in single copy	SIN lentiviral vector integrates as multiple copy	Hamaguchi *et al.*, 2000
No rev and no rev response element (RRE)	Presence of rev and rev response element (RRE), which functions as a strong nuclear export element allowing to package full-length unspliced RNA	Sadelain *et al.*, 1995
β-globin cassettes are less stable in γ-retroviral vectors	β-globin cassettes are more stable in lentiviral vectors	Sadelain *et al.*, 1995
Can hold small cargo	Can hold much larger cargo (9-10 kb). Therefore, can hold large fragments of LCR	Sadelain *et al.*, 1995

LCR, locus control region; NLS, nuclear localization signal; Vpr, viral protein R; Rev, regulator of expression of virion proteins; Tat, transcription activator; HSCs, hematopoietic stem cells; MLV, murine leukemia virus; HIV, human immunodeficiency virus; SIN, self-inactivating; kb, kilobase pair

I.4.3 Gene therapy assays in mouse models for β-thalassemia and sickle cell disease

Many gene therapy assays for β-thalassemia and SCD have been carried out in mouse models, often using high loads (multiplicity of infection, MOI) of viral vectors (Hanawa *et al.*, 2004; May *et al.*, 2000, 2002; Rivella *et al.*, 2003; Imrem *et al.*, 2002, 2004; Persons *et al.*, 2003; Pawliuk *et al.*, 2001; Levasseur *et al.*, 2003; Oh *et al.*, 2004), and rarely, low MOI (Puthenveetil *et al.*, 2004). Variable degrees of phenotype improvement and multiple integrations of the therapeutic transgene were often observed in these trials. **Table XII** illustrates the major attempts to express globin genes from retroviral vectors and the major assays in mouse models and the vectors used.

Table XII- Major gene therapy assays in mouse models for β-thalassemia and SCD

Vector	Disease	Tg	Globin-specific elts	Cytokines	Inc.	MOI	% Tg Pr.	Effect on phenotype	Ref.
γ-RV	wt	β-globin HG	β-globin promoter and 3' enhancer, core LCR elements	N.I.	N.I.	N.I.	4-8%	N/A	Dzirzak et al., 1988
γ-RV	wt	β-globin HG	β-globin promoter	10% serum, polybrene	24h	N.I.	Variable, Not quantified	N/A	Miller et al., 1988
γ-RV	wt	β-globin HG	β-globin promoter, core LCR	10% serum, polybrene	N.I.	N.I.	0.0098-0.04	N/A	Bender et al., 1989
γ-RV	wt	β-globin HG	β-globin promoter	N.I.	N.I.	N.I.	24	N/A	Novak et al., 1990
γ-RV	Erythro leukemia cells	β/δ hybrid globin, HG	β-globin promoter, LCR HS2 element	N.I.	N.I.	N.I.	N.I.	N/A	Takekoshi et al., 1995
γ-RV	wt	γ-globin HG	β-globin promoter and 3' enhancer, LCR HS2 & HS3, α-globin HS40	Polybrene	24	N.I.	73-165	N/A	Li et al., 1999
γ-RV	wt	β-globin HG (& EGFP)	β-globin promoter, LCR HS2, HBPRE	IL3, 6, SCF, Fibn, Prot, 15% serum	Pre: 48h Vect:48h	N.I.	0.1-20	N/A Preselection of EGFP positive cells	Kalberer et al., 2000
γ-RV	wt	γ-globin HG	Ankyrin 1 promoter	IL3, 6, SCF, polybrene	Pre: 48h Vect:48h	N.I.	3%	N/A	Sabatino et al., 2000
γ-RV	wt	γ-globin HG	β-globin promoter	IL3, 6, SCF, polybrene	Pre: 48h Vect:48h	N.I.	Undetectable	N/A	Lung et al., 2000
LV	β-Thal	β-globin HG	β-globin promoter and 3' enhancer,: HS2,3,4 core (RNS1) or large sections (TNS9); IVS2Δ	IL1α, 3, 6, SCF; β-ME; Gln; Polybrene	Pre: 18h Vect: 6h	N.I.	RNS1: 2-7 TNS9: 10-20	RNS1: almost no effect TNS9: moderate long term improvement of hematological profile	May *et al*, 2000
LV	SCD	$\beta^{A\text{-}T87Q}$-globin HG	β-globin promoter and 3' enhancer,: HS2,3,4 large sections; IVS2Δ	IL1α, 3, 6, SCF; Gln; Prot.; Ret.	5-6h	N.I.	16 ± 3.1	Intermediate long term improvement of hematological parameters; ↓RBC dehydration & sickling; correct SpG	Pawliuk *et al.*, 2001

Table XII- Major gene therapy assays in mouse models for β-thalassemia and SCD...continued

Vector	Disease	Tg	Globin-specific elts	Cytokines	Inc.	MOI	% Tg Pr.	Effect on phenotype	Ref.
LV, SIN`	wt	β/γ hybrid HG	β-globin promoter and 3' enhancer, HS2; woodchuck regulatory element; alternative promoters (ankyrin-1; α-spectrin; ζ-globin)	IL6, Flt3L, SCF, Tpo, Epo, dNTP, LDL	Pre: o/n Vect: 3x12h	100	11-28	Not assessed	Moreau-Gaudry et al., 2001
LV	β-Thal	β-globin HG	β-globin promoter and 3' enhancer,: HS2,3,4 large sections (TNS9); IVS2Δ	IL1α, 3, 6, SCF; β-ME; Gln; Polybrene	8h	N.I.	10-20	Intermediate long term improvement of hematological profile; ↓ iron deposits in various organs; correct SpG	May *et al.*, 2002
LV	β-Thal	β-globin HG	β-globin promoter and 3' enhancer,: HS2,3,4 large sections; IVS2Δ	Il11, Flt3L, SCF, Ins, Transf, β-ME ; Gln, Fibn	Pre : o/n Vect : 5h	N.I.	32 ± 4	Intermediate long term correction of RBC morphology and CBC; ↓ iron deposits in spleen & liver; correct SpG	Imren *et al.*, 2002
γ-RV	Hemoglobinopathies	γ-globin HG	β-globin promoter, α-globin enhancer; HS2,3,4 large sections; IVS2Δ; cHS4**	IL3, 6, SCF, Gln, NaPy, Polybrene	Pre: 48h Vect:48h	N/A	10.7-26.5	Not assessed; 3-fold increase in transgene expression with insulated versus non-insulated vector	Emery *et al.*, 2002
LV	β-Thal	β-globin HG	β-globin promoter and 3' enhancer,: HS2,3,4 large sections (TNS9); IVS2Δ	IL1α, 3, 6, SCF; β-ME; Gln; Polybrene	8h	N.I.	10-20	Rescue β-globin null mice Hbbth3/th3; mice survive at least 4 months instead of 2; improved hematological profile; ↓ iron deposits in spleen & liver	Rivella *et al.*, 2003
LV	β-Thal	γ-globin HG	β-globin promoter and 3' enhancer,: HS2,3,4 large sections; IVS2Δ	IL3, 6, SCF	Pre: 48h Vect:24h	40	12-25	Mild short term correction of RBC morphology & CBC.	Persons *et al.*, 2003
LV, SIN	SCD	$\beta^{A\text{-}S3}$-globin HG	β-globin promoter and 3' enhancer, HS2,3,4; large sections; IVS2Δ	N.I.	N.I.	30	20-25	Intermediate long-term correction of RBC morphology, CBC, & pathology of spleen, liver & kidney	Levasseur *et al.*, 2003
LV	β-Thal	Anti-sense snRNA	N/A	Epo, SCF, Gln, β-ME	N.I.	N.I.	N/A	In-vitro only; 25-fold increase in levels of HbA compared to non-treated cells	Vacek *et al.*, 2003

Table XII- Major gene therapy assays in mouse models for β-thalassemia and SCD...Continued

Vector	Disease	Tg	Globin-specific elts	Cytokines	Inc	MOI	% Tg Pr.	Effect on phenotype	Ref.
γ-RV	SCD	$\beta^{A\text{-}T87Q}$-globin HG	β-globin promoter and 3' enhancer, HS2,3,4; large sections; IVS2Δ	IL3, 6, SCF, G-CSF, Flt3L, LDL, β-ME ; Fibn , Prot,	Pre: 48h Vect:64h	N.I.	10	N/A: NOD/SCID recipient mice; Long-term expression after xenotransplantation of human CD34+ cells	Oh *et al.*, 2004
LV	β-Thal	γ-globin HG	β-globin promoter and 3' enhancer, HS2,3,4; large sections; IVS2Δ	IL3, 6, SCF, Polybrene	Pre: 48h Vect: 6h	100-300	8-22	Improved hematological parameters	Hanawa *et al.*, 2004
LV	SCD	γ-globin and shRNA HG	β-globin promoter and 3' enhancer, HS2,3,4; large sections; IVS2Δ; shRNA in intron 2	IL1α, 3, 6, SCF; Gln; Ret, Prot.	5-6h	N.I.	N/A	siRNA does not affect normal γ-globin transcripts; 29-fold reduction of HbS in CD34+ derived from SCD patients	Samakoglu *et al.*, 2006
LV	SCD	$\beta^{A\text{-}T87Q}$-globin HG	β-globin promoter and 3' enhancer, HS2,3,4; IVS2Δ	IL6, Flt3L, SCF, Tpo, Ins, Transf, β-ME ; Ret.	Pre: o/n Vect:6h	140	48-59	N/A: NOD/SCID recipient mice; Long term & efficient transduction of human cord blood stem cells	Imren *et al.*, 2004
LV , SIN	β-Thal	β-globin HG	β-globin promoter and 3' enhancer, HS2,3,4; large sections; IVS2Δ; cHS4	IL6, Flt3L, Tpo, SCF, Ret., Prot.	Pre: o/n Vect: 2x4h	1-14	7.3 ± 7	N/A: NOD/SCID recipient mice; Effective erythropoiesis 3-4 months after xenotransplantation of human CD34+ cells	Puthenveetil *et al.*, 2004

LV, lentiviral; RV, retroviral; β-thal, β-thalassemia; SCD, sickle cell disease; Ref, referemces; HG, human genomic;wt, wild type;Tg, transgene; elts, elements; Inc, incubation; Pr., protein; MOI, multiplicity of infection ;IVS2Δ, deletion of 272bp AT-rich region in intron 2 of the β-globin gene that interferes with vector production leading to low titers; cHS4, chicken hypersensitive site 4 as insulator; SpG, splenomegaly; SIN, self inactivating; N/A, not applicable; *, Anti-sense snRNA to block a specific aberrant splice site in a human β-globin; IL, interleukin; β-ME, β-mercaptoethanol; SCF, stem cell factor; Gln, glutamine; Pre, preculture; Vect, culture with vector; N.I., not indicated; Prot, protamine; Ret, retronectin; Ins, insulin; Transf, Transferrin; Fibn, fibronectin; NaPy, sodium pyruvate; N/A, not applicable; Epo, erythropoietin; Prot, protamine; LDL, low density lipoproteins; HBPRE, hepatitis B posttranscriptional regulatory element

I.5 Mouse Models for β-thalassemia and SCD

I.5.1 Mouse models for β-thalassemia

There are six murine models that reproduce various severities of the human β-thalassemia. One is naturally occurring, while the remaining five are experimentally generated.

- **Hbb^{th-1}**

The first mouse model for β-thalassemia resulted from a naturally occurring deletion of the adult β^{maj} globin gene and its promoter and was described by Skow in 1983 (Skow *et al.*, 1983). Most mice homozygous for the deficiency survived to adulthood and reproduced but were smaller at birth than their littermates and demonstrated a hypochromic, microcytic anemia with severe anisocytosis, poikylocytosis and reticulocytosis. Heinz bodies are observed in a high proportion of circulating RBCs. Mice heterozygous for the deficiency demonstrated a mild reticulocytosis but were not clinically anemic.

- **Hbb^{th-2}**

The second model for β-thalassemia was generated by Shehee (Sheshee *et al.*, 1993) by a disruption of the adult β^{maj} globin gene in mouse embryonic stem cells using homologous recombination to insert selectable sequences into the gene. Mice homozygous for this insertional disruption are severelly anemic and die perinatally, while heterozygotes show severe thalassemia intermedia phenotype.

- **Hbb^{th-3}**

In this model generated by Yang (Yang *et al.*, 1995), both adult cis β-globin genes have been deleted. Mice homozygous for this deletion die perinatally, similar to the most severe form of Cooley's anemia in humans. Mice heterozygous for the deletion show hematologic indices characteristics typical of severe thalassemia. Tissue and organ damage typical of β-thalassemia, such as bone deformities and splenic

enlargement due to increased hematopoiesis are present. Spontaneous iron overload in the spleen, liver and kidneys, are also seen in the heterozygous animals.

- **Hbb^{0}/Hbb^{S}**

This thalassemic model is the one selected for this study. In this model generated by Ciavatta (Ciavatta *et al.*, 1995), both adult cis β-globin genes, β^{maj} and β^{min}, have been deleted. Homozygous animals die in utero, however, heterozygous mice are fertile and transmit the deleted allele to progeny. Heterozygous animals reproduce the human β-thalassemia *intermedia.* That includes severe anemia with dramatically reduced hemoglobin levels, abnormal red cell morphology (microcytosis, anisocytosis, poikylocytosis, hypochromia), splenomegaly and markedly increased reticulocyte counts and many histological abnormalities. Further characterization revealed the presence of an ineffective erythropoiesis, increased numbers of immature hematopoietic precursors and stem cells, and reduced red blood cell half-life (Beauchemin *et al.*, 2004).

- **Hbb^{th-4}**

In this model generated by Lewis (Lewis *et al.*, 1998), two murine adult cis β-globin genes were replaced with a human β-globin gene containing a splice site mutation that results in no functional human β-globin chain. No homozygous mice survived postnatally, and heterozygotes showed classic signs of β-thalassemia including anisocytosis and poikylocytosis. Typical thalassemic anomalies are found in many organs including liver, spleen, heart and kidneys, as well as iron deposition.

- **$DH^{\Delta 4bp}$**

Generated by Jamsai (Jamsai *et al.*, 2005), this model consists of a double heterozygous for a mutant human β-globin gene containing a 4bp deletion and the $Hbb^{th3+/-}$ model. Mice are very anemic and have characteristic damage of severe β-thalassemia.

I.5.2 Mouse models for Sickle Cell Disease

A panoply of animal models for sickle cell disease exists. They could be broadly divided in two groups, based on whether or not they produce murine globin chains

I.5.2.1 Models producing both murine and human globin chains

The first models expressing moderately high levels of both human α- and β^S-globin chains were reported by Greaves (Greaves *et al.*, 1990) and Ryan (Ryan *et al.*, 1990). The former could not reproduce and the only reported characteristic is the presence of irreversibly sickled cells in 0.1% frequency. The latter was reported to present slight anemia and RBC sickling when crossed to thalassemic mice. Subsequently, many different lines of mice with variable degrees of severity reproduced, allowing more detailed studies of pathology.

- **SAD-1 mouse**

This is the model used in the current thesis project. Generated by Trudel (Trudel *et al.*, 1991, 1994) and extensively characterized (Trudel *et al.*, 1991, 1994; Blouin *et al.*, 1999; DePaepe & Trudel, 1994), this mouse expresses the human β^{SAD} and α-globin transgenes. β^{SAD} induces strong hemoglobin polymerization, and results in the formation of a new form of hemoglobin, human HbSAD ($\alpha_2^h \beta_2^{SAD}$), which has a high polymerization potential when diluted by the endogenous mouse hemoglobin. The β^{SAD} gene carries three mutations: β^S, $\beta^{S\text{-Antilles}}$, and $\beta^{DLos\text{-}Angeles}$, where the latter two exacerbate polymerization (Section II.4.6). The level of human HbSAD is at 19% (Trudel *et al.*, 1991). This model closely reproduces the human SCD on molecular, biological, pathological and clinical levels. That includes the presence of an ineffective erythropoiesis, increased RBC turnover, vaso-occlusions, organ damage, and shortened lifespan. **Table XIII** summarizes the major disease characteristics of the SAD-1.

Table XIII. Major disease characteristics in the SAD mouse model

HbSAD polymerization[a]
RBC sickling[a]
Increased RBC density[b]
Increased mean corpuscular hemoglobin concentration (MCHC)[a]
Chronic hemolysis[a, d]
Irreversibly sickled cells (ISC) 0.5-2.5%[a]
Ineffective medullary erythropoiesis[d]
Anemia in newborns that seems to be compensated for in the adults (who are not anemic)[a]
Microvascular occlusion in different tissues, especially lungs and liver[b]
Skin ulcers and underlying congestion and thrombosis[b]
Splenomegaly[b]
Splenic sequestration of RBCs[b]
Splenic extramedullary erythropoiesis[d]
Splenic fibrosis[b]
Loss of spleen architecture[b]
Glomerular hypertrophy and hyperplasia of mesangial cells[c]
Glomerulosclerosis[c]
Renal fibrosis and papillary necrosis[c]
Hyperplasia of kuppfer cells in the liver[b]
Iron deposits in tissues[b]
Shortened lifespan (survival ~15months)[b]

a, Trudel *et al.*, 1991; b, Trudel *et al.*, 1994; c, DePaepe & Trudel, 1994; d, Blouin *et al.*, 1999

- **The Costantini-Fabry-Nagel (NYC1) model**

Generated by Fabry (Fabry *et al.*, 1992a, b) this model, in which 75% of all β-like chains are human β^S, expresses murine β^{minor}, but not β^{major} which is achieved by breeding a mouse line carrying a deletion of β^{major} (Skow *et al.*, 1983) into the transgenic line. This model exhibits mild biological and clinical features of SCD (Fabry *et al.*, 1992a, 1992b, Lutty *et al.*, 1994; Shear *et al.*, 1993)

- **The S + S-Antilles model (Fabry *et al.*, 1995).**

This model is an NYC-1 mouse with the addition of the β^S-Antilles transgene (Rubin *et al.*, 1991) and homozygous deletion of mouse β^{major}. The product of the $\beta^{S\text{-Antilles}}$ gene is a hemoglobin with the sickle mutation coupled to a second mutation that enhances polymerization and reduces the oxygen affinity (Montplaisir *et al.*, 1986). The phenotype is more severe than the NYC1 model with homozygous β^{major} deletion. There is no adult anemia, but the model exhibits severe anomalies characteristic of SCD

I.5.2.2 Models producing exclusively human globin chains

These models are also called knock-out models because the transgenic mice expressing the sickling globin has been crossed to mice heterozygote knock out for murine α- and β-globin genes, progressively resulting in mice that produce only human α- and β-globin chains.

- **Transgenic mice with thalassemic characteristics**

Three models generated by Paszty (the Berkeley or BERK mouse) (Paszty *et al.*, 1997), Ryan (Ryan *et al.*, 1997), and Chang (Chang *et al.*, 1998) are all anemic with the presence of abundant irreversibly sickled cells. The organ damage reported is similar to the S + A-Antilles or SAD models. Human γ is incorporated into all three the knock-out mice aforementioned and is expressed at high levels during the fetal period. All three models present low mean corpuscular hemoglobin concentration (MCHC). The chain imbalance measured in the first model and the decrease in the mean corpuscular hemoglobin (MCH) in the other two models, strongly suggest the presence of thalassemic pathology (Fabry *et al.*, 1997), as has been described by Nagel (Nagel *et al.*, 2001) . The low MCHC protects against polymer formation and may be a factor which allows many of these mice to survive to adulthood.

- **Transgenic mice that are not thalassemic**

The NYC-1 mouse line (Fabry *et al.*, 1992a) expressing the human α-globin gene and a β^S gene has also been bred to mouse expressing human γ-globin (Gilman *et al.*, 1995; Arcasoy *et al.*, 1997), then to heterozygote knock-out mice for the α- and β-globin genes, resulting in full knock-out transgenic mice. In these mice, γ-globin levels ranges from 4% to 37% (Fabry *et al.*, 1997; Fabry *et al.*, 1999). The MCHC of many mice has an average value similar to control, and only that of the 37% γ-globin chain mice is elevated. Because of the higher MCHC, some of these mice are generally more severe than Berkeley mice (Fabry *et al.*, 1997; Fabry *et al.*, 1999).

CHAPTER II

AIMS OF THE PROJECT

As mentioned in the introduction, the myeloablation of the recipients preceeding bone marrow transplantation to allow donor cells to efficiently engraft is often lethal to permit a full replacement of the recipients bone marrow (BM). The severe side effects limit the range of patients that could potentially be treated, excluding the elderly and patients with co-morbidities. The alternative would be BMTs following only a partial myeloablation, which would lead to a partial replacement of the recipient's BM. On the other hand, β-thalassemia and SCD are characterized by excessive loss of erythroid cells. Based on this, my hypothesis was that normal red blood cells would have a survival and proliferative advantage over the thalassemic and sickle RBCs and predominate after BMT, and therefore, a partial replacement of the diseased BM with a normal BM would be enough to correct the disease phenotype.

On the basis of what preceded, the major goal of my project was to develop appropriate cellular therapeutic approaches by bone marrow transplantation that would allow the treatment of β-thalassemia and SCD with minimal requirements of myeloablation and donor cells. This was done using a mouse model reproducing the human β-thalassemia *intermedia*, and another model reproducing human sickle cell disease

The first specific aim was to determine the minimal percentage of normal bone marrow cells necessary for the treatment of both β-thalassemia and SCD. The determination of this percentage required the generation of hematologic chimeric mice that have different proportions of normal blood cells, by competitive repopulation assay (CRA). This section of the project allowed determining the minimal level of replacement of recipient's bone marrow with a normal one, required for long term phenotype correction of both diseases, and prepared for the second aim.

The second specific aim consisted on determining the minimal level of myeloablation (myelosuppression) that would reproducibly allow achieving the level of partial replacement of the recipient's BM determined in the first aim, for both diseases. To this end, five parameters were modulated: dose of irradiation, number of donor cells

transferred, number of cell doses, timing of cell transfer after irradiation, and route of cell transfer. Therapeutic levels of donor chimerism were achieved, and reproducible engraftment was obtained with many transplantation strategies. Also fully non-myeloablative strategies were performed and all mice engrafted in most animal groups.

The third specific aim consisted of an *in vivo* evaluation the efficiency of a novel lentiviral vector carrying the human γ-globin gene. After transduction of hematopoietic stem cells with low multiplicity of infections, the transduction efficiency, the long term persistence of expressing cells, and the level of γ-globin protein per RBC, were assessed. Based on this evaluation, the novel vector proved useful for future gene therapy studies aiming to achieve a therapeutic level of expressing ``corrected`` cells as determined in the first aim.

CHAPTER III

ARTICLE 1

FOREWORD

In order to determine the minimal percentage of normal bone marrow cells necessary to treat β-thalassemia (therapeutic threshold), a series of hematologic chimeric mice were generated, with different percentages of normal blood cells. Once the therapeutic threshold was determined, the minimal dose of myelosuppression and the minimal number of cells that allow reaching that threshold were determined.

The following article describes the generation of the different groups of chimerism by bone marrow transplantation (BMT), the full characterization of their phenotype and the determination of the therapeutic threshold.

Afterwards, the optimal therapeutic conditions of minimal myelosuppression and cellular requirements (number and transfer strategies) were determined, and phenotype correction confirmed.

The results of current article provide the first proof confirming that the different aspects of murine β-thalassemia *intermedia* can actually be significantly corrected for long term by partial replacement of the thalassemic bone marrow. It also provides the first standardization of BMT conditions, including myelosuppression and mode of cell transfer to achieve reproducible engraftment in all transplanted mice. The results of this article provide essential resources that could be used for the treatment of β-thalassemia in human clinics.

Contribution to the article

All the Figures (Figure 1, 2, 3, 4) preparation and results

All the Tables (Table 1, 2, 3, 4, 5, S1, S2, S3) preparation and results

The writing of this article was done in collaboration with my research supervisor

LONG TERM CORRECTION OF β-THALASSEMIA WITH MINIMAL CELLULAR REQUIREMENT AND TRANSPLANTATION MODALITIES

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Abstract

Determination of minimal criteria, pretransplantation regimens, and infusion modalities for effective and reproducible bone marrow (BM) therapy in β-thalassemia is of fundamental importance for clinical application. In this study, using repopulation assays, we first established the minimal proportion of normal bone marrow stem cells that would result in therapeutic benefit in this red blood cell (RBC) disorder. Eight groups of stable chimeric hemizygous β–thalassemic (hemi-βthal) mice (10-89%) were systematically subjected to cellular, molecular and patho-physiologic analyses for ~2 years. In the chimeric hemi-βthal groups containing 19-24% normal donor cells, all RBC parameters and consequent erythropoiesis were significantly improved. Mice in the 24% chimeric group and above had marked reduction in organ pathology including iron deposits, and survied to a normal lifespan. Altogether, these results established that a range of 19-24% normal BM cells is sufficient for long-term significant correction of hemi-βthal phenotype. We also determined concomitantly the minimal myelosuppression radiation doses, the number of cells to be infused, and number of infusions required in order to attain this therapeutic range in hemi-βthal mice. Importantly, with prior minimal myelosuppression with 1Gy or 2Gy, and using cell doses of 40 or 60 millions, 100% of the recipients were successfully engrafted at therapeutic levels, provided the cells were administered in two doses. This study has therefore determined the therapeutic chimeric level at 19-24% of normal cells, and has also defined the minimal transplantation modalities for the stable and successful correction of hemi-βthal phenotype.

Introduction

β-thalassemias are among the most frequently occuring human monogenic diseases, leading to high morbidity and mortality. This disorder is characterized by an abnormal level of hemoglobin (Hb) ($\alpha_2\beta_2$) production due to decreased levels or complete absence of β-globin chain synthesis.

In humans, the degree of imbalance in the ratio of β-globin chain to α-globin chain in red blood cells (RBCs) appears to be directly linked to the severity of β-thalassemia. The presence of excess unpaired α-globin chains in RBCs gives rise to aggregates or Heinz bodies, and morphologic changes including hypochromia, microcytosis, poikilocytosis and anisocytosis, resulting in osmotic fragility, shortened RBC half-life and anemia. This imbalance also hinders erythroid precursor maturation and promotes precursor destruction, rendering erythropoiesis ineffective. As a consequence of this primary defect, patients with severe β-thalassemia display general systemic alterations: significant levels of iron accumulation in multiple organs and tissues (due to erythroid cell destruction), massive erythroid hyperplasia, and extramedullary hematopoiesis to compensate the RBC loss, in addition to various pathologies mainly affecting spleen, kidneys and liver.

Individuals with severe thalassemia are dependent on repeated transfusion. However, this therapy causes additional iron overload in several organs and becomes life-threatening in the absence of continuous iron chelation. On the other hand, hematopoietic stem cell transfer by bone marrow transplantation (BMT), either from allogeneic sources or autologous following genetic correction, is potentially curative in thalassemia. Allogenic BMT treatment requires suitable histocompatible donors. Even with a compatible donor the patient is vulnerable to graft-versus-host disease, with risk of early mortality [1,2]. In principle, autologous BMT could prevent these complications. However, success in autologous BMT depends on the ability to achieve persistent therapeutic gene expression through stable transduction with a safe vehicle. In both BMT approaches, it was previously believed that complete bone

marrow (BM) replacement is necessary in order to overcome thalassemia. However, occasional evidence of the existence of transfusion-independent patients with stable BM chimerism [3,4] supports the need for further studies on this hematopoietic mechanism and, more importantly, on the development of reliable strategies to achieve efficient chimerism. In order to make the outcomes of autologous or even allogenic BMT clinically efficient and reproducible, a knowledge of the quantity of cells to be transplanted, optimization of the conditioning regimens, and the modality of transfer of the cells to the patients have to be established.

Only a few spontaneous or genetically induced animal models of β-thalassemia have been generated [5-8], and most of these have been used for assessment of gene therapy vectors [9-13]. While these studies did focus on the importance of developing specific vectors to transport the cells efficiently, they did not provide crucial information on the minimal level of "therapeutic" cells necessary for long-term correction of the disease.

The mouse from which the adult β-globin genes have been hemizygously deleted, so known as hemizygous β-thalassemic (hemi-βthal) mouse, is the most thoroughly analyzed animal model for β-thalassemia, with precise genomic targeting that specifically reproduces severe thalassemia *intermedia* [8]. An examination of the mechanisms of hematopoiesis and erythropoiesis in hemi-βthal mice showed stimulation of multipotent hematopoietic primitive cells and early erythroid progenitors, consequent to the shortened RBC half-life and ineffective erythropoiesis [14]. These thalassemic cellular characteristics are likely to confer selective advantage of normal or corrected RBC over thalassemic cells.

Using the hemi-βthal mouse model, we undertook a systematic and long-term study of the number of cells to be transplanted, optimization of the conditioning regimens, and modalities of transfer. The results from a competitive repopulation assay established the minimal level of normal donor at 19-24% chimerism, providing normal RBC amplification to 40-50% and thereby leading to significant long-term

phenotypic improvement. Moreover, in order to attain the required threshold of chimerism as well as stable and consistently reproducible engraftment for correction of the disease phenotype, we determined the minimal conditions of myelosuppressive preparative regimens and evaluated the effect of the modality of dose delivery. These studies will likely help the future development of gene therapy through BMT trials in human β-thalassemia *intermedia*.

Materials and Methods

Mouse strains

Hemi-β-thal mice were generated by deletion of the murine β^{major} and β^{minor} globin genes so as to closely reproduce the human β-thalassemia intermedia [8]. These mice had been bred onto C57BL/6J-*Gpi1*^{b}/*Gpi1*^{b} background for >21 generations, and were therefore considered to be syngeneic. Hemi-βthal mice were genotyped by polymerase chain reaction as previously described [14]. Congenic C57BL/6-*Gpi1*^{a}/*Gpi1*^{a} (carrying a hematopoietic cell marker) were obtained from Dr J. E. Barker (the Jackson labs, Bar Harbor, ME, USA). All mice were maintained in a specific pathogen free environment and experimental procedures were conducted in compliance with the guidelines of the Canadian Council on Animal Care (CCAC)

Production of chimeric mice

Nucleated BM cells were harvested from donor animals using Iscove's Modified Dulbecco's Medium (Gibco, Grand Island, NY, USA). The Competitive Repopulation Assays were carried out based on the principle described by David Harrison [21]. This assay consists in transplanting a fixed number of donor BM cells from normal and thalassemic populations mixed in reciprocal proportions. Ten hematopoietic chimeric groups were produced, having normal cells ranging from 0 to 100%. C57BL/6-*Gpi1*^{a}/*Gpi1*^{a} recipient mice (59 males and 34 females) of 2-4 months of age were exposed to a lethal dose of irradiation of 8.75 Gy (Mark I-68A-1 Research irradiator, JL Shephered & Associates, San Francisco, CA) at least 4 hours prior to injection of 2 x 10^6 donor cells in the lateral tail vein, thereby producing hematopoietic chimeric mice. In addition, hemi-βthal mice slightly myelosuppressed with a low irradiation dose of either 1 or 2 Gy were injected with 20, 40 or 60 x 10^6 donor cells eithrr in a single dose or in two doses at 24-hour intervals.

Analysis of chimerism

Chimeric mice were assessed for hematopoietic engraftment by *Gpi1* in both WBCs and RBCs, on a regular basis from the second month to the eighteenth month

following BMT. Because the two distinct isoforms of *Gpi1* have different electrophoretic mobilities, Gpi1 enzyme analysis served to determine the percentage of normal and thalassemic WBCs and RBCs. The electrophoretic assay involved protein Gpi1 separation onto a cellulose acetate membrane of WBC or RBC lysates in an alkaline buffer using Helena equipment (Beaumont, TX). The two isotype bands were revealed by an enzymatic colorimetric reaction, with the isoform $Gpi1^a$ migrating slower than $Gpi1^b$ (Szuber, N, Soe-Lin, S, Felfly, H, Buss, JL, Ponka, F, Trudel, M, manuscript submitted). The membranes were then scanned and bands quantified using ImageQuant software.

Hematologic analysis

Estimation of the hematological parameters of each chimeric mouse was performed with 250µl of blood using a Bayer Advia 120 cell analyzer with the mouse archetype of multispecies software version 2.2.06 (CTBR, Montreal, Canada). Gating of the mean cellular hemoglobin concentration (<22g/dl) and the mean cellular volume (MCV) (<25fl) was used in order to determine the percentage of hypochromic (Hypo) RBCs and the percentage of microcytic cells, respectively, as previously described [14]. Reticulocyte counts (retic) were obtained by specific RNA staining with the oxazine 750 dye, using the reticulocyte channels on the analyzer. On the basis of absorption intensities corresponding to RNA levels, the reticulocytes from the least to the most mature were classified as: H (high absorption), M (medium absorption), L (low absorption).

The murine RBC osmotic fragility test was performed by an adapted form from a previously described technique [22]. The osmotic fragility of RBCs was evaluated from their hemolysis pattern by suspending 5µL of blood with hypotonic solutions in a series of NaCl concentrations ranging from 0.85% to 0.15% at decreasing step gradient of 0.05%. After 30 minutes incubation at room temperature, tubes were centrifuged and light absorption of the supernatant was measured using a spectrophotometer at 540nm wavelength. The values obtained determined the point of initial and maximum hemolysis.

The morphology of RBCs was monitored on Romanowsky-stained peripheral blood smears prepared from heparinized blood, as previously described [23]. A total of ~1,000 RBCs was counted for each sample in order to quantitate cellular anomalies (target cells, fragmented cells). Crystal violet stain was used to detect Heinz bodies.

The RBC half-life was determined using a non-radioactive protocol that involved biotinylation of the entire RBC cohort and monitoring for RBC replacement, as previously described [14].

Hematopoeisis/erythropoiesis analysis

Flow cytometry analyses were performed on BM and spleens from chimeric mice, including controls. BM cells were harvested by flushing one femur with phosphate-buffered saline containing 2% fetal calf serum, and all the nucleated cells were counted. Single spleen cells were suspended in 2% fetal calf serum/phosphate-buffered saline. The cells (1×10^6) were incubated with antibodies for 30 minutes on ice with anti-mouse TER119-phycoerythrin (0.2ng) and biotin conjugated anti-mouse CD71 (transferrin receptor) (0.5 ng), and the latter was detected with streptavidin-allophycocyanin (0.2 ng) (BD Biosciences). Duplicate samples were analyzed with the flow cytometer FACSCalibur (BD Bioscience, Ontario, Canada) using CellQuest Pro version 4.0.2 software. Erythroid precursor subpopulations were identified on the basis of their respective CD71 and TER119 intensities and counted.

Multipotent CFU-S_{12} erythropoietic/hematopoietic cells from both BM and spleen were evaluated. Nucleated cells from BM (5×10^4) or spleen cells (10^6) were injected in lethally irradiated C57BL/6J recipient mice. CFU-S colonies were counted on day 12.

Pathological analysis

Chimeric mice (n=3-8) from each group were sacrificed for pathologic analysis. For evaluation of splenomegaly, the spleen weight was determined in proportion to total

body weight. Several organs including spleen, liver, kidney and heart were fixed in 10% phosphate-buffered saline formalin overnight and embedded in paraffin block. Four-micrometer thick sections were stained with hematoxylin and eosin for general evaluation, and with prussian blue for iron analysis. Iron deposits without counterstain were evaluated by μm^2 from a minimum of five fields at a magnification of X200, using the Northern Eclipse program.

Statistical analysis

Values were expressed as mean ± SD. Statistical analyses were performed using the two-sample student *t*-test with Microsoft Excel software and Fisher test with SigmaStat 3.1.

Results

Selective advantage of normal erythroid cell population in hemi-βthal chimeric mice

In order to determine the minimal percentage of normal BM cells necessary for the correction of the thalassemic phenotype, hemi-βthal mice previously backcrossed onto a homogenous C57BL/6J-*Gpi1*^{b} genetic background were used for competitive repopulation transplantation assay [14]. Mice lethally irradiated were infused with two genetically marked syngeneic BM cell populations, normal (C57BL/6J-*Gpi1*^{a}) and thalassemic, that could be distinguished by a specific glucose phosphate isomerase isotype (Gpi1). This Gpi1 marker served to determine by electrophoresis, the contribution of each normal and thalassemic cell population of the chimeric animals. Analysis of marrow stem cell engraftment and chimerism was performed on the transplant-recipient mice (n=93) at regular intervals from 2 months after BMT onwards. We generated 10 groups of hemi-βthal chimeras ($n \geq 5$ per group) stably engrafted with different reciprocal proportions of normal and thalassemic cells, consisting of 0, 10, 19, 24, 31, 39, 52, 60, 70, 89, 100% normal peripheral white blood cells (WBCs) at 7 months after BMT (**Figure 1**).

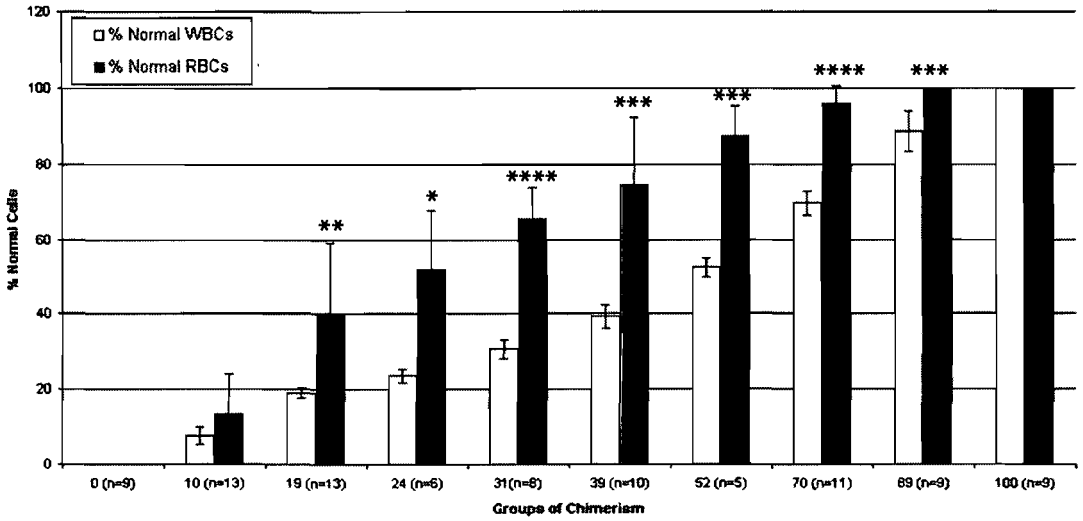

Figure 1. Chimerism in hemi-βthal mice. Comparison of engraftment levels of normal white blood cells (WBCs) (open bars) and normal red blood cells (RBCs) (black bars) for each of the 10 groups of chimeric mice at 7 months after transplantation. The *n* beside each group indicates the number of mice in the group. In hemi-βthal hematopoietic chimeric mice, amplification of the normal erythroid cell lineage relative to WBC was observed systematically. Data represent the mean values and standard deviation for each group of mice. *t*-test analysis of percentage of normal WBCs versus percentage of normal RBCs, *, p<0.01, **p<0.003, ***p<0.0003, **** p<3 X 10^{-6}.

The percentage of WBC in each recipient served to estimate the level hematopoietic stem cell engraftment. When compared to individual normal WBC groups, the proportion of normal RBCs in each group between 10 and 89% was significantly amplified as a result of losing thalassemic RBCs. As shown in the histogram, the percentage of normal RBCs reached ~2- to 2.5-times that of the normal WBCs in the peripheral blood in groups representing10-39%, thereby providing evidence of a selective advantage for the normal RBCs (**Figure 1**). Furthermore, regular assessment of these hemi-βthal chimeric groups showed persistent and stable long-term engraftment that was maintained from 2 to 18 months after BMT. Cellular, morphologic and pathologic analysis was carried out for all 10 chimeric hemi-βthal groups.

Improved hematology in hemi-βthal mice with low-level chimerism

In order to determine what level of WBC chimerism in hemi-βthal mice would have a positive impact on hematologic parameters, peripheral blood of these transplant-recipient mice was analyzed (**Table 1; Supplementary Table S1**). The hemi-βthal mice with 0% normal WBCs exhibited a severe decrease in their RBC counts and hemoglobin (Hb) concentrations in comparison to chimeric mice with 100% normal WBCs. This finding was in agreement with our earlier observations of severe anemia in non-transplant-recipient hemi-βthal mice in comparison to the controls [14]. Although a level of 10% normal WBCs was sufficient to ameliorate the abnormality of the hematologic indices, it was at the level of 19% normal WBCs that the RBC counts and Hb levels rose by ~ 50%. In addition, the mean cellular Hb and mean cellular volume of the RBCs in the 19% chimeric group showed a correction that correlated with a >50% decrease in number of hypochromic (Hypo) cells. In the groups representing chimerism of 31% and above, all parameters were significantly improved. In particular, there was a significant decrease in reticulocyte count (retic) associated with an improvement in reticulocyte maturation from high (H), to medium (M), to low (L) reticulocyte RNA, thereby suggesting less erythropoietic stimulation (**Table 1; Supplementary Table S1**)

Table 1. Hematological parameters of chimeric hemi-βthal mice

		Mature Erythrocytes					Reticulocytes			
Chimeric mice (% normal WBC)	**n**	**RBC** (10^6/μL)	**Hb** (g/dl)	**MCV** (fl)	**MCH** (pg)	**Hypo** (HC<22g/dL)	**H** (% / total retic)	**M** (% / total retic)	**L** (% / total retic)	**%** (total RBC)
0	7	5.1 ± 1.1	6.0 ± 1.2	45.3 ± 4.2	11.8 ± 0.4	28.5 ±15.0	36.9 ±10.2	26.6 ±8.0	29.3 ±10.6	15.7 ±5.6
10	12	6.3 ± 0.6[a]	7.7 ± 0.9[a]	47.3 ± 2.7	12.3 ±0.7[a]	17.6 ± 8.5	38.0 ± 6.2	29.1 ±2.9	32.9 ± 3.7	15.5 ±3.8
19	**12**	**7.7** ±2.8[a]	**8.9** ±1.2[c]	**48.1** ±3.3[a]	**12.9** ±0.8[b]	**10.9** ± 6.7[b]	**35.5** ±11.0	**29.6** ±4.1	**34.9** ± 7.1	**12.7** ±4.3
24	**6**	**7.7** ±0.6[d]	**10.0** ±1.5[d]	**46.6** ± 3.6	**13.0** ± 1.4	**5.6** ± 2.1[a]	**32.8** ± 7.1	**31.2** ±3.3	**36.0** ± 4.1	**10.2** ±2.6
31	8	7.6 ±0.8[d]	10.5 ±1.2[f]	49.9 ±2.1[e]	13.8 ±0.5[g]	5.2 ± 2.6[a]	26.4 ± 8.6	33.8 ±2.6	39.8 ± 6.2[a]	8.9 ±3.8[b]
52	5	8.6 ±0.4[e]	12.3 ±1.1[e]	51.5 ±1.6[d]	14.4 ±1.1[b]	4.5 ± 4.1[b]	22.9 ± 6.7[a]	35.4 ±2.7[a]	41.7 ± 4.8[a]	7.2 ±2.7[b]
100	5	9.6 ±0.9[f]	13.9 ±1.2[f]	50.3 ±2.4[a]	14.5 ±0.2[g]	0.7 ± 0.7[b]	9.7 ± 6.8[d]	36.8 ±1.2[a]	53.6 ± 8.0[c]	4.9 ±2.7[b]

Abbreviations: WBC, white blood cell; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin. *t*-test: sample vs chimeric 0%; a, $p<0.05$; b, $p<0.005$; c, $p<0.001$; d, $p<0.0005$; e, $p<0.0001$; f, $p<0.00001$; g, $p<0.0000001$

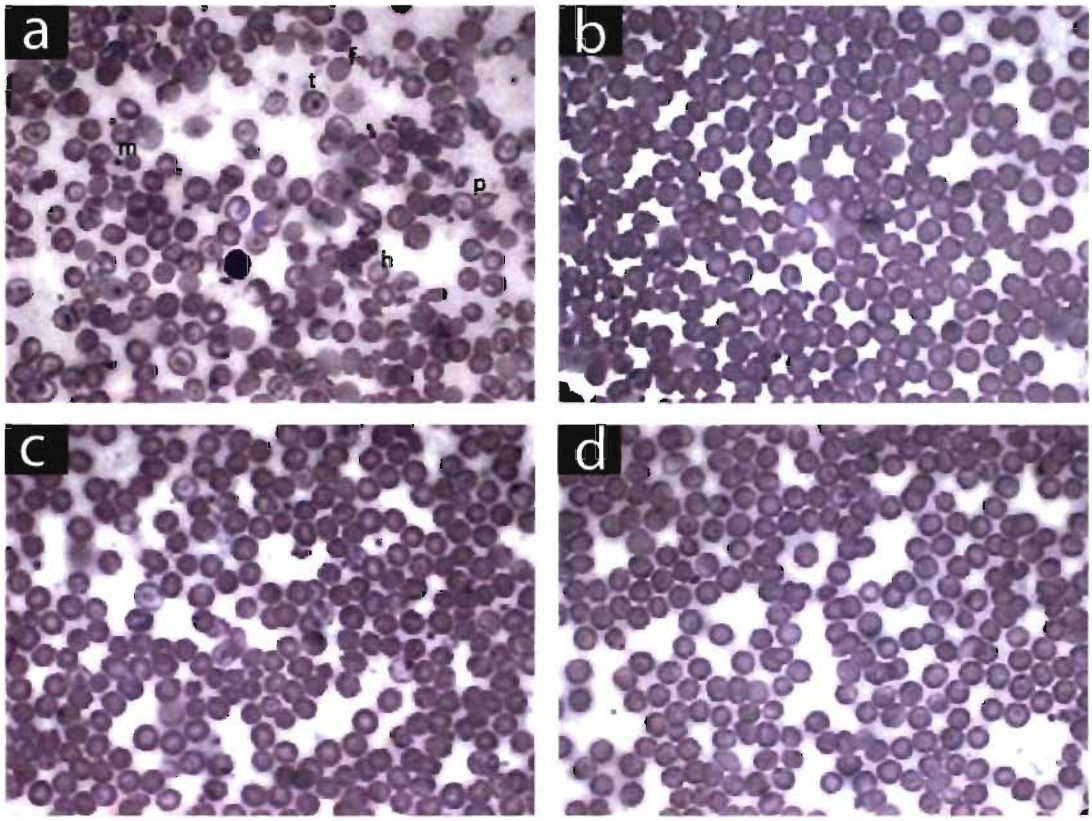

Figure 2. Peripheral blood cell smears of chimeric hemi-βthal mice.
a. Blood smear of a mouse stained with Wright from the 0% chimeric hemi-βthal group with 0% normal white blood cells (WBCs) contains red blood cell (RBC) fragments (f), microcytes (m), poikilocytes (p), hypochromia (h) and target (t) cells.
b. Blood smear of a mouse from the 100% chimeric hemi-βthal group with only normal WBCs.
c. Blood smear from the 24% chimeric group exhibited marked amelioration of the thalassemic anomalies in RBCs.
d. Blood smear from the 52% chimeric group displayed virtually normal RBC morphology. (Micrographs at x1000 magnification).

In order to determine what range of chimerism in the peripheral blood would have an impact on RBC morphology, we monitored peripheral blood smears of transplant-recipient hemi-βthal chimeric mice (**Figure 2**). In the 10% chimeric group, noticeable improvement was observed when compared with the typical RBCs of hemi-βthal mice with 0% normal WBCs (associated with the presence of RBC fragments and RBC poikylocytosis and anisocytosis) (**Supplementary Table S2**). Correction was even more pronounced in the 19% chimeric group, which displayed threefold fewer cell fragments. In addition, these chimeric groups displayed a similar ~2.7-fold

reduction in RBCs with visible α-globin chain precipitates or Heinz inclusion bodies which are typical of thalassemic RBCs because of their lowered proportion of β-globin chains (**Figure 2; Supplementary Table S2**).

The osmotic fragility of RBCs from hemi-βthal chimeric mice was assessed using a range of saline hypotonic solutions. Cell hemolysis displayed a two-step fragility curve separated by a plateau for each of the different hemi-βthal chimeric groups, carrying both normal and β-thalassemic RBC populations (data not shown). This pattern of discontinuous curve correlated with the degree of RBC chimerism detected in these mice. Half of the RBCs from hemi-βthal mice with 0% normal WBCs showed osmotic lysis at a concentration of 0.31±0.04% saline. In contrast, the mice with only 10% normal WBCs exhibited half of the RBC lysis at saline concentration of 0.53±0.02% similar to the chimeric mice with 100% normal WBCs which exhibited RBC lysis at concentration levels of 0.49±0.02%.

Following the finding that anemia was mitigated and the morphology of the RBCs was restored, we assessed the overall RBC turnover by measuring the half-life of biotin-labeled erythrocytes. The hemi-βthal mice with 0% normal WBCs exhibited a threefold lower RBC half-life (4.2±2.4 days; n=2) relative to hemi-βthal mice with only normal WBCs (12.9±1.6 days, n=3). The mice groups of 10 and 19% chimerism showed a strong improvement in the RBC half-life (7.0±2.6, n=2 and 7.3±0.6, n=3). With progressively increasing chimerism, the RBC half-life continued to improve.

Normalization of erythropoiesis efficiency in hemi-βthal chimeric mice

On the basis of our findings of stimulated hematopoiesis and ineffective erythropoiesis in non-transplant-recipient hemi-βthal mice [14], we examined the BM and spleens of these mice. Differentiation and maturation potentials of hematopoietic progenitors and erythroid precursors were evaluated by *in vivo* quantification of primitive multipotent CFU-S_{12} progenitor cells and of precursor cells, by flow cytometric analysis.

Analysis of BM in chimeric mice with 0% normal WBCs showed a mild (not significant) increase in number of CFU-S_{12} multipotent cells compared to control 100% chimeric mice, as also seen in non-transplanted mice [14]. Accordingly, chimeric mice with normal WBCs over the entire range produced very few changes in the levels of CFU-S_{12} multipotent cells and of BM cellularity (**Table 2**). By contrast, in the spleen the hematopoietic stimulation of chimeric mice with 0% normal WBCs was significant with an approximately tenfold increase in the number of CFU-S_{12} multipotent cells relative to chimeric mice with 100% normal WBCs. This is a frequent finding in mouse models of hemoglobinopathies because of the multipotent cells that proliferate in the marrow, become mobilized, and then colonize the spleen [14,15]. Splenic multipotent cell stimulation reverted significantly in the chimeric groups with 10% normal WBCs and above, toward values equivalent to those in the 100% chimeric group. Simultaneous with this regression in the multipotent progenitors, the splenic cellularity in these chimeric groups reached normal values (**Table 2**).

Because the non-transplant-recipient mice have anomalies in late stages of erythroid maturation in BM and spleen, we monitored the erythroid precursor cell population in these tissues. Four classes of erythroid precursors were identified and quantified upon maturation by fluorescence-activated cell sorting analysis based on the specific erythroid Ter119 and the transferin receptor CD71 surface markers. On the basis of the staining intensities observed, these precursors are (as arranged from the least to the most mature) the proerythroblast ($Ter119^{med}CD71^{high}$, region a), the basophilic erythroblast ($Ter119^{high}CD71^{high}$, region b), the late basophilic and polychromatophilic erythroblast ($Ter119^{high}CD71^{med}$, region c), and the orthochromatophilic erythroblast ($Ter119^{high}CD71^{low}$, region d) (**Figure 3**).

Table 2. Quantification of cellularity and multipotent cells in chimeric hemi-βthal mice

Chimeric mice		Femur	Spleen	Femur	Spleen
(% normal WBC)	n	(10^7 nucleated cells)		(10^2 $CFUS_{12}$)	
0	3	3.3 ±0.4	64.0 ±14.1	22.2 ± 6.2	55.4 ±13.5
10	3	5.1 ±1.0	21.0 ±15.0[a]	20.1 ± 4.0	9.9 ±8.0[b]
19	**4**	**4.2** ±1.3	**18.0** ±3.3[a]	**24.4** ± 6.0	**9.0** ±0.4[b]
24	**3**	**4.2** ±0.3[a]	**18.2** ±11.0[b]	**29.7** ±10.0	**7.1** ±3.9[b]
52	3	4.1 ±0.3[a]	16.0 ±7.9[b]	18.6 ± 8.1	4.3 ±2.6[b]
100	3	4.8 ±0.6[a]	14.5 ±3.6[b]	17.3 ± 5.8	5.0 ±2.9[b]

t-test: sample vs chimeric 0%; a, $p<0.05$; b, $p<0.02$

In the BM of chimeric mice with 0% normal WBCs, the proerythroblast and basophilic erythroblast population showed a significant increase when compared with control chimeric mice with 100% normal WBCs (**Figure 3; Supplementary Table S3).** In contrast, a significant (threefold) decrease in the orthochromatophilic erythroblast population relative to controls was inferred from the ratio of the polychromatophilic to the orthochromatophilic erythroblast BM population, thereby demonstrating ineffective erythropoiesis **(Supplementary Table S3).** In the 24% chimeric group, the proportion of orthochromatophilic erythroblast was significantly higher than in the chimeric mice with 0% normal WBCs.

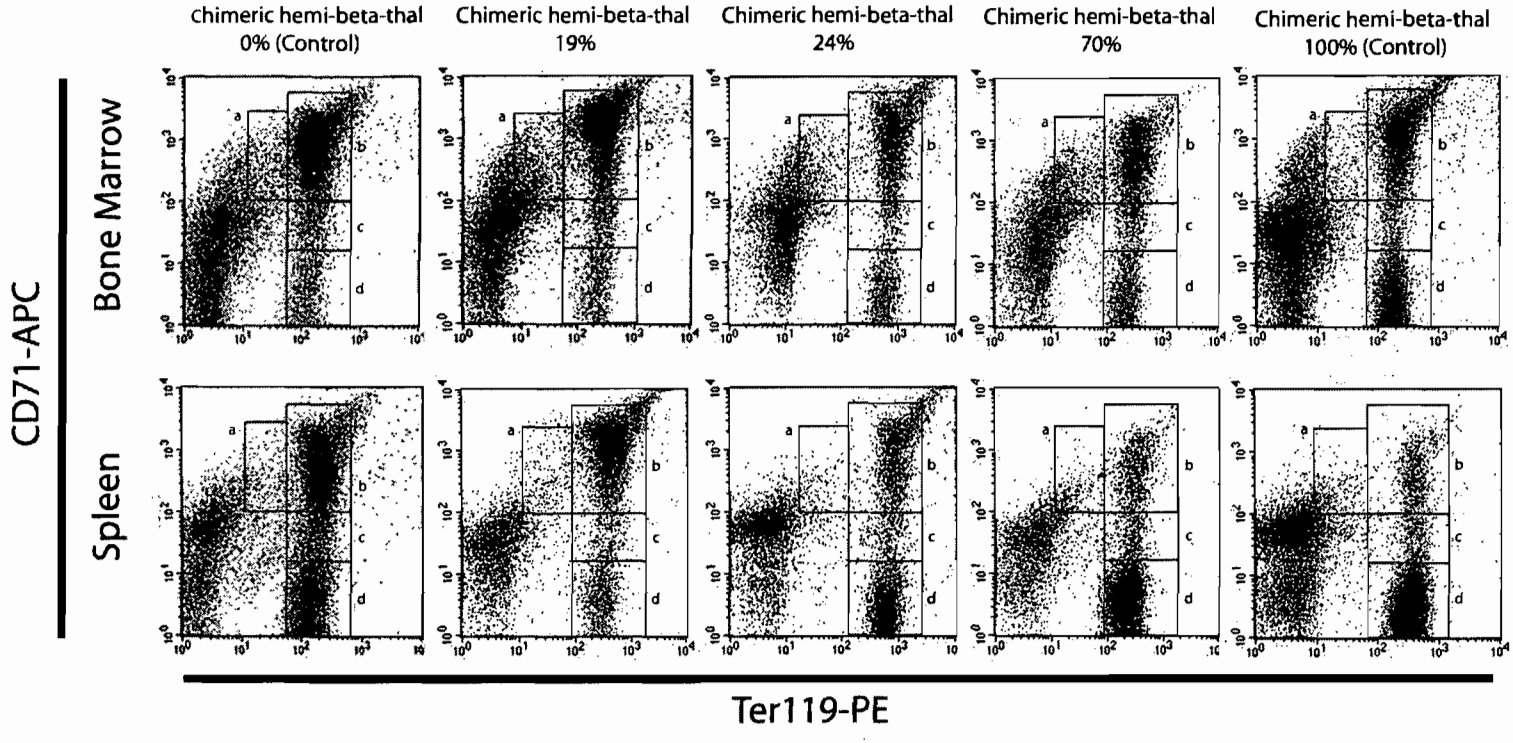

Figure 3. Analysis of erythroid cell precursor maturation in chimeric hemi-βthal mice.
Flow cytometry analysis of single erythroid cell from bone marrow (BM) (above) and spleen (bottom). Four regions are delineated by squares representing cell subpopulations: a, Ter119^{med}CD71^{high} proerythroblast; b, Ter119^{high}CD71^{high} basophilic erythroblast; c, Ter119^{high}CD71^{med} polychromatophilic erythroblast; d, Ter119^{high}CD71^{low} orthochromatophilic erythroblast. The 24% chimeric group displayed marked improvement in early to late erythroid precursors in BM and spleen, when compared with controls having 0% chimeric hemi-βthal.

In the spleen, the basophilic and polychromatophilic erythroblasts were significantly higher in the chimeric mice with 0% normal WBCs than in those with 100% normal WBCs (**Figure 3**). Moreover, the orthochromatophilic erythroblast population was decreased to below normal values (**Supplementary Table S3).** In correlation with the improvement of the BM erythropoiesis, the 24% chimeric group of mice showed a significant increase in the maturation of the basophilic erythroblast to the orthochromatophilic erythroblast populatuions leading to the correction of ineffective erythropoiesis in the groups with higher chimerism

Amelioration of pathology and lifespan in the 19-24% hemi-βthal chimeric groups

Thalassemia is characterized by splenic RBC sequestration and stimulated extra medullary hematopoiesis/erythropoiesis that result in massive splenomegaly, as observed in the chimeric mice with 0% normal WBCs (**Table 3**). The ratio of spleen weight to total body weight was significantly reduced even in the 10% group, and the ratio was restored to normal in the 19% group, to a value similar to that in the control 100% chimeric group. Along with this decrease in splenomegaly, there was a correlated decrease in hypercellularity as well (**Table 2**).

Histologic analysis of these hemi-βthal chimeric mice showed that the 0% chimeric group (11-16 month-old) develop typical severe thalassemic changes, with abundant iron deposits in several organs, especially the spleen, kidney, liver and heart (**Figure 4, Table 3**). The spleen pathology in the 5% chimeric group exhibited a completely disorganized red and white pulp, with RBC sequestration and significant erythroid hyperplasia associated with extramedullary hematopoiesis. In the 24% group of hemi-βthal chimeric mice (>18 months old), the spleen pathology showed a lower degree of disorganization, with significantly decreased iron deposits in the parenchyma as well as in macrophage and little extramedullary hematopoiesis (**Table 2**). Renal pathology in the 0% chimeric hemi-βthal mice was similar to that of human β-thalassemia major [16] by the presence of tubular iron deposits in the cortex and particularly in the glomeruli, that is associated with increase mesangial cells and focal sclerosis

Table 3. Pathological assessment in chimeric hemi-βthal mice

Chimeric mice (% normal WBC)	n	Spleen Wt / Body Wt (%)	n	Iron Deposits (%)			
				Spleen	Kidney*	Heart	Liver
0	8	1.20 ±0.32	7	5.2 ±2.6	2.3 ±1.9	0.5 ±0.4	1.3 ±0.9
10	8	0.79 ±0.35[a]	7	2.5 ±1.8[a]	1.5 ±2.5	0.3 ±0.1	1.4 ±1.4
19	**5**	**0.46** ±0.17[b]	**5**	**3.8** ±4.7	**0.6** ±0.6[a]	**0.2** ±0.2	**0.5** ±0.3
24	**5**	**0.36** ±0.09[c]	**5**	**1.5** ±1.2[e]	**0.5** ±0.5[b]	**0.3** ±0.1	**0.5** ±0.2
52	5	0.38 ±0.12d[d]	3	0.7 ±0.5[f]	0.2 ±0.1[c]	0.2 ±0.1	0.3 ±0.4[b]
100	5	0.43 ±0.26[e]	7	0.5 ±0.1[f]	0.3 ±0.6[c]	0.1 ±0.05	0.3 ±0.2[c]

* Iron deposits in kidney were measured in cortex
t-test: sample vs chimeric 0%; a, p≤0.05; b, p≤0.04; c, p<0.03; d, p<0.02; e, p<0.01; f, p<0.005

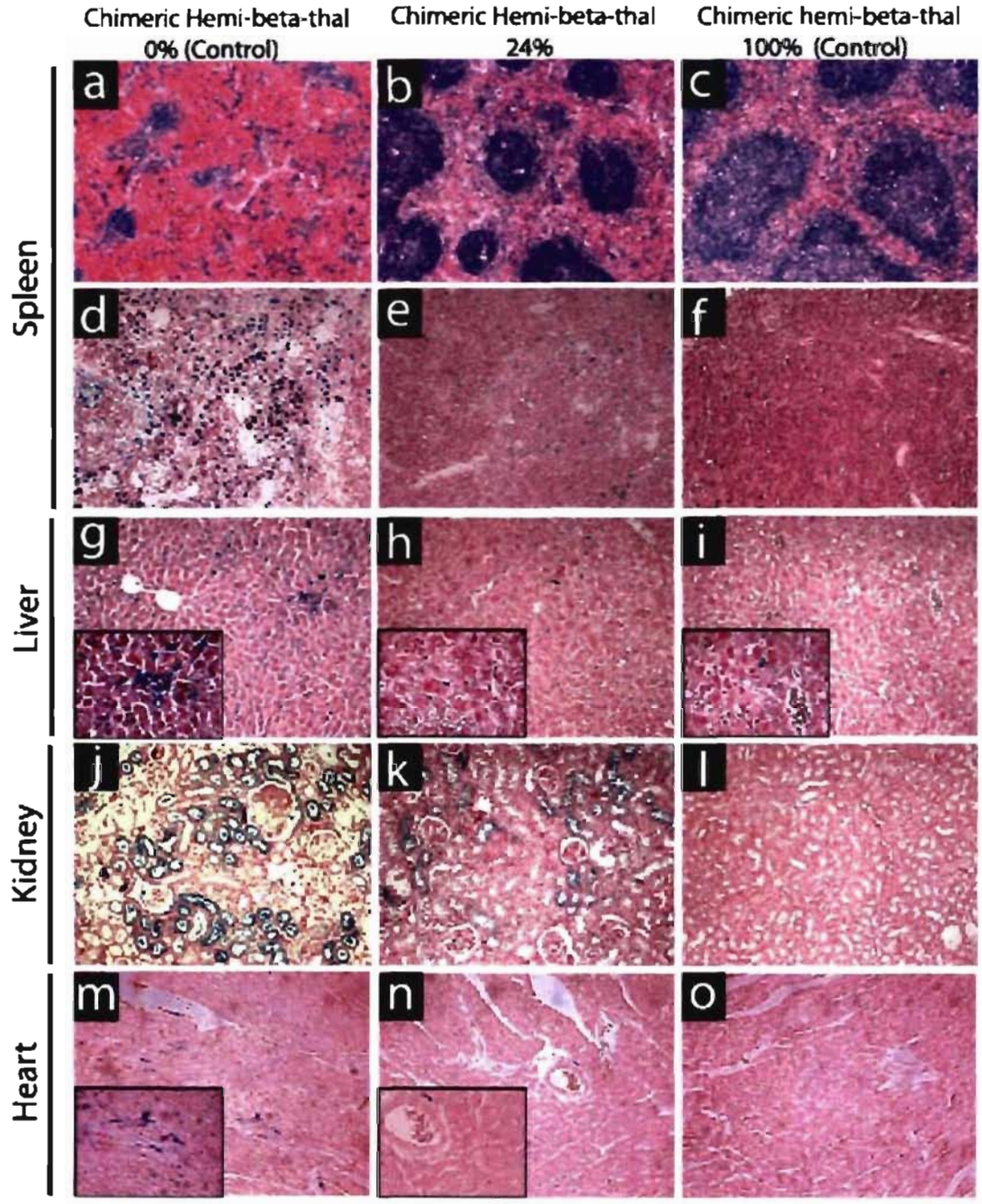

Figure 4. Histopathologic analysis of chimeric hemi-βthal mice (Legend on following page)

Figure 4. Histopathologic analysis of chimeric hemi-βthal mice.
Histologic analysis of tissue sections was performed on spleens stained with H & E of 16 month-old chimeric hemi-βthal with 0% normal donor cells as thalassemic control (a), 21 month-old chimeric hemi-βthal mice with 24% normal donor (b) and 20 month-old chimeric hemi-βthal mice with 100% normal donor cells as wild type control (c). In the 24% chimeric group, the spleen architecture was substantially ameliorated with reduced RBC sequestration and erythroid/hematopoietic hyperplasia. (x100). Hemosiderin analysis of chimeric hemi-βthal was carried out in various organs spleen (d, e, f), liver (g, h, i), kidney (j, k, l) and heart (m, n, o) stained with prussian blue and counterstained with nuclear fast red. Numerous iron deposits were readily observed in all tissues of the chimeric hemi-βthal with 0% normal WBCs (d, g, j at 11 month-old and m at 12 month-old). In contrast, a marked decrease in iron accumulation was noticeable in tissues of 24% chimeric hemi-βthal mice (e, h, k at 24 month-old and n at 20 month-old) tending toward the 100% chimeric hemi-βthal organs (f, i, l at 23 month-old and o at 20 month-old) particularly, in the hepatic Kupffer cells. Renal analysis also revealed a reduction in glomerular mesangial cells hypercellularity and in tubular dilatation. (magnification x200; Inset, x640).

The abnormality of renal pathology was markedly mitigated in the 24% chimeric group of mice. In the 0% chimeric group the liver showed hyperplasia of the Kupffer cells and of portal macrophages associated with stainable iron and erythrophagocytosis. These abnormalities were alleviated in the 19-24% chimeric group. A concomitant decrease in iron deposits in the heart was also readily detectable in the 19-24% hemi-βthal mice chimeric group (**Table 3**). This overall decrease in heme iron in the tissues probably results from the decrease in RBC turnover, because of the addition of a sufficiently large population of normal donor-derived RBCs with a longer half-lives.

In order to investigate the impact of normal blood cell populations on the overall physiology of chimeric mice, the survival of transplant-recipient mice was monitored. In comparison to the lifespan of chimeric mice with 0% normal WBCs (14.2 ±2.9, n=6), the 10% chimeric mice had a significantly improved lifespan (17.3±2.9, n=6; $p<0.05$), thereby providing evidence of a positive physiologic impact at this level of chimerism. Mice from the 19% chimeric group reached a lifespan (20.3±3.6, n=8; $p<0.02$) almost equal to the average normal lifespan of the control group with 100% normal WBCs (21.3±3.9, n=3), thereby indicating that the damage to organs in this group is minor.

Characterization of minimal myelosuppression and modalities for therapeutic engraftment

In view of the finding that a 19-24% chimerism of normal BM cells is sufficient for providing selective advantage for RBCs, and appears to be adequate for correction of the thalassemic phenotype in hemi-βthal mice, it is proposed that complete myeloablation in potential recipients is not an essential precondition for effective therapy of this disease. Two reports have documented that transplant-recipient mice, preconditioned with partial myeloablation, could occasionally be engrafted successfully [17, 18]. This indirectly emphasizes the importance of developing novel strategies for efficient and reproducible engraftment. On the basis of the chimeric threshold that we determined as sufficient for availing therapeutic benefit, we next sought to investigate different regimen conditions by modulating three parameters: the irradiation level, the quantity of normal donor cells to be infused, and the number of infusion doses of these normal cells.

For myelosuppression, two low levels of irradiation, 1 and 2 Gy were utilized in the study. For each irradiation condition, two different quantities of cells were transferred either in one dose 4 hours after myelosuppression, or in two equal doses, one at 4 hours and the other at 28 hours after myelosuppression (**Table 4**). The levels of chimerism in each recipient were stable and sustained long term, as evaluated in the totally myeloablated mice. Although not all recipient mice could be successfully engrafted, the engraftment efficiency improved not only at the higher irradiation levels but also when cell infusions were administered in two doses rather than one, at either of the two irradiation levels. Indeed, recipients receiving a given number of cells (60 million) even with low myelosuppression (1Gy) displayed a significant higher level of chimerism when the cells were administered in two infusions rather than one ($P<0.02$). With higher myelosuppression (2Gy), chimerism was significantly increased after 40 million cells were transplanted in one or two doses ($P<0.02$ or $P<0.001$) when compared with the group having minimal myelosuppression. Importantly, full engraftment efficiency was achieved in recipients conditioned with either 2 or 1 Gy, provided a defined minimal number of normal BM cells was

infused. When compared with the hemi-βthal chimerism after total body irradiation (**Figure 1**), the chimerism obtained with partial myelosuppression showed similar levels of amplification of the RBCs in peripheral blood (**Table 4**). Despite some variability among the animals in levels of chimerism achieved, there was a general tendency toward higher efficiency of engraftment and of chimerism when cells were transferred in more than one infusion.

Table 4. Engraftment and chimerism of hemi-βthal mice with minimal myeloablation

Irr. Level (Gy)	Cells (10^7)	Dose (#)	n (total)	n (engr)	% Chimerism WBC	% Chimerism RBC
2	**2**	**1**	**8**	**4**	**36.2** ±23.2	**77.3** ±29.3
	4	**1**	**7**	**6**	**61.5** ±34.9[a]	**87.1** ±28.8
	1	**2**	**5**	**4**	**63.3** ±25.7	**96.0** ± 4.7
	2	**2**	**5**	**5**	**82.5** ±21.7[a]	**100.0** ± 0.0
1	**4**	**1**	**6**	**0**	**0**	**0**
	6	**1**	**7**	**3**	**23.1** ±17.3	**65.0** ±31.1
	2	**2**	**7**	**3**	**48.1** ±38.8	**68.8** ±54.1
	3	**2**	**6**	**6**	**40.2** ±20.6[b]	**76.3** ±38.7

Abbreviations: WBC, white blood cell; RBC, red blood cell.
Data of chimeric levels at 7 months after transplantation. n (total), total number of transplanted mice; n (engr), number of transplanted mice engrafted; dose (#), number of infusion dose.
t-test : a, Level of chimerism at 200 rad in 1 or 2 doses versus 100 rad (2 doses) $P \leq 0.02$, $P \leq 0.001$ respectively
b, Level of chimerism (100 rad 1 vs 2 doses) $P \leq 0.02$;

We monitored the RBC parameters in these partially myelosuppressed chimeric mice, so as to verify whether they displayed improvements similar to those measured in myeloablated mice,. Although the mice exhibited variability in levels of chimerism, all the RBC parameters in the different groups were ameliorated in proportion to the level of chimerism (**Table 5**). It is noteworthy that the levels of correction achieved

Table 5. Hematology parameters of minimally myelosuppressed chimeric hemi-βthal mice

Conditioning Regimen					Hematological Parameters					
Irr. Level (Gy)	**Cells** (10^7)	**Dose** (#)	**Chimerism** (% normal WBC)	**n**	**RBC** ($10^6/\mu L$)	**Hb** (g/dl)	**MCV** (fl)	**MCH** (pg)	**Hypo** (HC<22g/dL)	**Retic** (%)
2			**0**	*5*	**6.5** ±0.8	**6.7** ±0.7	**43.3** ±3.0	**10.2** ±0.2	**45.4** ±18.9	**21.3** ±2.6
	2	**1**	**36.2** ±23.2	**3**	**9.5** ±0.8[c]	**13.1** ±2.2[a]	**47.2** ±2.6	**13.7** ±1.1[a]	**2.2** ±2.1[c]	**5.8** ±1.8[f]
	4	**1**	**61.5** ±34.9	**5**	**9.7** ±0.8[e]	**14.0** ±1.6[e]	**48.0** ±1.3[a]	**14.3** ±0.7[f]	**1.1** ±0.6[c]	**4.8** ±1.1[f]
	2	**2**	**63.3** ±25.7	**4**	**10.0** ±0.8[d]	**14.5** ±1.2[e]	**48.1** ±1.1[b]	**14.5** ±0.4[g]	**0.9** ±1.2[c]	**4.0** ±1.1[f]
	4	**2**	**82.5** ±21.7	**3**	**11.0** ±0.2[f]	**15.5** ±0.1[g]	**54.5** ±1.3[b]	**14.0** ±0.3[f]	**0.2** ±0.0[c]	**2.6** ±0.2[f]
1			**0**	*5*	**7.2** ±1.1	**7.4** ±1.7	**42.3** ±2.2	**10.2** ±0.9	**31.3** ±10.1	**16.1** ±6.1
	6	**1**	**23.1** ±17.3	**3**	**8.5** ±1.2	**10.7** ±2.0	**45.1** ±2.3	**12.5** ±0.7[c]	**5.4** ±2.4[d]	**8.3** ±2.0[f]
	4	**2**	**48.1** ±38.8	**3**	**9.1** ±2.1	**12.4** ±3.9	**47.9** ±0.6[d]	**13.4** ±1.6	**10.2** ±16.1	**5.8** ±3.3[b]
	6	**2**	**40.2** ±20.6	**5**	**9.2** ±1.1[a]	**12.3** ±2.3[c]	**47.0** ±6.0	**13.4** ±1.8[b]	**4.2** ±4.4[d]	**6.6** ±3.4[b]

t-test: sample vs chimeric 0% to 100R or 200R accordingly; a, $p \leq 0.05$; b, $p \leq 0.02$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 5 \times 10^{-4}$; f, $p < 10^{-4}$; g, $p < 10^{-5}$

were approximately within the range evaluated for chimeric mice which received cell transfusions after complete myeloablation **(Table 1).**

Discussion

In order to progress toward efficient and reproducible human BM transplantation trials for β-thalassemia, a characterization of the threshold level of normal or genetically corrected BM stem cells is necessary, and, pretransplantation regimens and modalities of cell transfer must be established. In this study, using hemi-βthal chimeric mice, we determined that a 19-24% level of normal bone marrow cells has significant benefit for appropriate improvement of the primary erythroid defects and secondary consequences of β-thalassemia. Moreover, on the basis of this threshold, we established the number of normal cells required to be infused, administered in two equal doses under minimal myelosuppression conditions, in order to achieve long-term full engraftment and high-level chimerism.

The marked increase in erythroid chimerism relative to non-erythroid or WBC cells in hemi-βthal mice after transfusion provided definitive and quantitative evidence of a selective advantage of normal or therapeutic BM cells over β-thalassemic cells. This preferential erythroid amplification is consistent with the findings from our previous studies showing a shortened RBC half-life and severely ineffective erythropoiesis, which also holds true in human β-thalassemia [14,19,20]. Our study showed that, as a result of erythroid differentiation, maturation and survival, there is a 2.0- to 2.5-fold amplification in the levels of normal donor cells, and this prevailed consistently until the 39% chimeric level. Thereafter it, decreased linearly with progressively higher donor chimerism.

Our study, using a systematic quantitative approach, determined that a chimerism of of between 19 and 24% of normal or therapeutically corrected donor cells significantly improved the typical thalassemic phenotypes. Correction of the different hematologic indices was noticeable even in the 10% chimeric groups. In the 19-24% chimeric cohorts with ~40-50% normal RBCs, hematologic analysis showed substantial amelioration of all parameters, including a significant increase (by ~4g/dl) in the Hb level. Concurrent with the improved hematologic indices, the significant

decrease of hemolysis and Heinz bodies in RBC indicated a lower incidence of premature RBC destruction, which correlates with the finding of increased RBC half-life. While aberrations in erythropoiesis differentiation/maturation were mitigated in both the BM and spleen in the 19-24% chimeric groups, the most noticeable impact was on the orthochromatophilic erythroblast, resulting in increase reticulocyte maturation. Normalization at the maturation stage of orthochromatophilic erythroblast correlated with the stage undergoing the most detrimental intracellular globin chain imbalance defect [14]. A similar erythropoietic response may be predicted in human β-thalassemia, because here too the orthochromatophilic erythroblast stage is the one most affected by the globin chain imbalance defect [19]. In parallel with these primary erythroid corrections, semi quantitative pathologic analyses were performed so as to determine the minimum level of donor chimerism that is therapeutic as regards to the secondary consequences of this disorder. From the increased number of RBCs and the higher Hb level, it may be inferred that more effective oxygen distribution to the tissues would mitigate ischemia and organ damage, and a decrease in RBC destruction would lead to decrease in iron overload. Indeed, splenomegaly is almost nonexistent in the 19-24% chimeric group. Therefore, the spleen can effectively eliminate the proportion of RBC destined to be removed from a ~50-60% severe thalassemic RBC population, thereby providing a threshold of the reticulo-endothelial sinusoid potential. Although the splenomegaly is reversed and erythropoiesis is almost corrected in the 19-24% chimericl group, the spleen displayed persistent though minor sequestration and iron deposits. Because the levels of chimerism that are most beneficial for different organs may vary, we have analyzed additional tissues that are most affected in thalassemia such as the kidneys, liver and heart. Importantly, in the 19-24% chimeric group, general improvements and decreased iron deposits were detected in all four tissues. This finding was quite impressive in the context of the cumulative pathology over their entire ~2 year lifespan of chimeric hemi-βthal mice of different groups. Evidence of significantly improved pathology and, consequently, improved physiology, was further obtained by the significantly increase in lifespan. The fact that the 19-24% chimeric group

attained normal lifespan, implies that total BM chimerism is not essential for substantially counteracting the adverse effect of thalassemia.

Interestingly, the hemi-βthal chimeric mice groups showed erythroid amplification response similar to those observed in rare human cases [3, 4]. Indeed, mixed chimerism inadvertently obtained in human β-thalassemia following allogeneic BMT has been described as free of symptoms and complications. Among these few patients, levels of normal BM chimerism at 25%, 50% or 73% led to peripheral blood erythroid amplification at 60-79%, 84-88% and 100%, respectively, which are comparable to the amplification obtained in hemi-βthal chimeric groups. In human β-thalassemia, however, given the limited experience and years of follow-up with a therapy of mixed chimerism, one cannot predict the outcome. This is because the selected transfusion or hematologic parameters may underestimate the levels of donor chimerism necessary for long-term correction of the most affected organs. This point was specifically addressed in our study by establishing the minimum percent of normal BM cells required for systemic correction of hemi-βthal mice. Keeping several criteria in mind, including pathology and survival, a minimum of 19-24% of donor chimerism was found to be required in order to produce a large beneficial impact, whereas 10% of normal donor chimerism was sufficient to improve hematologic indices significantly. One cannot extrapolate these results directly to human β-thalassemia; however, allowing for the limitations of species differences, and taking into consideration the occasional incidence of mixed human chimerism, the results of our study suggest that 20-25% normal bone marrow cells might constitute the minimum percentage necessary for partial but significant correction of β-thalassemia intermedia in humans.

Our data established an accurate quantitative estimate of the mixed chimerism necessary for a highly significant correction of β-thalassemia in mice, and supported the feasibility of limited/minimal myelosuppression approaches. The development of minimal myelosuppressive conditions will, in addition to being less toxic, probably improve outcomes in both allogeneic and autologous BMT gene therapy, particularly

since the latter has been seen to be far from 100% efficient. Our findings about chimerism levels prompted us to determine the optimal pretransplantation conditioning regimens and modalities of BM cell transfer in order to achieve full engraftment and appropriate chimerism. Optimal conditions were investigated by subjecting cohorts of hemi-βthal mice to different irradiation doses for myelosuppression, different numbers of normal donor cells, and different number of doses. These experiments showed that minor myelosuppression with irradiation of 1 or 2 Gy was sufficient to attain stable levels of chimerism for correction of thalassemia over the lifespans of the mice. Interestingly, all the mice were successfully engrafted when 60 million cells were transfused following 1 Gy radiation for myelosuppression, and also when 40 million cells were transfused after irradiation with 2 Gy provided the cells were transfused in two separate equal doses. The fact that splitting the cell infusion into two doses was seen to be more effective in achieving for engraftment suggests that medullary transepithelial passage of the homing BM cells becomes saturated above a certain threshold, and that the excess cells are being excluded from the marrow, and perhaps eliminated. Our results suggest that it would be desirable to adopt strategies involving multiple infusions so as to favor increased efficiency of engraftment. By measuring the hematologic parameters, we verified that correction of the thalassemic phenotype in the minimally myelosuppressed hemi-βthal chimeric mice had indeed been achieved, to the levels expected in the fully myeloablated group. In the matter of variability of chimerism, mice irradiated with 1 Gy for myelosuppression before being engrafted with 60 million cells, and those irradiated with 2 Gy before receiving 40 million cells, with the infusion being split into two equal doses in both cases, showed a significant correction of the thalassemic phenotype. These practical strategies involving minimal myelosuppression followed-up with infusion of a reasonable number of cells were highly successful, and could possibly be adapted to human clinical trials.

In summary, our study determined a strong selective ~2- to 2.5-fold enrichment of the erythroid cells. We also established the basic threshold of 20-25% normal donor BM cells for achieving significant long-term improvement of the general systemic

phenotype in hemi-βthal mice. The alternative therapeutic strategies for human achieving stable level of chimerism and complete engraftment under conditions of minimal myelosuppression provide the basis for human pre-clinical trials, both in allogeneic BMT and autologous with gene therapy BMT.

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Table S1. Hematologic parameters of chimeric hemi-βthal mice

		Mature Erythrocytes					Reticulocytes			
Chimeric mice (% normal WBC)	**n**	**RBC** (10^6/μL)	**Hb** (g/dl)	**MCV** (fl)	**MCH** (pg)	**Hypo** (HC<22g/dL)	**H**	**M**	**L**	**%** (total RBC)
							(% / total retic)			
0	**7**	**5.1** ± 1.1	**6.0** ± 1.2	**45.3** ± 4.2	**11.8** ± 0.4	**28.5** ±15.0	**36.9** ±10.2	**26.6** ±8.0	**29.3** ±10.6	**15.7** ±5.6
10	**12**	**6.3** ± 0.6[a]	**7.7** ± 0.9[a]	**47.3** ± 2.7	**12.3** ±0.7[a]	**17.6** ± 8.5	**38.0** ± 6.2	**29.1** ±2.9	**32.9** ± 3.7	**15.5** ±3.8
19	**12**	**7.7** ±2.8[a]	**8.9** ±1.2[c]	**48.1** ±3.3[a]	**12.9** ±0.8[b]	**10.9** ± 6.7[b]	**35.5** ±11.0	**29.6** ±4.1	**34.9** ± 7.1	**12.7** ±4.3
24	**6**	**7.7** ±0.6[d]	**10.0** ±1.5[d]	**46.6** ± 3.6	**13.0** ± 1.4	**5.6** ± 2.1[a]	**32.8** ± 7.1	**31.2** ±3.3	**36.0** ± 4.1	**10.2** ±2.6
31	**8**	**7.6** ±0.8[d]	**10.5** ±1.2[f]	**49.9** ±2.1[e]	**13.8** ±0.5[g]	**5.2** ± 2.6[a]	**26.4** ± 8.6	**33.8** ±2.6	**39.8** ± 6.2[a]	**8.9** ±3.8[b]
39	**10**	**8.1** ±0.5[d]	**11.0** ±0.8[e]	**49.5** ±1.3[d]	**13.6** ±0.3[g]	**4.2** ± 2.9[b]	**26.0** ± 9.6[a]	**34.0** ±2.9[a]	**39.9** ± 7.4[a]	**7.5** ±4.1[a]
52	**5**	**8.6** ±0.4[e]	**12.3** ±1.1[e]	**51.5** ±1.6[d]	**14.4** ±1.1[b]	**4.5** ± 4.1[b]	**22.9** ± 6.7[a]	**35.4** ±2.7[a]	**41.7** ± 4.8[a]	**7.2** ±2.7[b]
70	**5**	**8.6** ±1.2[c]	**12.5** ±1.4[e]	**52.7** ±1.5[e]	**14.7** ±0.9[c]	**2.1** ± 0.4[b]	**17.5** ± 8.5[b]	**37.1** ±1.6[a]	**45.5** ± 7.1[a]	**6.3** ±2.2[a]
89	**3**	**9.4** ±1.1[b]	**13.7** ±0.6[f]	**50.3** ±3.6	**14.7** ±1.1[a]	**0.7** ± 0.5[b]	**15.3** ± 2.4[c]	**37.3** ±1.6[a]	**47.3** ± 3.4[b]	**4.5** ±0.9[a]
100	**5**	**9.6** ±0.9[f]	**13.9** ±1.2[f]	**50.3** ±2.4[a]	**14.5** ±0.2[h]	**0.7** ± 0.7[b]	**9.7** ± 6.8[d]	**36.8** ±1.2[a]	**53.6** ± 8.0[c]	**4.9** ±2.7[b]

t-test: sample vs chimeric 0%; a, p<0.05; b, p<0.005; c, p<0.001; d, p<0.0005; e, p<0.0001; f, p<0.00001; g, p<0.000001; h, p<0.0000001

Table S2. Quantification of red blood cell morphologic anomalies in chimeric hemi-βthal mice

Chimeric mice (% normal WBC)	n	RBC Fragments (per 100 cells)	RBCs with Heinz Bodies (%)
0	3	**10.9** ±3.3	**51.0** ±12.8
10	5	**4.4** ±0.9	**21.6** ±15.0[a]
19	7	**2.9** ±1.2[a]	**18.9** ±3.9[a]
24	5	**3.1** ±4.4[a]	**18.6** ±7.8[a]
31	4	**2.2** ±1.0[a]	**12.5** ±3.1[a]
39	4	**1.7** ±1.7[a]	**14.1** ±5.7[a]
52	3	**0.7** ±0.5[a]	**9.2** ±8.4[b]
70	4	**0.4** ±0.4[a]	**6.2** ±6.1[b]
89	3	**0**[a]	**0.8** ±0.1[b]
100	3	**0**[a]	**0**[b]

t-test: sample vs chimeric 0%; a, $p<0.05$; b, $p<0.02$

Table S3. Quantification of erythroid precursors in chimeric hemi-βthal mice

Chimeric mice (% normal WBC)	n	Proerythroblasts (% of total cells)		Basophilic erythroblasts (% of total cells)		Late basophilic / Polychromatophilic erythroblasts (% of total cells)		Orthochromatophilic erythroblasts (% of total cells)	
		Bone marrow	Spleen	Bone marrow	Spleen	Bone marrow	Spleen	Bone marrow	Spleen
0	3	6.9 ±1.9	2.6 ±0.4	44.6 ±6.3	44.0 ±7.2	4.1 ±0.5	8.2 ±2.6	5.0 ±0.5	19.6 ±3.4
10	3	4.7 ±0.5	2.3 ±0.5	45.2 ±0.5	39.0 ±7.9	4.9 ±3.0	5.1 ±2.8	6.8 ±3.1	26.8 ±5.6
19	**4**	**5.0** ±1.9	**2.2** ±0.6	**40.9** ±6.7	**41.7** ±19.2	**5.9** ±1.5[a]	**6.4** ±1.9	**6.2** ±1.3	**15.1** ±7.3
24	**3**	**5.5** ±1.3	**1.8** ±0.3	**38.7** ±6.9	**24.8** ±7.5[a]	**5.9** ±2.6	**6.7** ±3.6	**10.2** ±0.2[d]	**31.9** ±1.8[b]
52	3	6.7 ±1.8	2.6 ±1.9	30.8 ±4.1[a]	8.1 ±3.0[c]	5.2 ±2.8	7.4 ±7.8	12.3 ±0.3[d]	38.6 ±14.9
100	3	3.2 ±1.4	1.3 ±0.5[a]	23.2 ±2.7[b]	3.5 ±1.5[c]	7.0 ±5.0[a]	2.1 ±1.0[a]	20.2 ±4.5[a]	32.8 ±7.2

t-test: sample vs chimeric 0%; a, p≤0.05; b, <0.02; c, p<0.01; d, p<0.005

CHAPTER IV

ARTICLE 2

FOREWORD

As described for β-thalassemia in Chapter 3, I also similarly determined the minimal percentage of normal bone marrow cells necessary to correct sickle cell disease (therapeutic threshold), by generating a series of hematologic chimeric mice, with different percentages of normal blood cells. On the basis of that threshold, the minimal dose of myelosuppression and the minimal number of cells that allow reaching that threshold were determined.

Using similar approach as in Chapter 3, the following article describes the generation of the different groups of chimerism for SCD by bone marrow transplantation (BMT), the full characterization of their phenotype and the determination of the therapeutic threshold. This threshold was used as a basis to optimize the therapeutic conditions of minimal myeloablation and cellular requirements (number and transfer strategies) that allow equivalent phenotype correction.

The results of current article demonstrate for the first time that murine sickle cell disease can actually be significantly corrected for long term by partial replacement of the sickle bone marrow, and therefore a partial chimerism of normal RBCs. It also provides the first standard conditions of BMT for SCD, including myelosuppression and mode of cell transfer, to achieve reproducible engraftment in all transplanted mice. The results of this article provide valuable information for planning human pre-clinical trials in BMT for SCD.

Contribution to the article

All the Figures (Figure 1, 2, 3) preparation and results

All the tables (Table 1, 2, 3, 4, 5, S1, S2, S3) preparation and results

The writing of this article was done in collaboration with my research supervisor

CORRECTION OF MURINE SICKLE CELL DISEASE BY OPTIMIZATION OF CELLULAR FUNCTIONAL REQUIREMENTS

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Running title: Therapeutic requirements for SCD

Key words: sickle cell disease, erythropoiesis, cellular therapy, conditioning regimen, BMT

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Abstract

Minimal criteria requirements of stem cell replacement, conditioning regimen and modalities of infusion essential for permanent cure of sickle cell disease (SCD) by bone marrow (BM)/stem cell transplantation or gene therapy must be established prior to clinical trials. The minimal percentage of normal BM stem cells was determined for therapeutic correction of this red blood cell (RBC) disorder by transplantation repopulation assay in the SCD SAD mice. Eleven groups of stable chimeric SAD mice (5-92%) were analyzed thoroughly over ~2 years by various cellular, molecular and physio-pathologic approaches. Chimeric mice with 26-31% normal donor stem cells showed improvement of RBC parameters and consequent erythropoietic responses. Mice in the 26% chimeric groups and above showed for all organs substantial amelioration of pathologies with generalized decreased iron deposits, fibrosis and thereby reached a normal lifespan. These results established the minimal range of 26-31% normal BM cells as the threshold for long-term significant correction of murine SCD. Subsequently, we investigated the minimal myelosuppression doses concurrently with the number of BM cells to infuse, the number and timing of infusions following myelosuppression to achieve the therapeutic threshold in SAD mice. Significantly, 100% recipients mildly myelosuppressed with 1 or 2Gy irradiation reproducibly engrafted at therapeutic levels with three equivalent cell infusions by 28h from the myelosuppression. These studies determined the minimal transplantation requirements concomitant with mild myelosuppression for significant correction of SCD SAD mice and established the long-term therapeutic chimeric threshold of healthy/normal BM stem cells to 26-31%.

Introduction

Sickle Cell Disease (SCD) is one of the most common single-gene disorders encountered worldwide leading to high mortality and morbidity. The molecular basis for this hemoglobin (Hb) defect was the first to be described several decades ago but still there is no specific therapy for this disease. The presence of abnormal Hb, HbS, results in intracellular Hb polymerization in red blood cells (RBC) and causes RBCs to become rigid and deformed or sickled (1).

SCD patients display severe secondary manifestations from these sickled RBCs. Production of abnormal RBCs leads to altered hematologic indices and to their marked destruction or shortened survival that in turn stimulates erythropoiesis and extramedullary hematopoiesis. In severe SCD, patient even display impaired erythroid maturation or ineffective erythropoiesis (2). Sickled RBCs can also become trapped in the circulation resulting in vaso-occlusions that induce in SCD patient painful crises, causes ischemia and damage to several tissues or organs. Systemic complications in patients are due to iron overload and deposits, fibrosis as well as particular alterations affecting the kidneys, spleen, liver, and lungs (3). These SCD patients suffer from chronic and acute complications leading to progressive multisystemic organ damage and, reducing by ~40% their median life expectancy.

Individuals with severe SCD are dependent on chronic transfusion. However, this therapy causes additional iron overload in several organs and becomes life threatening without continuous iron chelation. Presently, bone marrow transplantation (BMT) from allogeneic or autologous source provides, if safe and efficient, a promising therapeutic alternative. Allogeneic BMT was demonstrated to be curative in SCD patients (4). However, the toxicity of the intensive preparative regimens associated with myeloablative BMT and the limited histocompatible stem cell donors have restricted the application to patients. Alternatively, these complications could be prevented by autologous BMT following gene transfer into hematopoietic stem cells (HSCs) but it depends on availability of a transduced therapeutic gene with persistent

expression and vehicle safety. For either BMT approach, it was previously assumed that complete bone marrow replacement is necessary for correction of SCD. Among the BMT clinical intervention for SCD, few patients with stable but partial bone marrow chimerism became transfusion-independent and did not display SCD related complications (5,6). Such clinical experience support development and design of mixed hematopoietic chimerism rather than complete bone marrow replacement for SCD improvement. To develop reliable and efficient therapeutic strategies, it would be important to first determine the adequate levels of chimerism and second to establish optimal transplantation strategies including conditioning regimens and cell transfer modalities.

Many mouse models for SCD have been generated over the years (7-11). Although some with the elegant use of YACs expressing only human sickle Hb, these transgenic mice displayed thalassemic phenotype with significant globin imbalance that confuse pathology interpretation (12,13). By contrast, the SAD transgenic mouse is among the best analyzed hematologically, histo- and physio-pathologically and importantly, SAD RBC do not have globin chain imbalance or thalassemic characteristics (14-16). The SAD mice exhibit the complex multi-organ SCD defects and reproduce all the typical features of human SCD with stimulation of erythropoiesis and hematopoiesis, premature RBCs destruction and ineffective erythropoiesis (16). This phenotype is likely, as described in human SCD, to confer selective advantages of normal or corrected BM cells over the sickle cells.

As initial attempts to investigate the threshold of mixed RBC chimerism for SCD correction, a SCD model was used with substantial thalassemic phenotype (17-20). Although this model was reported problematic for therapy testing (21), it was observed that normalization of hematologic parameters did not correlate with correction of organ pathology. However, many questions remain about patho-physiological analysis including long-term chronic complications or typical thalassemic rather than SCD features. Together with the small number of mice with

intermediate mixed BM chimeras, the threshold level could not be addressed adequately.

To establish the minimal level of normal/healthy stem cells to cure SCD, we perform a competitive repopulation assay with the SAD mouse model to produce a panel of numerous mixed BM chimeras over the entire range. Detailed systematic and long-term analysis defined the minimal therapeutic level of normal donor stem cells at 26-31%, and was associated with normal RBC amplification to ~55-65% providing evidence of selective advantages. Most importantly, 26-31% BM chimerism resulted in significant long-term phenotypic correction of SCD and normal life expectancy. Because of the adverse toxic effect of myeloablation, we determined the minimal conditioning regimens, the number of cells to infuse and modality of dose delivery for stable systemic correction of SCD. Our results provide the first chimerism threshold for BMT and optimized conditions for minor myelosuppressive regimens that could serve as basis to future therapeutic trials for mixed chimerism in human SCD.

Materials and methods

Mouse strains

The sickle cell mouse model (SAD-1 mouse) expresses a modified human β-globin chain, the β^{SAD} and the human α-globin genes (14). These mice were bred onto C57BL/6J-*Gpi1*^{b}/*Gpi1*^{b} background for >21 generations, therefore considered syngeneic. SAD-1 mice were genotyped as previously described (14). Congenic C57BL/6-*Gpi1*^{a}/*Gpi1*^{a} (Glucose phosphate isomerase isotype 1a) mice carry a hematopoietic cell marker and were obtained from Dr J. E. Barker (the Jackson labs, Bar Harbor, ME, USA). All mice were maintained in a specific pathogen free environment and experimental procedures were conducted in compliance with the guidelines of the Canadian Council on Animal Care (CCAC).

Production of chimeric mice

Nucleated bone marrow (BM) cells were harvested from donor animals using IMDM (Iscove's Modified Dulbecco's Medium, GIBCO, Grand Island, NY, USA). Competitive Repopulation Assays were carried out based on the principle described by David Harrison (22). Briefly, a fixed number of donor bone marrow cells from normal and SAD populations mixed in reciprocal proportions were transplanted into recipients. Thirteen hematopoietic chimeric groups were produced with 0 to 100% normal cells. Recipient mice were lethally irradiated and transplanted as previously described (23). In addition, for mice that were partially myelosuppressed with a dose of 1 or 2 Gy, 20, 40, 50, 60 or 80 x 10^6 cells were injected in one dose at variable times after irradiation (4h, 28h, or 52h), and 40 or 60 x 10^6 cells were injected in two, three, or four doses within the first 24-28 hours following irradiation.

Analysis of mice hematopoietic chimerism

Hematopoietic engraftment was assessed by the relative proportion of the two Gpi1 isotypes in both white and red blood cells on a regular basis from the second month to the 18th month following BMT. Gpi1 quantification was determined from the different electrophoretic mobilities onto a cellulose acetate membrane of white or red

blood cell lysates in an alkaline buffer using Helena equipment (Beaumont, Texas, USA), as previously described (23). The membranes were then scanned and bands quantified using ImageQuant software.

Hematological analysis

Hematological analysis of chimeric mice was performed on 250µl blood using a Bayer Advia 120 cell analyzer with the mouse archetype of multispecies software version 2.2.06 (CTBR, Montreal, Canada). Gating of the cellular hemoglobin concentration mean (>35g/dl) and the mean cellular volume (<25fl) was used to determine the percentage of hyperchromic RBCs and the percentage of microcytic cells, respectively, as previously described (24). Reticulocyte counts were obtained by specific RNA staining with the oxazine 750 dye using the reticulocyte channel on the analyzer. The osmotic fragility of murine red blood cells (RBCs) was evaluated by their hemolysis pattern when suspended in hypotonic solutions using decreasing NaCl concentration ranging from 0.85% to 0.15% in a 0.05% step-gradient as previously described (23). The RBC half-life was determined using a biotinylation protocol of the total RBCs and evaluated by RBC replacement as previously described (23).

Protein level of various hemoglobins molecules was quantified from RBC population by High Performance Liquid Chromatography (HPLC) (Varian) as previously described (25).

Hematopoiesis/erythropoiesis analysis

Cells from bone marrow and spleen of chimeric mice, as well as of C57BL/6J and SAD controls were subjected to flow cytometry analysis. Bone marrow cells were harvested from one femur and single spleen cell suspensions were prepared as previously described (23). Cells were stained with anti-mouse TER119-phycoerythrin, and biotin conjugated anti-mouse CD71 (transferrin receptor) that was detected with streptavidin-allophycocyanin as previously described. Samples were further processed and analyzed as previously described (23).

Multipotent CFU-S_{12} hematopoietic cells were evaluated from both bone marrow and spleen. Nucleated bone marrow (5×10^4) or spleen cells (10^6) were injected in lethally irradiated C57BL/6J recipient mice. CFU-S colonies were counted on day 12, as previously described (23).

Pathologic analysis

Mice (n=3 to 9) from each group were sacrificed for pathological analysis. Spleen weight was determined relative to body weight. Several organs including spleen, liver, kidney, and lungs were fixed in 3.7% phosphate-buffered saline-formaldehyde overnight and embedded in paraffin block. Four-micron thick sections were stained by hematoxylin and eosin, Sirius Red for collagen quantification, and with Prussian blue for iron analysis, as previously described (23).

Statistical analysis

Values were expressed as mean ± standard deviation. Two samples student T-test was used to determine statistical analysis with Microsoft excel software and Fisher test with SigmaStat 3.1 software.

Results

Expansion of normal erythroid cell population in SAD chimeric mice

To determine the minimal proportion of normal bone marrow cells required to correct the sickle cell phenotype, a competitive repopulation assay was performed on SAD mice previously backcrossed onto a homogeneous C57BL/6J genetic background (14,15). Mice lethally irradiated were transplanted with mixtures of marked syngeneic normal (C57BL/6J-*Gpi1*^{a}) bone marrow cells and SAD (C57BL/6J-*Gpi1*^{b}) bone marrow cells, distinguishable by a specific Glucose Phosphate Isomerase isotypes. The Gpi1 marker served to determine the relative proportions of normal and SAD cell populations in peripheral blood of chimeric mice. Bone marrow cell engraftment and chimerism were analyzed in recipient mice (n=147) at regular intervals after transplantation from two months onwards. We produced 13 groups of SAD chimeras ($n \geq 3$ per group) stably transplanted with reciprocal proportions of normal (0, 5, 11, 16, 21, 26, 31, 39, 49, 64, 80, 92, 100%) and SAD white blood cells (WBCs) at 7 months post-bone marrow transplantation (BMT) (Figure 1). The percentage of WBC in each recipient mice corresponded to their level of hematopoietic stem cell engraftment. In comparison to the WBC in the peripheral blood, the proportion of normal red blood cells (RBCs) was amplified significantly in each group from 5% to 80% due to loss of SAD RBCs. Amplification of normal RBCs attained for the 5-31% chimeric groups 2- to 4-fold relative to WBCs, supporting a selective advantage for the normal RBCs (Figure 1). Regular assessement of the recipient chimerism showed stable long term engraftment maintained up to 23 months after BMT. Cellular, physiologic and pathologic characterization was performed for all 13 chimeric SAD groups.

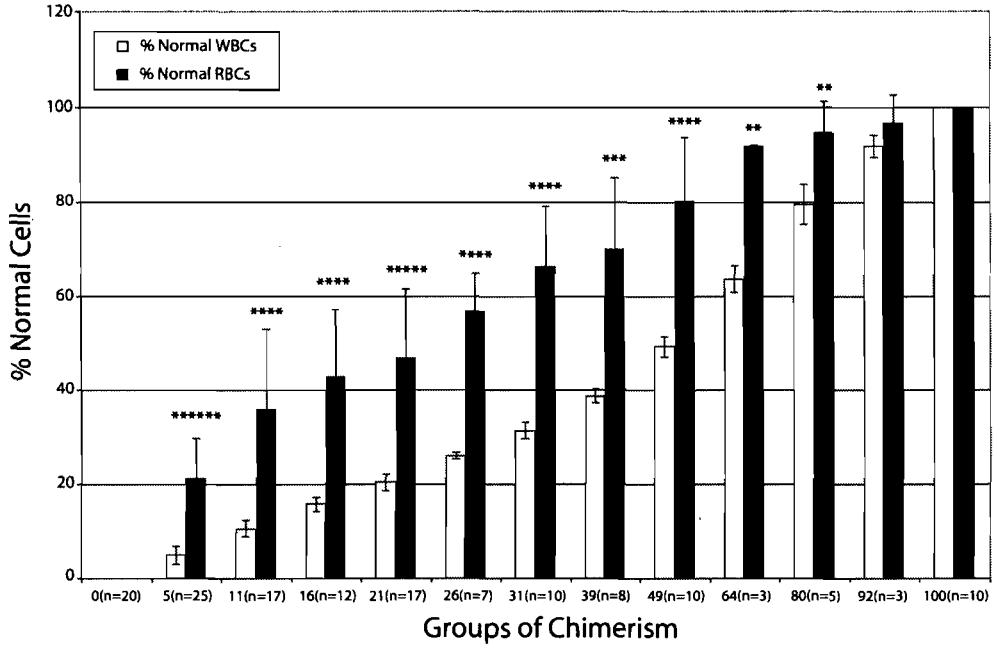

Figure 1. Chimerism in SAD mice.
Comparison between chimerism levels of normal WBCs (open bars) and normal RBCs (black bars) for each of the 13 groups of chimeric mice 7 months post-transplantation. The n below each group refers to the number of mice per group. Amplification of the normal erythroid cell population in peripheral blood of SAD chimeric mice relative to WBCs was observed systematically. *t*-test: p = normal RBCs vs normal WBCs; *, $p<0.05$; **, $p<0.005$; ***, $p<10^{-3}$; ****, $p<10^{-4}$; *****, $p<2 \times 10^{-6}$; ******, $p<10^{-7}$

Improved erythrocyte parameters in SAD mice with low-level chimerism

Based on the amplification of normal RBCs, levels of hemoglobin SAD (HbSAD) were monitored in RBCs of chimeric mice by HPLC on peripheral blood lysates. The levels of HbSAD were inversely proportional to the percentage of normal RBCs. In the 26% chimeric SAD group, HbSAD levels were decreased by half consistent with the presence of ~50% normal RBCs (data not shown; Figure 1). To determine the percentage of chimerism that can ameliorate hematological parameters previously shown altered in SAD mice (15,24), peripheral blood was analyzed in chimeric SAD mice. With increasing normal donor chimerism, the analysis showed progressive amelioration of the typical features of SCD, the cellular hemoglobin concentration

mean (CHCM), the hyperchromic or dense cells and the mean cellular volume (MCV) that was significant from the 31% or 39% chimerism and above (Table 1).

To define the range of chimerism that would ameliorate the SAD erythrocyte osmotic fragility, the erythrocytes were resuspended in a range of saline hypotonic solutions. Cell hemolysis displayed a two-step fragility curve separated by a plateau for each of the different SAD chimeric groups that carried both normal and SAD RBC populations (data not shown). This pattern of discontinuous curve correlated with the degree of RBC chimerism detected in these mice. Half of the RBCs of the 0% SAD chimeric group (n=3) displayed osmotic lysis at 0.35±0.01% saline concentration whereas those of the 100% SAD chimeric group (n=2) occurred at 0.46±0.01%. Consistently, the 26% SAD chimeric group (n=3) exhibited half RBC lysis at saline concentration of 0.40±0.01%, midway between controls that corresponds to the amplification at ~57% normal RBC.

Table 1. Hematological parameters of SAD chimeric mice

Chimeric Mice (% normal WBCs)	n	CHCM (g/dl)	MCH (pg)	MCV (fl)	Hyper (HC>35g/dl)	n	RBC half-life (days)
0	15	30.5 ±1.4	14.3 ±0.6	46.3 ±2.6	6.2 ±0.49	4	6.6 ±1.6
26	7	30.4 ±1.3	14.3 ±0.6	46.5 ±2.9	5.8 ±0.37	2	9.0 ±1.2
31	9	30.1 ±1.1[d]	13.8±0.6	45.3 ±2.7[c]	3.6 ±0.31		n/d
39	8	28.8 ±1.2[c]	14.1 ±0.8	48.5 ±3.5	2.1 ±0.18[b]	2	14.3 ±1.1[c]
100	9	28.2 ±1.3[c]	14.5 ±0.9	50.2 ±3.9[b]	0.4 ±0.08[c]	2	16.6 ±3.5

t-test: sample vs chimeric 0%; a, $p<0.05$; b, $p<0.02$; c, $p<0.01$; d, $p<0.001$

Because it has been shown that SAD RBCs have a very high turnover (), we evaluated the RBC half-life in the SAD chimeric groups by biotin labeling. In table 1, RBC half-life of the 0% chimeric group was decreased by 2.5-fold in comparison to the 100% chimeric group. Even with the 11% chimeric group, an important increase in RBC survival was attained at 9.4±0.1 (n=2; $p<0.04$) and almost reached normal values in the 39% chimeric group.

Normalization of erythropoiesis efficiency in SAD chimeric mice

Based on our previous finding of SAD mice stimulated medullary and splenic hematopoiesis as a compensatory response to damaged erythroid precursors/inefficient erythropoiesis, we monitored bone marrow and spleen hematopoietic/erythropoietic function in the chimeric groups. Differentiation and maturation potential of hematopoietic progenitors and erythroid precursors were evaluated by in vivo quantification of primitive multipotent CFU-S_{12} progenitor cells and by flow cytometric analysis of precursor cells.

Analysis of bone marrow and spleen in 0% SAD chimeric group showed a significant increase in the number of CFU-S_{12} multipotent cells compared to control 100% SAD chimeric group (Table 2). The important stimulation of splenic hematopoiesis of ~ 2-2.5 fold in CFU-S_{12} multipotent cells is concordant with our hematopoietic model that SAD marrow proliferating multipotent cells become mobilized in peripheral blood to colonize the spleen (25,26). Significant decrease of splenic multipotent cell stimulation was observed in the 5% SAD chimeric group and above to values similar of the 100% SAD chimeric group. Parallel to this decrease in the multipotent progenitors, the splenic cellularity in the 5% chimeric group and above reached normal values (Table 3 and data not shown).

Table 2. Quantification of medullary and splenic multipotent cells in chimeric SAD mice

Chimeric Mice (% normal WBCs)	n	Femur (10^2 CFU-S_{12})	Spleen (10^2 CFU-S_{12})
0	**3**	**23.4** ±9.0	**12.1** ±6.0
26	**3**	**8.8** ±1.4	**4.3** ±1.2[a]
31	**3**	**11.8** ±1.6	**7.3** ±8.0[a]
39	**3**	**10.8** ±7.4	**2.2** ±1.2
100	**3**	**15.2** ±2.3[a]	**5.1** ±3.2[a]

Abbreviations: WBCs, white blood cells; CFU-S_{12}, colony forming unit in the spleen at day 12 after transplantation;
t-test: sample vs chimeric 0%; a, $p<0.05$

Table 3. Semi-quantitative analysis of pathology in SAD chimeric mice

Chimeric Mice (% normal WBCs)	Spleen								Kidney					Liver				Lungs	
	Spleen wt / Body wt		Nucleated Cells		Iron		Fibrosis		Glomeruli Area Outer	Glomeruli Area Inner		Fibrosis		Iron		Fibrosis		Fibrosis	
	n	(%)	n	(10^7)	n	(%)	n	(%)	n	($x10^2\mu m^2$)		(%)		n	(%)	n	(%)	n	(%)
0	9	**0.48** ±0.36	3	**16.1** ±4.5	5	**3.3** ±1.1	5	**7.3** ±3.8	5	**33.4** ±5.3	**63.1** ±15.4	**5.1** ±2.3		4	**0.7** ±0.40	4	**1.4** ±0.4	4	**2.6** ±0.3
26	5	**0.32** ±0.09[a]	3	**12.6** ±1.9[a]	5	**0.6** ±0.3[e]	5	**1.6** ±0.6[a]	5	**31.6** ±2.80	**51.5** ±9.5	**2.2** ±1.1[b]		4	**0.3** ±0.40	4	**0.8** ±0.3[a]	4	**1.6** ±0.7
31	4	**0.27** ±0.08[a]	3	**14.1** ±7.3	4	**0.8** ±0.3[b]	4	**2.1** ±0.8	3	**30.3** ±7.20	**48.0**± 11.8	**2.5** ±1.0[a]		4	**0.1** ±0.08	4	**0.6** ±0.1[c]	4	**1.3** ±0.7[a]
39	5	**0.33** ±0.10	3	**8.5** ±3.5	3	**1.2** ±0.4[a]	4	**1.7** ±0.6	3	**26.5** ±3.10[a]	**38.7** ±12.0[a]	**1.5** ±0.5[b]		5	**0.1** ±0.04	5	**0.6** ±0.3[c]	4	**1.4** ±0.5[e]
100	5	**0.24** ±0.06[d]	3	**13.6** ±3.7[a]	4	**0.3** ±0.2[c]	5	**1.5** ±0.4	6	**26.9** ±3.80[a]	**49.2** ±11.3	**0.9** ±0.2[b]		5	**0.1** ±0.08	4	**0.4** ±0.2[a]	4	**1.1** ±0.3[e]

Abbreviations: WBC, white blood cells; wt, weight

t-test: sample vs chimeric 0%; a, $p<0.05$; b, $p<0.02$; c, $p<0.01$; d, $p<0.005$; e, $p<0.0005$

To determine the level of chimerism that could correct the damaged medullary erythroid precursors/ineffective erythropoiesis, the maturation potential of erythroid precursor cell population in spleen and bone marrow was analyzed. Four classes of erythroid precursors were identified and quantified by FACS analysis with the specific erythroid Ter119 and transferin receptor CD71 surface markers. Based on staining intensities, these precursors from the least to the most mature are: the proerythroblast ($Ter119^{med}CD71^{high}$), the basophilic erythroblast ($Ter119^{hgh}CD71^{high}$), the late basophilic and polychromatophilic erythroblast ($Ter119^{high}CD71^{med}$), and the orthochromatophilic erythroblast ($Ter119^{high}CD71^{low}$) (Figure 2). While in bone marrow of 0% SAD chimeric group, the earlier proerythroblast and basophilic erythroblast populations showed some increase in comparison to 100% SAD chimeric group, the orthochromatophilic erythroblast population in contrast exhibited a significant decrease by ~2.5 fold (Figure 2; Supplementary Table S1). This inefficient erythropoiesis (Supplementary Table S1) was significantly improved in the 16% and 26% chimeric groups onwards (**Figure 2).**

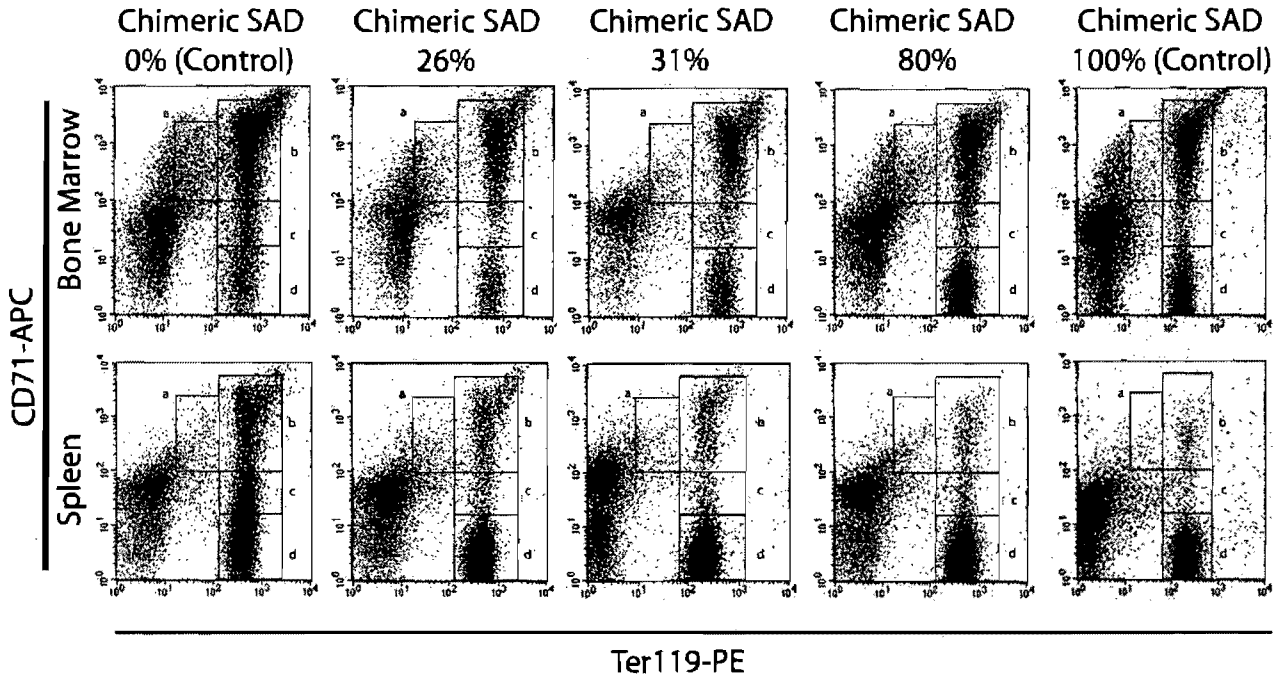

Figure 2. Analysis of erythroid cell precursor maturation in chimeric SAD mice.
Flow cytometry analysis of erythroid cells from bone marrow (top) and spleen (bottom). Four regions are delineated by rectangles representing cell subpopulations: a, $Ter119^{med}CD71^{high}$, proerythroblasts; b, $Ter119^{high}CD71^{high}$, basophilic erythroblasts; c, $Ter119^{high}CD71^{med}$, late basophilic / polychromatophilic erythroblasts; d, $Ter119^{high}CD71^{low}$, orthochromatophilic erythroblasts. The 31% group of SAD chimeric mice displayed marked improvement of the bone marrow and spleen early to late erythroid precursors in comparison to control with 0% normal WBCs.

In the spleen, the basophilic and polychromatophilic erythroblasts were significantly increased in the SAD chimeric mice with 0% normal WBCs compared to those with 100% normal WBCs. By contrast, the orthochromatophilic erythroblast displayed normal population levels (Supplementary Table S1), and suggested that the earlier splenic erythroid precursors are increased to maintain normal levels of the orthochromatophilic erythroblast and counterbalance the loss due to ineffective erythropoiesis. In correlation with the improvement of bone marrow erythropoiesis, the 16-26% SAD chimeric group showed significant increased in the basophilic and polychromatophilic populations, leading to correction of ineffective erythropoiesis in these groups and above.

Amelioration of pathology and lifespan in the 26-31% SAD chimeric mice

Because we showed that SAD mouse model as in sickle cell disease displayed pathologic features in many organs including spleen, kidneys, liver and lungs (15), these were investigated in all the SAD chimeric groups at the end of their lifespan (Figure 3). Spleen is a major organ affected in SAD and in the 0% normal WBCs chimeric group with 2-fold enlargement (Table 3). The 0% chimeric group exhibited histologically complete loss of the lymphoid follicular structure of the red and white pulp, with RBC sequestration, iron deposition, focal areas of necrosis, extramedullary hematopoiesis, congestion and interstitial fibrosis (Figure 3a, e; Table 3). By the 26% SAD chimeric group, the spleen to body weight ratio was significantly reduced with decreased iron deposits and RBCs sequestration and the hypercellularity and fibrosis reached normal values (Table 3; figure 3b, f). The renal pathology in the 0% SAD chimeric group similar to SAD and human SCD (20, 21, 22, 23), developed cortical infarcts, glomerular hypertrophy, segmental and/or global glomerulosclerosis, tubular iron deposits and segmental interstitial fibrosis (Table 3; Figure 3m). The important glomerular hypertrophy from both the inner and outer cortex caused by expansion of mesangial cells decreased progressively as well as glomerulosclerosis with increasing chimerism (Figure 3 n, o; Table 3). Renal fibrosis was significantly reduced and absence of detectable vascular thrombi in the 26% SAD chimeric group (Table 3). Hepatic pathology of 0% SAD chimeric group similar to SAD and human SCD

exhibited hyperplasia of the Kupffer cells and of portal macrophages associated with iron deposits and fibrosis (Figure 3i). In the 26-31% SAD chimeric group both iron deposits and fibrosis had declined substantially and the Kupffer cells regressed in size (Figure 3 j, k Table 3) almost comparable to the 100% SAD chimeric group (Figure 3l). Analysis of lung histology in the 0% SAD chimeric group showed septal thickening, pulmonary fibrosis, vascular attenuation/occlusions and increased vasculature wall thickness (Figure 3q). In contrast, the 26% SAD chimeric group had reduced pulmonary vascular congestion and occlusions with almost normal septa (Figure 3r) and the 31% SAD chimeric group had significantly improved fibrosis similar to the 100% SAD chimeric group (Figure 3s, t).

Since we previously reported that SAD mice had a reduced lifespan of 15.4 ± 7.3 (15), we determined the level of chimerism that could impact on the overall physiology of the transplanted chimeric mice (Table 4). In comparison to the 0% SAD chimeric group, the lifespan of SAD chimeric mice was significantly increased in 26% SAD chimeric group and reached normal survival expectancy similar to control 100% SAD chimeric group, indicating that the organ pathology still observed had minimal consequences.

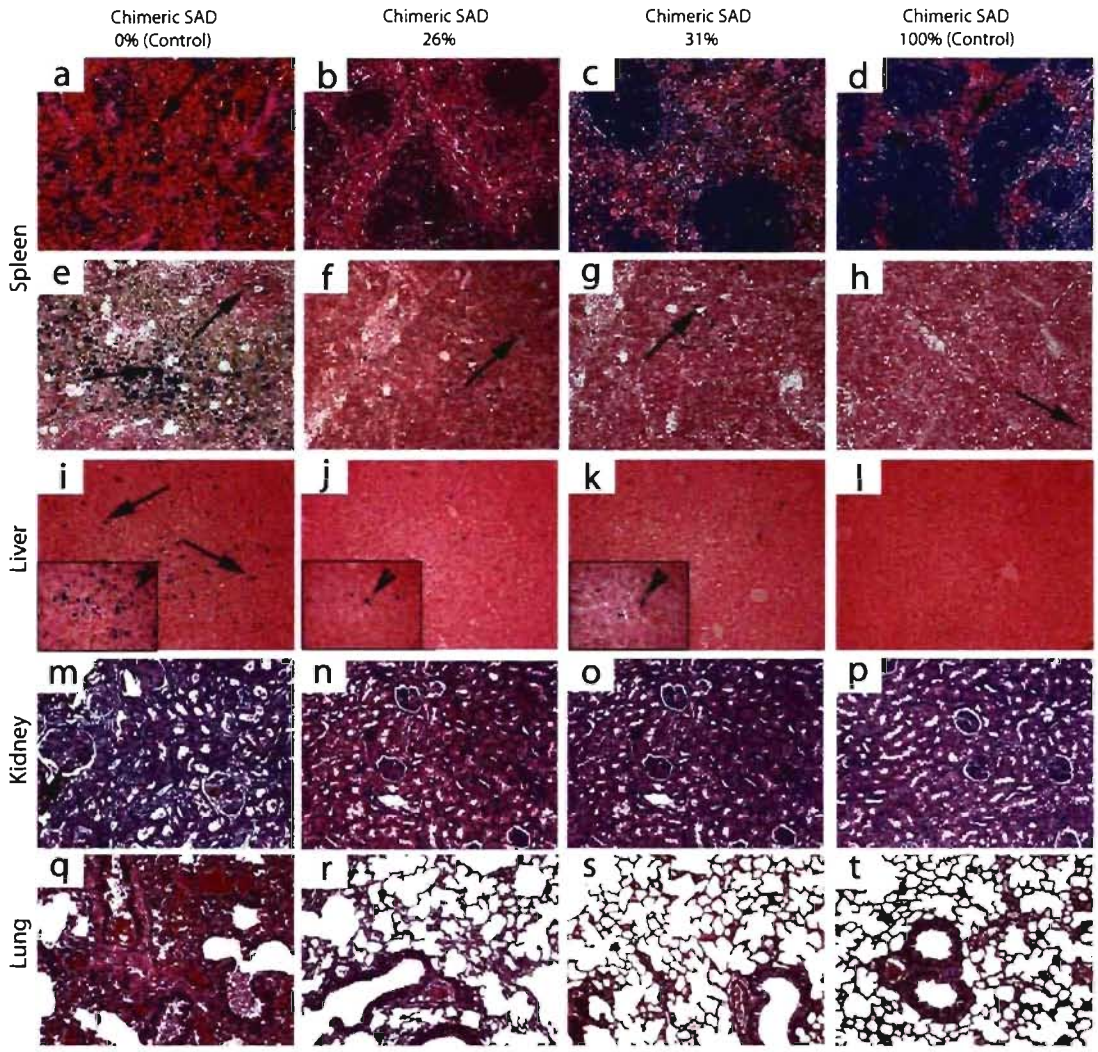

Figure 3. Histopathologic analysis of chimeric SAD tissues.
Histologic analysis of tissue sections was performed on spleen, kidneys and lung stained with H & E and spleen and liver with Prussian blue counterstained with nuclear fast red. The 0% chimeric SAD mice as sickle controls were of 14-month-old (a, e) and 18-month-old (i, m, q), the 26% chimeric SAD mice of 19-month-old (b, f) and 21-month-old (j, n, r), the 31% chimeric SAD mice of 18 months-old (c, g) and 26-month-old (k, o, s) the 100% chimeric SAD mice as wild-type controls were of 21-month-old (d, h) and 17 months old (l, p, t). In the 26 and 31% chimeric groups, the spleen architecture was substantially improved relative to the 0% chimeric group with reduced RBC sequestration (arrowhead in a) and erythroid/hematopoietic hyperplasia (arrow in a) (x100) with discernible white pulp (arrowhead in d) and red pulp (arrow in d). Spleen hemosiderin analysis (arrow e-h) showed substantially less iron deposits in the 26% chimeric SAD group (f) (Magnification x200). Numerous liver iron deposits were detected in the 0% chimeric SAD group (i) and their abundance was significantly reduced correlating with size normalization of Kupffer cells in the 26% chimeric SAD group (j) (Magnification x200; Inset, x640). The glomerular

Figure 3. Histopathologic analysis of chimeric SAD tissues ...(continued) hypertrophy (arrow) and tubular dilatation in 0% chimeric SAD kidneys (m). were noticeably reduced in the 26 or 31% chimeric SAD groups (n, o) (Magnification x200). Severe pulmonary septal and alveoli thickening, vascular wall and congestion of 0% chimeric SAD group (q) were much less observed in the 26% (r) and 31% (s) chimeric SAD mice (Magnification x200).

Table 4. Survival of SAD chimeric mice

Chimeric Mice (% normal WBCs)	n	Age (months)
0	8	15.5 ±4.5
26	4	19.5 ±1.0[a]
31	4	17.3 ±2.9
39	2	19.8 ±1.8
100	6	18.7±1.5

Abbreviation: WBCs, white blood cells;

t-test: sample vs chimeric 0%; a, $p<0.05$

Characterization of minimal myelo-suppression and modalities for therapeutic engraftment

Based on the results of selective advantage of normal RBC over those of SAD/SCD at 26-31% chimerism, complete BM replacement is not necessary for significant correction of the phenotype in SAD mice. It can be hypothesized that preconditioning regimen with mild myelo-suppression could be sufficient to attain an efficient therapy instead of complete myeloablation of recipients. Accordingly, we have investigated different regimen conditions by modulating four parameters: irradiation level, number of transplanted cells, number of cell doses and time of cell transfer after irradiation, necessary to reach the determined therapeutic window.

For this analysis, two low-levels of irradiation at 1 or 2 Gy were selected for myelo-suppression. For each condition, three different quantities of cells were infused in one dose at different time-points following myelo-suppression, 4h or 28h (1Gy), 4h or 52h (2Gy) (Table 5 A) or in two, three or four equal doses within 28h of myelosuppression (Table 5B). Analysis of each recipient mouse for chimerism showed long-term stable level as determined for myeloablated mice above. As higher numbers of cells are transferred at 4h following either level of myelosuppression, a general increase in levels of chimerism was observed in the recipients (Table 5A). Noticeably, infusion of cells at 28h or 52h following myelosuppression displayed no significant advantage over transfer at 4h on the levels of chimerism. With higher myelosuppression (2Gy), chimerism was significantly increased when 40 million cells were transplanted in comparison to the group with lower myelosuppression (1Gy), attaining the therapeutic range. However, the one-cell dose infusion protocol lead to a fraction of engrafted recipients irrespective of infusion time, number of cells and both irradiation levels. Subsequently, we determined whether more than one-cell dose could provide for higher recipient engraftment efficiency with the same levels of myelosuppression and cells infused. Importantly, full engraftment for all recipients was obtained when cells were administered in three or four equal cell dose within the 28h for both irradiation levels (Table 5B). At higher level of myelosuppression, chimerism was higher than those receiving milder myelosuppression. Nevertheless, these recipient mice achieved therapeutic levels of donor chimerism (Table 5B).

As observed for chimerism of SAD mice with total body irradiation (Figure 1), chimerism obtained with mild myelosuppression showed similar amplification of normal RBCs in peripheral blood (Table 5A, 5B). In general, the levels of chimerism tend to increase with higher cell number and myelosuppression (2Gy vs 1Gy) whereas the engraftment efficiency appeared dependent on cell dose.

Table 5A-Engraftment and chimerism of myelosuppressed SAD mice with one cell dose

Irr. Level (Gy)	Cells (10^7)	Trans. Time (h)	n (total)	n (engr)	% Chimerism WBC	% Chimerism RBC
2	2	4	21	4	**37.0** 0±9.5	**84.2** ±15.1[d]
	4	4	12	5	**48.1** ±21.4**	**88.4** ±26.0[a]
	5	4	8	5	**76.6** ±11.1[A,B,C]	**97.3** ± 4.0[b]
	2	52	13	2	**38.3** ±14.6	**73.5** ± 9.2[e]
	5	52	8	3	**27.8** ±20.6	**50.1** ± 34.1
1	4	4	19	6	**21.1** ±15.5	**66.0** ±31.7[b]
	6	4	7	4	**21.7** ±14.0	**69.5** ±4.9[c]
	8	4	8	4	**52.6** ±14.4*#	**87.9** ±1.7[b]
	4	28	17	6	**32.0** ±20.6	**65.7** ±23.6[a]
	8	28	10	4	**59.1** ±32.2	**90.7** ±18.6

Abbreviations: WBC, white blood cells,; RBC, red blood cells; n (total), total number of mice transplanted; n (engr), numbr of transplanted mice that engrafted;
t-test: RBC vs WBC chimerism; a, p<0.05; b, p<0.02; c, p<0.01; d, p<0.005; e, p<0.0001;
Level of WBC chimerism, %WBC in one group vs %WBC in another group; *1Gy, 4h: 8 x 10^7 cells vs 4 x 10^7, p<0.02; #1Gy, 8 x 10^7 vs 6 x 10^7, p<0.05; **2Gy, 4h, 4 x 10^7 vs 1Gy, 4h, 4 10^7, p<0.05; A, 2Gy, 4h, 5 x 10^7 vs 2 x 10^7, p<0.001; B, 2Gy, 4h, 5 x 10^7 vs 4 x 10^7, p<0.05; C, 2Gy, 5 x 10^7, 4h vs 52h, p<0.05;

Table 5B-Engraftment and chimerism of myelosuppressed SAD mice with multiple cell doses

Irr. Level (Gy)	Cells (10^7)	Inject (#)	n (total)	n (engr)	% Chimerism WBC	% Chimerism RBC
2	4	2	7	6[b]	**73.5** ±20.0	**89.5** ±10.8[a]
		3	5	5	**50.7** ±26.0	**70.4** ±21.8
		4	6	6	**71.8** ±24.0	**93.2** ±10.0
1	6	2	7	1	**34.4**	**77.8**
		3	7	7	**28.7** ±20.6	**52.3** ±22.3
		4	7	7	**26.5** ±20.6	**43.2** ±38.5

Abbreviations: WBC, white blood cells,; RBC, red blood cells; n (total), total number of mice transplanted; n (engr), number of transplanted mice that engrafted.
t-test: RBC vs WBC chimerism; a, p<0.05; b, 2Gy, 2 x 2 x 10^7 vs 1Gy 2 x 3 x 10^7, p<0.05

To verify whether the levels of chimerism in these minimally myelosuppressed mice displayed similar correction as in fully myeloablated mice, we monitored their RBC half-life and parameters as well as hematopoiesis/erythropoiesis. Although these myelosuppressed mice exhibit variable levels of chimerism, all RBC half-life and parameters were ameliorated with 20-30% chimerism (Supplementary Table S2). In addition, the hematopoietic progenitor (CFU-S_{12}) and erythroid precursor cells of spleen and bone marrow were improved within the range monitored for the myeloablated chimeric mice (Supplementary Table S3) as well as the spleen size (data not shown), confirming the chimeric level therapeutic window.

Discussion

To implement successful and reproducible BM stem cells transplantation for SCD, major issues must be resolved. First the threshold level of normal or genetically corrected BM cells necessary for therapeutic benefit and second, the minimal requirements of pre-transplantation regimens, frequency and timing of infusions must be determined. Our experiments in the SAD mice defined the normal BM cells threshold at 26-31% that is fundamental and must be reached as a therapeutic index to achieve cure or improvement of the primary erythroid defect and secondary multisystemic disorder of SCD. In addition, we characterized the minimal functional requirements, of 40-60 millions cells in multiple equal subdoses with mild myelosuppression for total engraftment at the established threshold of chimerism.

Our study provides the first systematic quantification of the level of normal erythroid amplication relative to the normal non-erythroid/WBC in SAD mice. Our results on this important difference of erythroid cells provided proof for a marked selective advantage of normal over sickle erythroid cells. This favored amplification of the erythroid compartment resulted most likely from the cumulative effects of the dramatically reduced sickle RBC half-life and defective erythroid maturation or ineffective erythropoiesis. This selective advantage was evaluated at ~2- to 4-fold for 39% SAD chimeric cohorts and below. Interestingly, similar erythroid selective advantage was also detected for the rare inadvertently obtained mixed chimerism in human SCD (5). These findings of erythroid cells selective advantage corroborate also with another hemoglobinopathy β-thalassemia, in murine model as in human (25,27,28). For thalassemia however, the ineffective erythropoiesis was much more prevalent than in SCD (23). From the 49% chimeric SAD cohorts and above, the selective advantage waned linearly with increasing normal donor chimerism.

Our analysis determined that the 31% normal donor cells chimerism with corresponding 66% normal RBCs significantly improve critical SCD hematological indices. Together the increased RBC half-life and decrease in hyperchromic or dense

cells likely resulted in decrease of hemolysis and RBC sequestration. Because correction of hematologic parameters may not be indicative of organ damage as previously stated (18,19), studies were focused on hematopoietic/erythropoietic defects and on histo-pathologic analysis of the main organs affected in SCD. Consequent to SAD mice hematologic anomalies and to production of hemoglobin polymers in erythroid precursors, splenic and also medullary erythropoiesis are severely altered (16). Interestingly, in the 26-31% chimeric groups both spleen and bone marrow were markedly ameliorated from the defective basophilic erythroblasts and onwards. Noticeably, comparable erythropoietic response in human sickle cell disease has also been reported with similar proportional defects in erythroblast precursors (2). Further support to the 26-31% chimeric groups was also provided from the substantial improvement of general hematopoiesis status through normalization of the multipotent cells. The apparent discrepancy with the lower ~18% chimerism required to ameliorate another SCD model with thalassemia may be explained by the mixed phenotype (18). Indeed, a lower chimerism of 19-24% in murine thalassemia improved significantly hematologic and hematopoiesis/erythropoiesis (23) whereas in the restricted SCD murine SAD model, therapeutic requirement is shifted to higher range.

One of the most critical point to consider in evaluating the threshold necessary to cure SCD is correction of organ pathology and the physiologic impact on lifetime. Consistent with the erythropoiesis/hematopoiesis amelioration in the 26-31% chimeric groups, the splenic extramedullary hematopoiesis as determined by nucleated cells is virtually undetectable and the splenomegaly is significantly corrected. In fact, the minor RBC sequestration of the 26-31% chimeric groups with ~55-66% normal RBC inferred from the decrease splenomegaly and histopathology highlighted the spleen effective clearance potential. Accordingly, virtual normalization of iron deposits and fibrosis in the spleen of these 26-31% chimeric groups confer these chimeric ranges as beneficial. Since SCD is a multi-systemic disease, therapeutic threshold of pathology was investigated for other targeted organs, kidneys, liver, and lungs, due to potential different metabolic responses (29). While

kidneys of the 26% SAD mice chimeric group exhibit decrease fibrosis typical SCD and glomerular size, normalization was reached from the 39% SAD mice chimeric group. The pronounced improvements detected in both lung and liver was observed in the 26-31% SAD mice chimeric group. These data on the pathology were quite striking considering the cumulative damage over the entire ~2 years of the chimeric SAD mice lifespan for the different groups. Most importantly, support for improved pathology and consequent physiology was obtained by the significantly increased lifespan. Indeed, mice from the 26-31% SAD chimeric group survived normal lifespan, this result implied that total bone marrow chimerism is not required to impede the primary or secondary adverse effects of SCD.

In human sickle cell disease, the transfusion of hematological parameters may underestimate the required level of donor chimerism for long-term correction of the most affected organs, and therefore, the therapeutic outcome cannot be predicted due to limited experience and years of follow-up with a therapy of mixed chimerism. This point was specifically addressed herein by characterization of the minimal percentage of normal bone marrow cells needed for systemic correction of SAD mice. When considering several criteria including pathology and survival, a minimal 26-31% of donor chimerism was required for a large beneficial impact whereas 11-16% of normal donor chimerism was sufficient to improved many hematological indices. Although it is difficult to extrapolate to human sickle cell disease and within the limitation of species differences, together our study and the occasional mixed chimerism suggest that 25-30% normal bone marrow cells might constitute the minimal percent of normal BM cells necessary for partial but significant correction of sickle cell disease.

Our data established an accurate quantitative estimate of mixed chimerism for a highly significant correction of sickle cell disease, and supported the feasibility of limited/minimal myelosuppression approaches. Development of minimal myelosuppressive conditions in addition to being less toxic, will likely benefit both allogenic BMT and autologous BMT with gene therapy, particularly since the latter is

predicted to be far from 100% efficient. Our results prompted the characterization of the pretransplantation conditioning regimen and modalities of bone marrow cell transfer to achieve full engraftment and appropriate chimerism. Optimal conditions were investigated from cohorts of SAD mice receiving different irradiation doses, number of cells transferred, number of cell doses, and timing of cell transfer after partial myelosuppression. These experiments showed that minor myelosuppression at 1 or 2Gy was sufficient to attain stable levels of chimerism for correction of sickle cell disease over the entire lifespan. Interestingly, all mice engrafted after 1Gy or 2Gy with 60 and 40 million cells respectively administered in three doses within the first 28h after irradiation, and 85.7% of mice engrafted after 2Gy with 40 million cells administered in 2 equal doses at 4h and 24h after irradiation. Importantly, cell infusion in two and three relative to one dose was more effective for engraftment, suggesting both the potential clinical benefit of multiple injections of donor cells, and the requirement for a mild (2Gy) rather than a minimal (1Gy) dose of irradiation. Correction of the sickle phenotype of these minimally myelosuppressed SAD chimeric mice was verified by erythrocyte parameters and analysis of hematopoiesis/erythropoiesis and confirmed by similar improvement as predicted from the fully myeloablated SAD chimeric mice groups. These practical strategies of minimal myelosuppression with a reasonable number of cells were highly successful, and similar adapted approaches may be relevant to human clinical trials.

Overall, the results of this study are similar to our previous findings with β-thalassemia (23). Both the therapeutic threshold and the parameters correction are very similar. This is surprising because the two diseases have different basis, but also encouraging because it implies that many hemoglobin or erythrocyte diseases would potentially be treatable with partial replacement of the recipient BM.

A previous report has suggested that 100% normal RBC chimerism should be sought to achieve full correction of all parameters, in particular the hematological organs parameters (18). Our study confirms this, but also demonstrates that full correction of all parameters is not required for therapeutic benefit, on the contrary, full correction of some parameters and partial but significant amelioration of many other parameters,

confer lifelong correction of murine SCD, with almost 50-60% normal RBC chimerism (26-31% normal WBC).

In summary, our study determined a strong selective of ~2- to 4-fold enrichment of the erythroid cells. We also established the therapeutic window of 26-31% normal donor bone marrow cells for significant long-term improvement of the general systemic phenotype in sickle cell disease. Development of alternative therapeutic strategies for stable successful levels of chimerism and complete engraftment with minor myelosuppression provided the basis for setting human pre-clinical trials in both allogenic BMT and autologous BMT with gene therapy. Also the success of low myelosuppression in allowing full engraftment raises the possibility of investigating strategies with no myeloablation to attain the therapeutic window.

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Table S1. Quantification of erythroid precursors in SAD chimeric mice

Chimeric Mice (% normal WBC)	n	Proerythroblasts (% of total cells)		Basophilic Erythroblasts (% of total cells)		Late basophilic / Polychromatophilic erythroblasts (% of total cells)		Orthochromatophilic erythroblasts (% of total cells)	
		Bone marrow	Spleen	Bone marrow	Spleen	Bone marrow	Spleen	Bone marrow	Spleen
0	3	6.5 ±1.7	1.7 ±1.0	39.0 ±6.1	15.4 ±6.9	5.7 ±2.2	14.8 ±3.5	6.3 ±1.2	43.0 ±12.1
26	3	5.1 ±1.4	1.4 ±0.5	31.8 ±6.3	8.9 ±9.6[c]	6.4 ±1.0	9.5 ±4.9[a]	10.8 ±0.3[b]	41.5 ±5.8
31	3	4.4 ±0.4	0.8 ±0.8	33.9 ±4.1	6.8 ±3.1[b]	6.8 ±3.1	11.8 ±5.4	12.7 ±4.7	52.8 ±11.8
39	3	5.6 ±0.2	1.6 ±0.5	30.6 ±4.5	7.8 ±8.7	4.7 ±0.4	6.9 ±3.2[b]	17.1 ±3.7[a]	40.5 ±14.3
100	3	4.7 ±1.0	1.7 ±0.2	29.2 ±7.4	7.1 ±2.0[c]	4.3 ±3.1	2.6 ±0.9[c]	16.7 ±4.4[a]	43.8 ±1.3

Abbreviations: WBC, white blood cells; *t*-test: sample vs chimeric 0%; a, $p \leq 0.05$; b, $p < 0.02$; c, $p < 0.005$

Table S2. Hematological parameters of myelosuppressed SAD chimeric mice

Chimerism (%)	n	CHCM (g/dl)	MCH (pg)	MCV (fl)	Hyper (HC>35g/dl)	n	RBC half-life (days)
0	**13**	**31.5** ±2.1	**14.4** ±0.5	**44.5** ±2.0	**6.1** ±3.5	**3**	**5.8** ±1.2
10	**5**	**29.8** ±0.7[b]	**15.3** ±0.5	**48.8** ±1.3[f]	**3.4** ±1.5	**2**	**13.6** ±1.7[a]
20	***5***	***29.9*** ±0.7[a]	***14.8*** ±0.4	***47.9*** ±2.3[a]	***0.6*** ±0.5[g]	**3**	***13.8*** ±5.8
30	**8**	**30.1** ±0.4[a]	**14.7** ±0.8	**47.4** ±1.7[e]	**0.4** ±0.2[g]	**3**	**12.9** ±1.3[e]
50	**13**	**30.3** ±0.7[a]	**14.2** ±0.6	**47.3** ±1.2[d]	**1.08** ±0.4[h]	**3**	**16.2** ±5.9
Control	**16**	**27.6** ±1.1[g]	**14.6** ±0.5	**50.8** ±2.8[h]	**0.6** ±0.8[g]	**3**	**15.2** ±3.5[a]

t-test, sample vs chimeric 0%; a, $p<0.05$; b, $p<0.02$; c, $p<0.01$; d, $p<0.005$; e, $p<0.002$; f, $p<0.0002$; g, $p<10^{-5}$; h, $p<10^{-6}$

Table S3. Analysis of hematopoiesis/erythropoiesis in myelosuppressed SAD chimeric mice

Chimeric Mice (% normal WBCs)	n	Spleen wt/ Body wt (%)	n	CFU-S_{12} ($\times 10^2$) Spleen	n	Basophilic Erythroblasts (% of total cells) Bone marrow	Basophilic Erythroblasts (% of total cells) Spleen	Late Basophilic/ Polychromatophilic Erythroblasts (% of total cells) Bone marrow	Late Basophilic/ Polychromatophilic Erythroblasts (% of total cells) Spleen	Orthochromatophilic Erythroblasts (% of total cells) Bone marrow	Orthochromatophilic Erythroblasts (% of total cells) Spleen
0	59	**0.56** ±0.31	5	**20.8** ±22.1	5	**22.9** ±4.6	**8.9** ±4.9	**3.0** ±1.0	**13.1** ±6.1	**5.7** ±1.8	**53.8** ±7.0
10	5	**0.35** ±0.06[g]	3	**6.0** ±3.5	3	**23.8** ±4.9	**6.6** ±2.6	**4.2** ±0.8	**16.6** ±9.5	**9.8** ±2.9[a]	**47.8** ±11.0
30	5	**0.35** ±0.17[a]	4	**6.6** ±5.8	4	**26.5** ±5.4	**5.2** ±2.4	**5.4** ±1.5[b]	**10.0** ±2.4	**13.5** ±9.0	**52.0** ±6.6
50	4	**0.33** ±0.08[d]	4	**5.6** ±2.6	4	**25.7** ±6.2	**5.5** ±2.6	**6.9** ±2.5[c]	**9.5** ±5.8	**13.6** ±3.0[f]	**44.3** ±3.5[a]
Control	4	**0.42** ±0.23	4	**5.3** ±3.3	8	**20.7** ±5.6	**4.8** ±3.8	**4.6** ±1.7	**3.8** ±1.0	**18.1** ±4.3	**53.6** ±7.6

Abbreviations: WBCs, white blood cells; wt, weight; CFU-S_{12}, colony forming unit at day 12;
t-test: sample vs chimeric 0%; a, $p \leq 0.05$; b, $p \leq 0.02$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.002$; f, $p < 0.001$; g, $p < 0.0005$

CHAPTER V

ARTICLE 3

FOREWORD

The results of the previous sections showed that partial myelosuppression was sufficient to allow therapeutic chimerism to be reached and even exceeded after the transfer of relatively mild amount of cells to recipient mice. These results were encouraging to perform BMTs on SAD mice without any myelosuppression, to determine whether a therapeutic level of chimerism could be obtained with no prior conditioning, and with the transfer of a reasonable number of donor cells.

This article describes the efficiency of intravenous BMT, as well as the efficiency of BMT done by intra-bone marrow (IBM) cell transfer into SAD mice, without prior conditioning. It demonstrates that high and reproducible engraftment efficiency could be achieved since all mice engrafted under particular transplant conditions. On the other hand, this study also suggests that achieving therapeutic levels of donor chimerism without myelosuppression would require substantial amounts of donor cells.

Contribution to the article

All the Tables (Tables 1, 2, 3, 4) preparation and results

The final writing of this article will be done in collaboration with my research supervisor

HIGH ENGRAFTMENT EFFICIENCY OF NORMAL DONOR CELLS IN NON-MYELOSUPPRESSED SAD RECIPIENTS

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Running Title: Efficient engraftment in non-myelosuppressed recipients

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Abstract

Sickle cell disease (SCD) is a monogenic blood disorder caused by abnormal hemoglobin and characterized by anemia and tissue damage. Using the SAD transgenic mouse model of SCD, we have previously demonstrated that the therapeutic donor chimerism of ~25-30% normal bone marrow cells could be reproducibly attained with only minimal myelosuppression under particular transplantation conditions. In order to further minimize the intensity of conditioning regimens, we investigated the engraftment efficiency and the levels of donor chimerism that could be achieved in non-myelosuppressed SAD mice by bone marrow transplantations (BMT). In one transplantation strategy, after intravenous (IV) cell transfer, all mice engrafted long term after receiving a single dose of 120 million total bone marrow (BM) cells. These mice presented a level of 11% normal donor white blood cell chimerism in peripheral blood and showed amelioration of erythrocyte parameters. Using a different transplantation strategy, intra-bone marrow (IBM) injection of BM cells from 5-FU-treated donors allowed long-term engraftment of all mice in most animal groups and levels of normal WBC chimerism averaged between 1.5% and 5.4%. Together, these results demonstrated high efficiency of engraftment without myelosupression, after both IV and IBM BMT. This study also suggests that a certain level of myelosuppression would still be required to achieve a therapeutic level of donor chimerism with a reasonable number of donor cells.

Introduction

Sickle cell disease (SCD) is one of the most common single gene disorders and widespread throughout the Mediterranean region, Africa, Middle East, the Indian Subcontinent, Bruna and Southeast Asia (**1**). SCD is characterized by the presence of abnormal hemoglobin resulting in elongated RBCs that block microvasculature resulting in ischemia and multi-organ disease (**2, 3, 4**).

Bone marrow transplantation (BMT) represents a curative approach for SCD. Several studies showing high rates of engraftment in non-myeloablated mice have challenged the concept that complete myeloablation is a pre-requisite to open spaces for bone marrow stem cell engraftment (**5, 6, 7, 8, 9**).These studies imply that high levels of donor chimerism might be possible with bone marrow transplantations (BMTs) without prior myeloablation/myelosuppression. Furthermore, studies of the homing process have shown that while homing efficiency to the bone marrow after intra-venous injection of the cells is ~17% in non-irradiated mice (**10**), it is no more than 10% in irradiated recipients (**10, 11, 12, 13, 14, 15**). The cells not reaching marrow niches are probably sequestrated by other organs with large capillary beds like the liver and lungs and are lost (**16**). To overcome this problem, another strategy of transplantation has been developed: the intra-bone marrow (IBM) injection of hematopoietic stem cells. This technique has been successfully used in rats (**17**), mice (**18**), and monkeys (**19**). Intra-bone injections were also cited in humans (**20, 21, 22**).

Using the SAD mouse which reproduces the features of the human disease (**23, 24**), we have previously corrected the sickle cell phenotype with only minimal myelosuppression (**25**). We now aim to explore fully non-myeloablative BMT strategies for potential therapeutic benefit for SCD. We herein report the analysis of different transplantation strategies involving the injection of various numbers of cells intravenously (IV) and intra-bone marrow (IBM). Our results show that 100% engraftment could be achieved, both after IV and IBM BMT. Our study also implies that with the currently available transplant strategies, and in order to achieve levels of

donor chimerism of therapeutic value with reasonable amounts of cells, some (mild) myelosuppression of recipients would still be required.

Materials and Methods

Mouse strains

The sickle cell mouse model (SAD-1 mouse) was generated by co-injecting into fertilized eggs the β^{SAD} and the human α-globin genes, each fused to the locus control region (LCR) (**23**). These mice were bred onto C57BL/6J-*Gpi1*^{b}/*Gpi1*^{b} background for >21 generations, therefore considered syngeneic. SAD-1 mice were genotyped as previously described (**23**). Congenic C57BL/6-*Gpi1*^{a}/*Gpi1*^{a} (Glucose phosphate isomerase isotype 1a) mice carry a hematopoietic cell marker and were obtained from Dr J. E. Barker (the Jackson labs, Bar Harbor, ME, USA). All mice were maintained in a specific pathogen free environment and experimental procedures were conducted in compliance with the guidelines of the Canadian Council on Animal Care (CCAC)

Production of chimeric mice

Nucleated bone marrow (BM) cells were harvested from donor animals C57BL/6-*Gpi1*^{a}/*Gpi1*^{a} using IMDM (Iscove's Modified Dulbelco Medium, GIBCO, Grand Island, NY, USA). Intravenously injected recipient SAD mice (34 males and 35 females) (**24**) received 40, 80 or 120 million total nucleated bone marrow cells in 1, 2, or 3 doses at 48h interval, by lateral tail vein injection(s). Intra-bone marrow (IBM) injections were performed as previously described (**26**). IBM-injected SAD mice (34 males, 33 females) received 1, 2, 3, 4, 6, 8 or 10 million bone marrow cells from normal donors treated with 5-FU (0.15mg/g weight) 6 days prior to cell harvest. Cells were injected in the femoral cavity of both femurs, in either one or three doses at 48h interval. In the latter group, some recipients were bled ~500µl 4 hours to the first cell transfer.

Analysis of chimerism

Chimeric mice were assessed for hematopoietic engraftment by *Gpi1* in both white and red blood cells on a regular basis from the second month to the 18th month following BMT, using Helena equipment as previously described (**27**). The

membranes were then scanned and bands quantified using ImageQuant software as previously described (**27**).

Hematological analysis

Complete blood counts of chimeric mice were performed on 250µl blood using a Bayer Advia 120 cell analyzer with the mouse archetype of multispecies software version 2.2.06 (CTBR, Montreal, Canada). Gating of the cellular hemoglobin concentration mean (>35g/dl) and the mean cellular volume (<25fl) was used to determine the percentage of hyperchromic RBCs and the percentage of microcytic cells, respectively as previously described (**27**). Reticulocyte counts were obtained by specific RNA staining with the oxazine 750 dye using the reticulocyte channel on the analyzer.

Statistical analysis

The statistical significance was determined using the student T-test available in Microsoft excel software

Results:

To determine levels of normal donor cell chimerism that could be achieved in SAD recipient mice without pre-transplantation conditioning, SAD mice previously backcrossed to a homogeneous C57BL/6J genetic background (C57BL/6J-*Gpi1*^{b}) (**24**) were used as recipients for normal donor cell population (C57BL/6J-*Gpi1*^{a}). Bone marrow graft and recipient cell population were distinguishable by a specific Glucose Phosphate Isomerase marker isotypes. The quantification of each isotype of the Gpi1 marker was used to determine the relative proportions of normal and SAD cell populations in peripheral blood of chimeric mice. Stem cell engraftment based on the presence of donor white blood cells (WBCs) and chimerism levels of both donor WBCs and red blood cells (RBCs) were analyzed in recipient mice (n=136) at regular intervals from two months after transplantation onwards.

High engraftment efficiency after intravenous (IV) cell transfer

Six groups of SAD chimeras ($n \geq 8$) were generated and chimerism was maintained 7 months following bone marrow transplantation (BMT) (**Table 1**). The number of engrafted mice increased with the amount of donor cells infused and reached 100% with one dose of 120 million cells. The level of WBC chimerism increased proportionally with the number of donor cells infused whether cells were injected in 1, 2 or 3 doses. The proportion of normal RBCs in the peripheral blood was amplified relative to normal WBCs in each group due to the loss of SAD RBCs (**Table 1**). Long term engraftment was maintained up to 18 months after transplantation. The highest level of RBC chimerism was ~30%, which, according to our previous studies (**25**) provides a mild therapeutic benefit. Since homing efficiency is low after intravenous injection of donor cells (**10**), we decided to explore the intra-bone marrow (IBM) injection of donor cells would allow higher levels of donor chimerism.

Table 1- Engraftment and chimerism of intravenously transplanted SAD chimeric mice

Cells ($\times 10^7$)	Inj.	n (total)	n (engr)	% Chimerism WBC		% Chimerism RBC	
4	1	15	8	**2.1**	±1.4	**11.0**	±12.4
8	1	12	11	**9.3**	±8.2	**19.7**	±17.1
12	1	8	8	**11.2**	±13.7	**29.6**	±21.6
8	2	13	7	**5.9**	±3.5	**15.9**	±11.1[a]
12	2	10	7	**8.6**	±3.9	**28.0**	±14.2[a]
12	3	11	9	**14.6**	±8.3	**29.8**	±21.6

Abbreviations: n (total), total number of mice transplanted; n (engr), number of transplanted mice that engrafted; WBC, white blood cells; RBC, red blood cells; *t*-test, normal RBCs vs normal WBCs; a, $p<0.01$

Full engraftment and low donor chimerism after single intra-bone injection

We used the IBM strategy to generate 7 groups of mice ($n\geq5$), without any pre-conditioning of recipients, and chimerism was analyzed as before. In all but one group, 100% of recipient mice engrafted (**Table 2**). The examination of the level of donor chimerism in peripheral blood of chimeras showed that the percentage of normal WBCs averaged 1.5% to 5.4%, while RBC chimerism averaged 5.1% to 11.1%, thus demonstrating an amplification of the normal erythroid cells as we previously observed (**25**). The transfer of donor cells in multiple doses provided no change to the level of chimerism (**Table 3**).

Table 2-Engraftment and chimerism of SAD chimeric mice intra-bone marrow transplanted in single cell doses

Cells ($\times 10^6$)	n (total)	n (engr)	% Chimerism WBC		% Chimerism RBC	
1	10	8	**1.5**	±1.2	**5.1**	±5.0[a]
2	10	10	**1.8**	±1.0	**5.6**	±2.8[d]
3	4	4	**2.2**	±1.1	**9.7**	±5.2
4	5	5	**2.2**	±1.3	**8.1**	±3.0[b]
6	5	5	**3.1**	±2.0	**7.9**	±4.9
8	5	5	**2.7**	±1.4	**11.1**	±4.1[c,f]
10	7	7	**5.4**	±3.8	**9.1**	±4.1[e]

Abbreviations: n (total), total number of mice transplanted; n (engr), number of transplanted mice that engrafted; WBC, white blood cells; RBC, red blood cells; t-test, normal WBCs vs normal RBCs; a, $p<0.05$; b, $p<0.009$; c, $p<0.008$; d, $p<0.002$; WBCs, 2×10^6 vs 10×10^6, e, $p<0.04$; RBCs, 1×10^6 vs 8×10^6, f, $p<0.04$

Table 3-Engraftment and chimerism of SAD chimeric mice intra-bone marrow transplanted in three cell dose

Cells ($\times 10^6$)	n (total)	n (engr)	% Chimerism WBC		% Chimerism RBC	
1	7	6	**2.5**	±1.7	**10.3**	±7.7
2	7	6	**2.1**	±1.8	**8.8**	±8.0[a]

Abbreviations: n (total), total number of mice transplanted; n (engr), number of transplanted mice that engrafted; WBC, white blood cells; RBC, red blood cells; *t*-test, RBCs vs WBCs; a, $p<0.05$

No effect on engraftment and donor cell chimerism after bleeding pre-conditioning

In an attempt to stimulate hematopoietic regeneration, we bled mice 500µl 4 hours prior to first cell transfer, then carried out 3 IBM injections at 48 hour-intervals, of a total of 2 (n=7) and 3 (n=7) millions cells. Normal cell chimerism and the percentage of engraftment remained unchanged relatively to the untreated recipients (data not shown). Thus, any stress induced by bleeding before transplantation did not show any significant impact on the outcome.

Improved hematological parameters with 10-15% normal WBC chimerism

To determine whether levels of chimerism between 10 and 15% confer a phenotypic benefit as we previously demonstrated with competitive repopulation assays (**25**), erythrocyte parameters were analyzed. Improvement of MCV and CHCM were observed in all chimeric mice with 9% or more normal WBCs (**Table 4**).

Table 4-Hematological parameters of intravenously transplanted SAD chimeric mice

Cells ($x10^7$)	Inj	Chimerism % (WBCs)	n	Hematological Parameters								
				RBC ($x10^6/\mu l$)		Hb (g/dl)		MCV (fl)		CHCM (g/dl)		% Hyper (HC>35g/dl)	
		0	7	**10.7**	±0.9	**14.9**	±0.8	**44.0**	±1.0	**29.9**	±1.0	**0.9**	±0.6
4	1	2.1	3	**10.9**	±0.2	**15.0**	±0.8	**42.7**	±1.5	**31.0**	±0.5	**1.5**	±1.3
8	1	9.3	5	**10.8**	±0.4	**14.9**	±0.8	**43.5**	±1.1	**30.7**	±0.4	**1.0**	±0.2
12	1	11.2	3	**10.0**	±1.5	**14.6**	±1.5	**48.0**	±5.3	**29.8**	±1.5	**0.8**	±0.3
8	2	5.9	6	**10.4**	±1.1	**15.4**	±0.6	**46.9**	±4.1	**30.4**	±0.3	**0.6**	±0.2
12	2	8.6	3	**9.9**	±0.1[c]	**14.6**	±0.3[a]	**46.9**	±0.5[b]	**30.4**	±0.1	**0.6**	±0.2
12	3	14.6	5	**10.7**	±0.5	**15.0**	±0.8	**45.7**	±1.2	**29.8**	±0.7	**0.6**	±0.1
Wild-type control			5	**10.9**	±0.7	**15.6**	±1.1	**49.6**	±1.9[d]	**27.9**	±0.6[e]	**0.05**	±0.05

Abbreviations: WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; MCV, mean cell volume; CHCM, cell hemoglobin concentration mean; % hyper, percentage of hyperchromic cells; *t*-test: sample v/s chimeric 0%; a, $p<0.02$; b, $p<0.005$; c, $p<0.002$; d, $p<0.00005$; e, $p<0.000001$

Discussion

To render clinical bone marrow transplantation for sickle cell disease possible without any myelosuppression, reproducible and efficient non-myeloablative bone marrow transplantation (BMT) strategies have to be set. In this report, we have demonstrated the possibility to attain engraftment in 100% of recipients after either intravenous (IV) or intra-bone marrow (IBM) injection of BM cells. Moreover, we investigated several transplantation strategies, based on the number of cells injected, number of injections, route of cell transfer and pre-transplant bleeding of mice.

BMT by intravenous injection of donor cells showed a significant increase in the percentage of engrafted mice, as well as important increase of donor chimerism levels with increasing cell number. However, beyond 80 million cells, this increase was very mild and engraftment efficiency was almost unchanged, implying a possible plateau, or saturation of recipient bone marrow. Transfering the cells in two or more doses showed comparable results. The highest level of normal RBC chimerism averaged 29% after 120 million total BM cells. This level it allowed noticeable improvement of erythrocyte parameters on complete blood count (**Table 4**) as we previously observed with a similar percentage of normal cells (**25**). Attaining the therapeutic threshold with ~50-60% normal RBCs which requires 25-30% normal WBCs seems to be more demanding in amount of donor cells and might not be rapidly attained if bone marrow saturation level could not be expanded. Therefore, we decided to explore alternative strategies. Since homing/seeding efficiency is low after IV injection (**10**), this might be a possible cause for the low chimerism in peripheral blood.

We therefore investigated the IBM BMT, hoping it would confer a repopulation advantage since stem cells are directly introduced in bone marrow, especially that recent reports have shown successful engraftment after IBM injection of donor cells following partial myelosuppression of recipients. IBM transplantation require the use of different strategy than IV BMT. We performed IBM transplants with different

amounts of cells after enrichment by treating donors with 5-FU, including 1, 2, 3, 4, 6, 8, 10 millions (**Table 2**) by intra-femoral injection of cells. Although levels of WBC chimerism doubled with 10 million cells relative to 3-8 million cells, the requirement for such a large number of cells to increase the level of chimerism suggests that most injected cells are not having high selective seeding advantage. This might be due either to the low availability of spaces within the bone marrow of non-ablated recipients, or to the high number of stem cells in the donor graft that might have saturated the limited capacity of one femur. Performing multiple IBM injections led to similar results. It was previously shown (**31**) that following IBM injection, some donor cells will leave the injected femur and home to other bones. Our results suggest that this process is not sufficient to allow therapeutic levels of chimerism with reasonable amounts of cells transferred.

We finally sought to determine whether inducing a non-ablative hematopoietic stress in the recipient BM would have an effect on engraftment and chimerism. We therefore bled mice prior to transplantation. There was no difference with the previous strategies, suggesting that such stress did not provide an advantage for donor graft cells.

While both cell tramsfer routes have their special effect, comparing the two of them would not be possible given the difference in the cell number and preparation used in each strategy. Performing IV and IBM BMTs with equal numbers of purified HSCs would allow comparing the efficiency of the two strategies in the SAD model. A combined IV/IBM BMT would also further answer the question regarding the levels of chimerism in non-ablated recipients. This study also suggests that, until further optimization allows otherwise, some levels of mild myelosuppression will still be required to achieve therapeutic levels of donor cell chimerism with reasonable amounts of donor cells

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CHAPTER VI

ARTICLE 4

FOREWORD

As mentioned in the introduction, most gene therapy trials in mouse models for globin diseases have used large amounts of viral vectors ("multiplicity of infections, MOI") and often obtained multiple integrations per cell. These studies, although leading to variable degrees of phenotype correction, pose some difficulties for clinical applications, especially due to the requirements for very large amounts of viral vector that would result in only very few patients that could be treated. At the same time, applications in globin gene therapy require sustained and efficient expression, which in turn require efficient regulation. A novel insulated globin lentiviral vector with adult globin targeting regulatory elements, demonstrated in vitro efficiency, and its globin construct demonstrated efficiency and specificity in transgenic mice. Therefore, it constituted a good candidate to be tested for hematopoietic stem cell (HSC) transduction, and subsequent *in vivo* efficiency after transplantation of transduced cells, into lethally irradiated recipient mice.

This article described an *in vivo* evaluation of a novel vector, in comparative analysis with a control (EGFP) vector on the same vector backbone, both for transduction of HSC at low MOI and follow up in mice after transplantation of transduced cells

Contribution to the article
Figures 2, 3, 4, 5 preparation and results
Figure 6, preparation
Tables 1, 2 preparation and results

The final writing of this article will be done in collaboration with my research supervisor

IN VIVO EVALUATION OF NOVEL LENTIVIRAL VECTORS IN SICKLE CELL MOUSE MODEL

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Abstract

Gene therapy is a promising approach for hematopoietic disorders like sickle cell disease. Several trials using lentiviral vectors carrying globin genes resulted in variable degree of success, after the use of often large amounts of vector infectious units, therefore limiting their clinical applications, in part due to the large industrial requirements of vector production. We thus evaluated novel SIN lentiviral vectors for transduction efficiency and expression levels with minimal vector requirements. Hence, using vectors carrying either human β/γ hybrid globin gene with globin-specific regulatory elements (globin vector) or an EGFP gene (control vector), titers of up to 3 x 10^9 IU/ml were obtained. Heterogeneous expression profile with populations of high expression intensities were observed with MEL cells. In hematopoietic cells, long term follow-up in mice showed sustained expression for both vectors. Furthermore, the percentage of γ-globin protein per cell was within the therapeutic range demonstrating the efficiency of the globin vector. Overall, this study suggests that the novel γ-globin lentiviral vector is able to transduce hematopoietic cells, and seems promising for future testing in SCD gene therapy trials. Exploitation of various transduction conditions would help ameliorate transduction efficiencies with moderate requirements of vectors.

Introduction

Hemoglobinopathies constitute a major group of blood disorders affecting millions of people around the world. Sickle cell disease (SCD) is a blood disorder due the production of a mutated β-globin (**1, 2**) leading to the formation of an abnormal hemoglobin S (HbS; $\alpha_2\beta_2^S$). HbS polymerizes upon deoxygenation (**3, 4**) , distorting the red blood cells shape, leading to anemia, vaso-occlusions and multi-organ disease (**5-10**).

Current therapeutic measures focus on induction of fetal hemoglobin to inhibit polymer formation, treatment and prevention of infections, palliative measures to control pain, and surgical treatments of complications (**11**). Allogenic bone marrow transplantation is the only available cure, but this procedure is limited to a minority of patients with an available, histocompatible donor (**12**).

Attempts to correct globin diseases including β-thalassemia and sickle cell disease (SCD) by gene therapy using various retroviral and lentiviral vector constructs resulted in variable degrees of phenotype correction often after the use of very high MOI (up to MOI 140) (**15-19; 21-26**), and was accompanied by multiple integrations. These studies, although showed that correction of these hemoglobinopathies is possible by gene therapy, would, however, be difficult to extrapolate to human clinics given the impracticability posed by the requirements for very high amounts of vector, and the potential resulting high risk of oncogenesis. Therefore, investigating gene therapy strategies that would ultimately result in high transduction efficiency with relatively low MOIs and with permanent expression at levels high enough for phenotype correction, would be of clinical value. In addition, the therapy of SCD is largely based on preventing HbS polymerization. Therefore, γ-globin expression would be preferred due to the anti-sickling potential of the resulting HbF.

Autologous transplantation of bone marrow stem cells that are transduced with a stably expressed, antisickling globin gene would benefit a majority of patients with

SCD. One difficult aspect of gene therapy is in safely and reproducibly obtaining high level, tissue specific and long term expression from of the transgene, which require adequate globin-specific regulatory elements **(13, 14)**. Therefore, we aimed to use a novel vector harboring various regulatorty elements that could provide expression advantage relative to previously tested vectors.

We herein report the testing of transduction efficiency of lentiviral vectors with the VSV-G surface glycoprotein at various but moderate MOIs using novel lentiviral vectors. The γ-globin expressing vector (BGT158) **(27, Buzina *et al., submitted*)** includes the human γ-globin gene along with regulatory elements and insulator. We also characterized the transduction efficiency and expression of a doubly insulated lentiviral vector carrying the EGFP **(27, Buzina *et al., submitted*)** for comparative analysis and evaluation of transductions at various MOIs, and the long term steady expression with both vectors. The vectors were able to infect hematopoietic cells at low MOI and allow sustained expression of the transgenes. The γ-globin levels per RBC were within the therapeutic range. This study demonstrated the efficiency of BGT158 lentiviral globin vector for applications in SCD gene therapy. Future modulation of transduction conditions would allow therapeutic levels of expressing cells with moderate MOI.

Materials and Methods

Vector production

The γ-globin expressing plasmid (BGT158) and the EGFP-expressing plasmid were constructed in the lab of Dr James Ellis (Hospital for Sick Children, Toronto, Canada) (**Buzina *et al., submitted***) and kindly provided, along with the packaging plasmids and 293T cell line. Production of each lentiviral vectors consisted on transiently co-transfecting producing 293T cell line with 5 plasmids (gag/pol, tat, rev, VSV-G, and either BGT158 or EGFP) in 10ml Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 20mM HEPES (Gibco). After O/N incubation at 37°C and 5%CO_2, the medium was replaced with 25ml fresh DMEM with 10% serum, and incubated for 24h, after which the cell-free supernatant was centrifuged at 25000rpm using SORVALL SURESPIN 630 rotor in Beckmann L8-55M ultracentrifuge. Pellets were resuspended in 50µl serum-free X-VIVO-15. In few preparations, pellets were resuspended, pooled, and centrifuged a second time, then resuspended in up to 100µl.

MEL cell infection and vector titration

Various volumes of concentrated viral suspensions were used to infect 2 x 10^5 MEL cells (kindly provided by Dr James Ellis, Hospital for sick children, Toronto, Canada). After 48h, EGFP infected cells were analyzed by flow cytometry to determine the percentage of EGFP-expressing cells. BGT158 infected cells were induced after 3 day-infection to differentiate using HMBA (N-hexamethylene Bis Acetamide, Aldrich), at a final concentration of 5mM. Six days later, 5 million induced cells were processed for γ-globin staining. Briefly, cells were washed with HBSS, fixed with 4% formaldehyde for 30 minutes at RT, followed by washing with HBSS. Then, three 3-min incubations were done in acetone (50%, 100%, 50%), then washed with 2% FBS-HBSS, and incubated with FiTC-coupled anti- γ-globin antibody for 30 minutes on ice, in the dark, washed twice with HBSS-serum and analyzed with a flow cytometer FACSCalibur (BD Bioscience, Ontario) using CellQuest Pro version 4.0.2 software.

Mouse strains

The sickle cell mouse model (SAD-1 mouse) was generated by co-injecting into fertilized eggs the β^{SAD} and the human α-globin genes, each fused to the locus control region (LCR) (**28**). These mice were bred onto C57BL/6J-*Gpi1*^{b}/*Gpi1*^{b} background for >21 generations, therefore considered syngeneic. SAD-1 mice were genotyped as previously described (**28**). Congenic C57BL/6-*Gpi1*^{b}/*Gpi1*^{b} (Glucose phosphate isomerase isotype 1b) mice carry a hematopoietic cell marker and were obtained from Dr J. E. Barker (the Jackson labs, Bar Harbor, ME, USA). All mice were maintained in a specific pathogen free environment and experimental procedures were conducted in compliance with the guidelines of the Canadian Council on Animal Care (CCAC)

Bone marrow cell harvesting, infection protocol, and transplantations

Briefly, donor mice (2-5 months) were intravenously injected with 5-fluorouracil at 150μg per gram of mouse weight. Nucleated bone marrow (BM) cells were harvested from donor animals 6 days later using IMDM (Iscove's Modified Dulbecco's Medium, GIBCO, Grand Island, NY, USA) supplemented with 5% serum. Red blood cells (RBCs) were lyzed and nucleated cells resuspended in serum-free X-VIVO-15 supplemented with 10 ng/ml IL-1α, 100 IU/ml IL-3, 150 IU/ml IL-6, 10 ng/ml SCF obtained from Genzyme (Cambridge, MA), 0.5mM β-mercaptoethanol (Sigma, St Louis, MO), 200 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Transductions of 1.5 x 10^5 nucleated bone marrow cells were carried out in 1ml final volume X-VIVO-15 with same supplements as above, and 8μg/ml polybrene (sigma), with MOIs ranging from 0.5 to 20 for 12 hours. Recipient mice (2-5 months old SAD or C57BL/6J) were irradiated 9.25Gy (JL Shephered & Associates, Mark I-68A-1 Research irradiator, San Francisco, CA, USA) 4h or 24h before transplantation and transduced cells were transferred to recipients by either intra-venous injection in lateral tail vein, or intra-bone marrow (IBM) injection, respectively.

Analysis of CFU-S$_{12}$

12 days after transplantation of EGFP-transduced HSC, recipient mice were sacrificed and spleens dissected out. The number of colonies (CFU-S_{12}) was counted, and individual colonies were dissected delicately, converted into single cell suspension in 1X PBS through nylon mesh, washed twice with PBS and processed for FACS. For evaluation of EGFP+ erythroid cells per colony, EGFP+ clones were further processed by staining with Phyco-Erythrin-conjugated anti-Ter119 antibody in the standard procedures for 30 minutes on ice in the dark, washed twice then processed for FACS. FACS analysis was done with a flow cytometer FACSCalibur (BD Bioscience, Ontario) using CellQuest Pro version 4.0.2 software.

Analysis of percentage of expressing red blood cells

At various times after transplantation, recipient mice were regularly screened for the presence of EGFP or γ-globin positive cells in peripheral blood. For EGFP, 1-2μl whole blood were withdrawn on heparin, washed twice with 1X PBS, diluted in 1-2 ml 1X PBS and processed for flow cytometry. For γ-globin, 8-10μl whole blood was processed for staining with FiTC-conjugated anti- γ-globin antibody (Cortex Biochem) and flow cytometry as described above. FACS analysis was performed with flow cytometer FACSCalibur (BD Bioscience, Ontario) using CellQuest Pro version 4.0.2 software.

γ -globin protein quantification

Globin chains were separated by urea-triton PAGE as previously described (41) using total blood lysates of transplanted mice, transgenic mouse line expressing the human Aγ globin chain as positive control (29), and negative control (mock) (30μg each). Globin chains were quantified by densitometry using Alpha Imager 2000 software.

Results

Characterization of infection efficiency and intensity of expression in MEL cells

To evaluate infectivity of the vectors, MEL cells were infected with increasing volumes of concentrated vectors (**Table 1, Figure 1**). Both vectors showed high efficiency and strong signal as detected by flow cytometry (**Figure 2**), as previously described (**Buzina *et al.*, *Submitted*).**

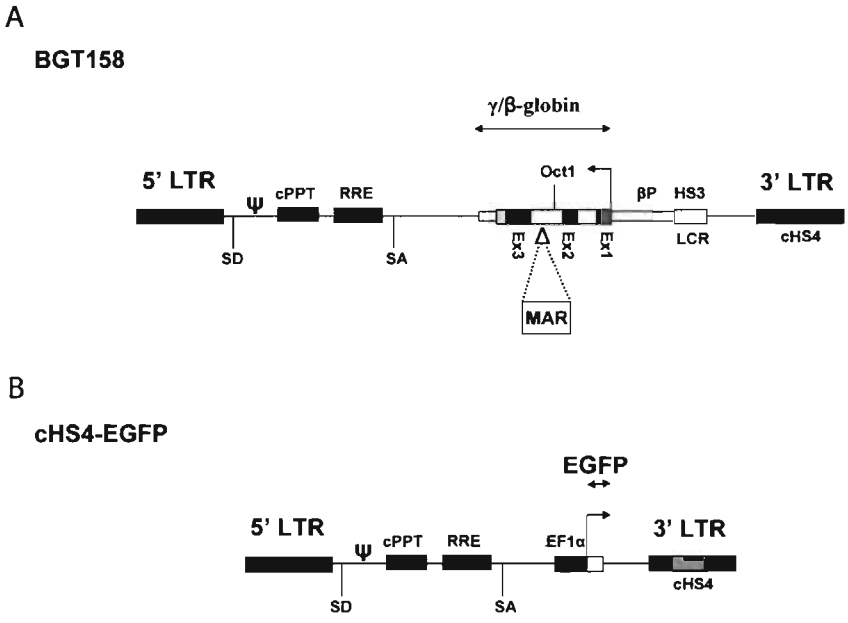

Figure 1.
Self-inactivating lentiviral vectors
A-BGT158 is the γ-globin expressing vector. The globin gene is a γ/β hybrid with γ-globin exons and intron 1, and β-globin intron 2. cHS4 insulator is included in the construct, replacing the U3 promoter. The β-globin promoter and 3`regulatory elements are included. The AT-rich region in intron 2 has been deleted and replaced with the matrix attachment region (MAR) of the μ chain of immunoglobulin, and the Oct-1 binding site was included in intron 2. B-cHS4-EGFP is the control vector. The enhanced GFP is under the control of EF1α promoter. The vector backbone is similar to BGT158 (Adapted from Ref. 27, Buzina *et al.*, Submitted)

Successful transductions with low MOI

To determine whether reproducible transductions of hematopoietic cells could be obtained with the novel vectors at low MOIs, and given the two vectors have the same backbone and cell surface glycoprotein (VSV-G), we first performed EGFP transductions with MOI ranging from 0.5 to 10 (short term) or 10 to 20 (long term). Transduced cells were intravenously injected into lethally irradiated recipient mice. The presence of EGFP expression was detected by flow cytometry either from peripheral blood, or by dissecting the spleen and extracting the colonies at day 12 (CFU-S_{12}). Overall, starting MOI 8, all transductions allowed detectable EGFP expressing cells at variable levels (**Figure 3A**).

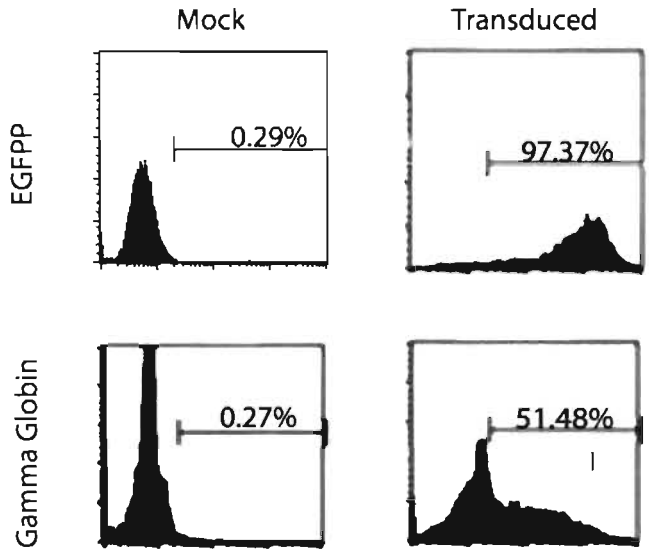

Figure 2
Intensity of transgene expression from lentiviral vectors in MEL cells.
EGFP and BGT158 efficiency in MEL cells transduced with increasing volume of concentrated virus was used to both titrate the vectors and examine the intensity of transgene expression by flow cytometry. For γ-globin, cells were stained with FiTC-coupled anti-gamma globin antibody. Both vectors allow high intensity transgene expression.

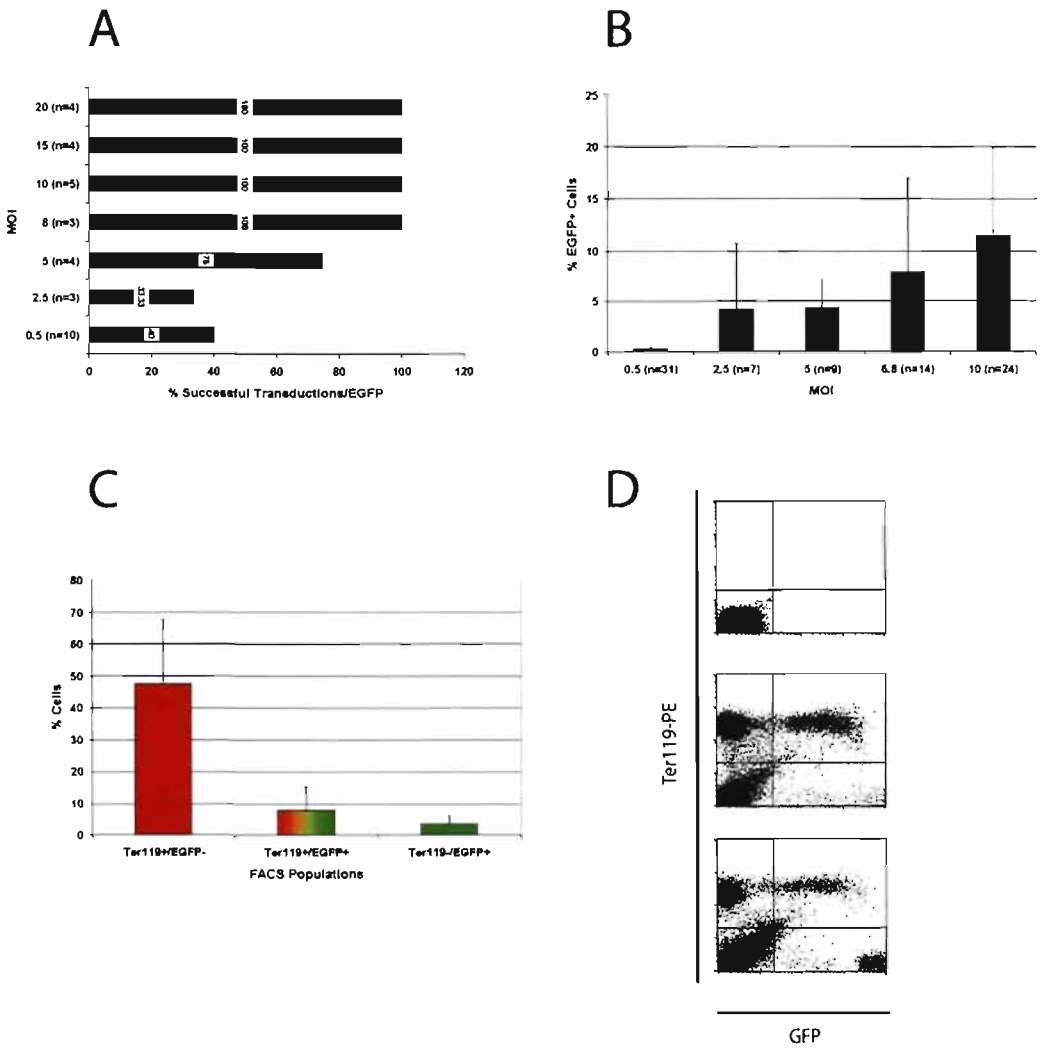

Figure 3

Flow cytometric functional evaluation of cHS4-EGFP lentiviral vector transduction efficiency.

5-FU-enriched HSC were transduced O/N with EGFP lentivector preparations with different MOIs, transplanted into lethaly irradiated C57BL/6J mice, which were sacrificed on day 12 and individual CFU-S_{12} evaluated for EGFP espression by flow cytometry. A, All transductions became successful when the MOI used was 8 or higher. B, The percentage of EGFP+ cells per CFU-S_{12} increased with MOI; C,D, Staining EGFP+ CFU-S_{12} cells with anti-Ter119 revealed almost equivalent expression of EGFP in erythroid and non-erythroid cells.

Short term analysis of EGFP expression from cHS4-EGFP vector

One of the advantages of EGFP is the ease of detection which makes it a valuable tracer for several analyses, especially that it is expressed from a ubiquitous promoter **(Figure 1)**. CFU-S_{12} colonies obtained from cells transduced with MOI 0.5 to 10 were analyzed by flow cytometry. EGFP+ colonies showed a low percentage of EGFP+ cells, which presented some variation with MOI **(Figure 3B)**. Staining of the EGFP+ cells obtained from CFU-S_{12} with anti-Ter119 erythroid specifc marker revealed almost equivalent expression of EGFP among erythroid and non-erythroid cell populations **(Figure 3C, D)** as expected

Table 1- Titers of the lentiviral vectors

Vector	Titer (IU/ml)	
	One spin	Two spins
BGT158	3.02×10^7	3.0×10^8
EGFP	1.56×10^8	1.3×10^9

IU, infectious unit; EGFP, enhanced green fluorescent protein

Long-term steady *in vivo* expression of γ-globin and EGFP in peripheral blood

To characterize the ability of the doubly insulated vectors to allow sustained expression in vivo, 5-FU-enriched hematopoietic cells from SAD donors were infected with various MOI (0.5, 1.5, 5, 8, 10, 15, and 20) then transplanted into lethally irradiated SAD and C57BL/6J mice. At various times after bone marrow transplantation (BMT) flow cytometry was used to detect and follow γ-globin and EGFP expression in peripheral blood in transplanted mice. At low MOIs (0.5, 1.5 and 5), the percentage of γ-globin or EGFP positive RBCs were low but sustained from 19 days till 6.5 months post-BMT, indicating that long term repopulating cells were infected **(Figure 4)**. γ-globin protein was detected only in the red blood cells, confirming the specificity of expression previously described **(Buzina *et al., Submitted*)**.

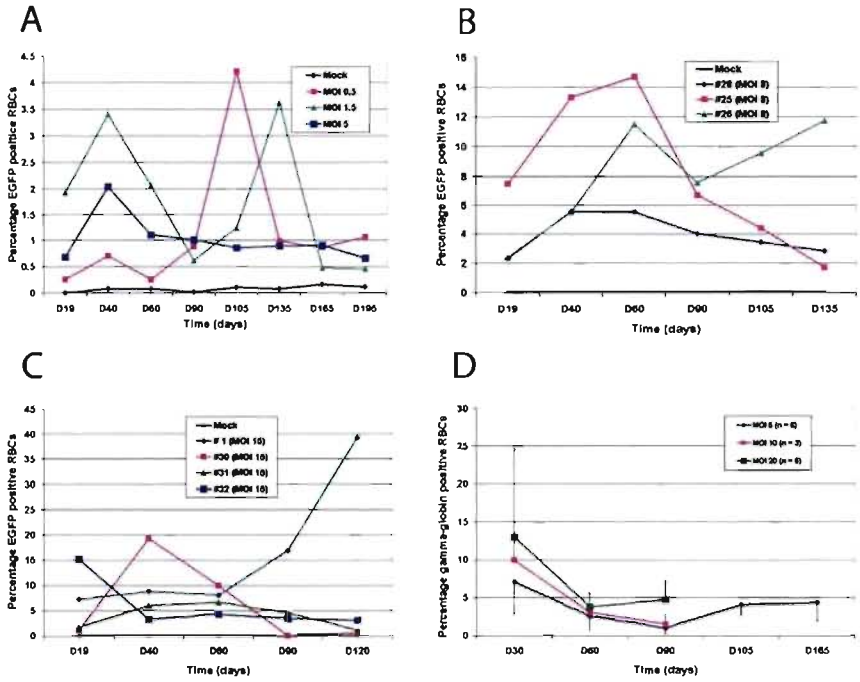

Figure 4- *In vivo* long-term time-course follow-up of EGFP and γ-globin positive RBCs. Peripheral blood RBCs of transplanted mice were assessed for the presence of EGFP and γ-globin proteins at many intervals after BMT. A-EGFP+ RBC population resulting from low MOI (0.5, 1.5, 5) showed constant levels overtime, while RBCs from higher MOI (8, 15) showed high levels shortly after transplantation, then decrease and stabilized by 3 months onwards (B, C). Each line represents one animal (A, B, C). D-Low and high MOI γ-globin transduction also gives an early peak of expression that stabilizes in two months (n = 3 for each of MOI 5, 10, and 20).

At higher MOIs (8, 10, 15, 20), the overall percentage positive RBCs was higher, however, that level declined and stabilized by day 60 (γ-globin) and 90 (EGFP) afterwards. This result suggests that long-term and short-term repopulating stem and progenitor cells were infected, allowing higher contribution early after transplantation, then the percentage of positive RBCs decreased as progenitors are eliminated. The γ-globin and EGFP positive RBC levels observed 2-3 months after BMT result almost exclusively from the infected long term repopulating stem cells, while those resulting from progenitors are cleared from blood around day 60. Overall, with the same MOI, the percentage of expressing cells was similar for both γ-globin

and EGFP vectors (**Table 2**). This result suggests that both the globin and the EGFP vectors have similar efficiencies with low MOIs. The route of cell transfer into the mice (intra-venously versus intra-bone marrow) had no effect on the percentage of peripheral blood RBCs with EGFP or γ-globin (data not shown).

Table 2- Percentage of RBCs with γ-globin or EGFP detected by flow cytometry in periphertal blood of recipients

MOI	γ-globin		EGFP	
	n	% positive RBCs	n	% positive RBCs
5	10	2.0 – 9.0	1	0.7 – 2.0
8	2	4.0 – 10.0	3	0.3 – 4.0
10	3	2.0 – 13.0	3	1.0 – 15.0
15	7	4.0 – 10.0	7	2.0 – 39.0
20	5	4.0 – 34.0	8	4.0 – 21.0

MOI, multiplicity of infection; EGFP, enhanced green fluorescent protein

Variable expression intensities detected by flow cytometry

Flow cytometry analysis of peripheral blood allowed not only to determine the percentage of expressing RBCs, but also to detect the presence of RBC populations of different intensities (**Figure 5**). Data from mice transplanted with transduced cells showed two RBC populations with low and high intensities, within the same sample (**Figure 5**). Interestingly, when levels of EGFP+ cells decreased by D60, the population with lower EGFP intensity was the most affected where most cells seem to have become EGFP negative (data not shown). In γ-globin expressing cells, few mice showed populations with very high intensity of expression (**Figure 5, B**)

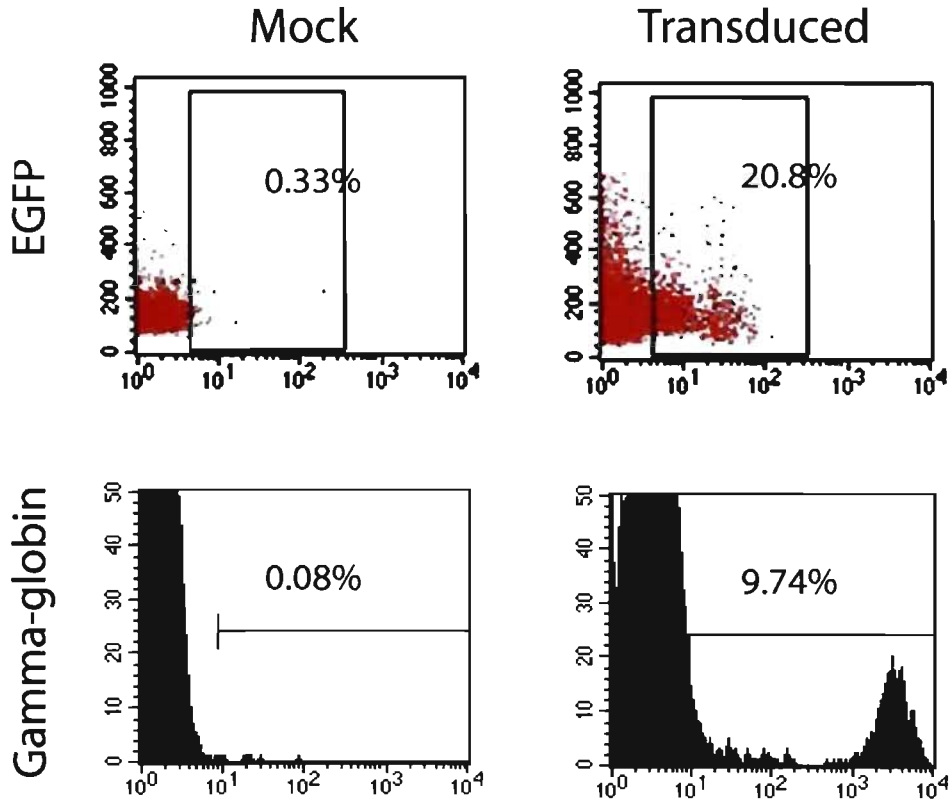

Figure 5

Cell populations of distinct fluorescence intensities detected by flow cytometry. RBCs obtained from mice transplanted with hematopoietic cells transduced with EGFP (MOI 15 or 20) showed two populations of high and low intensities within the same sample. γ-globin positive RBC population with very high intensities were detected in the peripheral blood of few transplanted mice; The percentage indicate the percent of RBCs with flow cytometry-detectable EGFP or γ-globin.

High γ-globin expression per cell (RBC)

One of the important aspects of gene therapy for SCD is the level of expression per cell (RBC). Using urea-triton PAGE, we determined the level of γ-globin protein per RBC between 7.54% to 9.03% (n = 2), 14.16% to 19.43% (n = 3), 27.51% to 66.35% (n = 3), regardless of MOI (**Figure 5 and data not shown**). This clearly demonstrates that the level of γ-globin expression from BGT158 vector is within the range previously determined to be therapeutic (**40**).

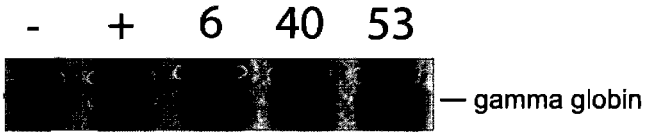

Figure 6
Protein analysis of globin chains.
Separation of globin chains was performed on total RBC lysates by urea-triton polyacrylamide gel electrophoresis (UT-PAGE). γ-globin is diluted in the total RBC lysate because it is expressed in only a small percentage of RBCs as detected by flow cytometry (in 4.25% of RBCs in sample 6; 8.4% in sample 40, and 2.86% in sample 53), therefore resulting in a faint band. The percentage of γ-globin on gel was then used to calculate its percentage per RBC taking into account the percentage of RBCs positive for γ chain. -, negative control (mock); +, positive control (a mouse transgenic line expressing the Aγ globin in erythroid cells, Ref. 29).

Discussion

We have performed an analytical study of the effect of various low-to-mild MOIs on the transduction efficiency with lentiviral vectors. Our aim was to test novel lentiviral vectors and to determine the transduction efficiency with acceptable amounts of viral vector, as an effort to render transductions more practical as far as large scale applications are concerned where reduced amounts of vectors required would be preferred. We analysed EGFP and γ-globin expression cassettes using novel SIN, doubly insulated, lentiviral vectors (**Figure 1**). These vectors were the cHS4-EGFP and BGT158, the latter carrying the human γ-globin gene. Both vectors were previously characterized for efficient expression *in vitro*, and γ-globin construct studied in transgenic mice and its expression specificity demonstrated (**27**). After optimization, very high titers of vectors were achieved, with double centrifugation far more effective than a single one (**Table 1**).

BGT158 is a self-inactivating (SIN), doubly insulated with cHS4, and containing an 850bp HS3, β-globin promoter and 3' regulatory element. This is also the first vector that includes advanced construct with a β/γ hybrid gene, including γ-globin exons and intron 1, and β-globin intron 2 containing the Oct-1 binding site, and the immunoglobin (Ig) μ chain matrix attachment region (MAR) replacing the AT-rich region (ATR). The ATR that is normally present in the β-globin intron 2 results in low titers in the context of vector production. In the BGT158 vector, the ATR was deleted, and replaced with a candidate element that could have equivalent role in improving the transcription. This element is the Ig μ MAR. A recent study in transgenic mice have demonstrated that the Oct-1 binding site cooperates with the Ig μ MAR to boost expression levels (**27, Buzina *et al., submitted***).

Transduction of MEL cells revealed decent expression profile and higher intensities were obtained with higher MOIs, which correlates with the *in-vivo* experiments where the signal obtained showed populations with different intensities (**Figure 4 A, B**), Possible reasons include (a) presence of cell populations with multiple integrations

and (b) loss of the insulator during the integration process as previously described (Dr James Ellis, personal communication). This effect was not observed at low MOIs such as 2.5, or 5, but with higher MOIs of 10 and above, and was not constant. Whether the VSV-G temporarily appearing on the surface of infected cells might have facilitated the entry of more viral particles into the transduced cells remains open to discussion

Our *in vivo* results suggest that with higher MOIs, although more hematopoietic cells cells are infected, short-term repopulating progenitors seemed to be the major targets, and their progeny rapidly cleared from the circulation, while the progeny cells of the long-term repopulating stem cells were sustainably detectable. Low MOI transduction of hematopoietic stem cells allowed both short-term expression in up to 34% (γ-globin) and 39% (EGFP) of RBCs, and long-term expression in up to 10% RBCs (both γ-globin and EGFP). Thus the globin vector appears equally efficient in transduction compared to EGFP vector, and allows sustained expression of γ-globin. Furthermore, BGT158 and cHS4-EGFP vectors showed other similarities: the in vivo percentage of expressing RBC population overtime was similar for cells expressing γ-globin and EGFP, and both vectors showed RBC populations with strong signal as detected by flow cytometry. This makes the BGT158 efficient for use in long-term in vivo applications in hematopoietic cells. Further optimization of transduction conditions would determine whether therapeutic level of expressing cells could be obtained for long term with low MOI. Our results along with the encouraging findings previously reported with low MOIs (**20**) suggest that this is likely to be possible.

In addition, the level of γ-globin per RBC (7.54% to 66.5%) is within the range capable of efficiently preventing HbSAD polymerization. In fact, our lab has previously demonstrated that a γ-globin level per RBC between 9 and 16% is sufficient to prevent polymerization of HbSAD (**29**). Given appropriate percentage of RBCs (almost 50%) with this level of γ-globin, a therapeutic effect should be expected, as we previously demonstrated (**40, 42**). Furthermore, the γ-globin positive

RBCs were detected for long term after transplantation of transduced hematopoietic cells. Therefore, the BGT158 vector is well promising for future applications in sickle cell disease gene therapy.

Altogether, our results have demonstrated the in vivo efficiency of the novel globin lentiviral vector tested. Furthermore, this study has shed light on several issues that might have been more or less overlooked in the current understanding of gene therapy planning for globin disorders, and we thus suggest several checkpoints that should be re-assessed for better efficiency:1, knowing that very high MOIs do not seem appropriate for clinical application, testing low and moderate MOIs (8- 20) would therefore be important for future optimizations; 2, the cytokine cocktail used during transduction should be carefully re-assessed; 3, the time of transduction could be changed and multiple transductions explored as previously described (20); 4, transduction of purified HSCs could be considered with low MOI; 5, testing transductions with the same MOIs, but with various concentrations (low versus high) of vector within the transduction mix would be helpful, as previously described (**20**); 6, although not directly implied from the current study, oxygen levels during transduction should probably be modified to get closer to the hypoxic microenvironment of bone marrow (**36, 37, 38, 39**), which might allow stem cells to remain in their "native" status and be easier to infect; whether their transduction in such conditions would be easier, remains to be elucidated.

Acknowledgements

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CHAPTER VII

DISCUSSION

β-Thalassemia and sickle cell disease (SCD) are among the most common single gene disorders, both associated with high levels of morbidity and mortality. Worldwide spreading has made them major health concern. Several symptomatic and palliative treatment modalites have been implemented over time, including blood transfusion. While these treatments relief the symptoms, they do not cure the actual disease. In addition, iron accumulates overtime, resulting in iron intoxication or hemochromatosis, necessitating iron chelation therapy. Furthermore, while administration of agents that induce HbF leads to some improvement, not all patients respond to the therapy, and long-term exposure to the chemotherapeutic agent(s) used expose patients to potential toxicities. On the other hand, bone marrow transplantation (BMT) provides the means to replace the diseased bone marrow by a normal one, therefore leading to production of normal RBCs, and a cure of the disease. Although few BMTs are carried out in human clinics for β-thalassemia and SCD (Kapelushnik *et al.*, 1995; Bernaudin *et al.*, 1993), when I started my project, there were many unanswered questions regarding the BMT strategies, and standard settings were not established, resulting in non-reproducible outcomes with only sporadic cases of successful transplants, often after conditioning with strong myeloablative regimens. In addition, as mentioned earlier, high-intensity myeloablation is very toxic, and reduces the range of patients that could be treated by BMT. Therefore, preconditioning with minimal myelosuppression would be preferred, provided it allows enough selective advantage for donor cells to correct the disease phenotype. Furthermore, the difficulties to find appropriate compatible donors limit the number of patients that could benefit from allogenic BMTs. As a result, gene therapy approach would have a strong impact, whereby the patient's own hematopoietic stem cells (HSCs) would be "corrected" by receiving a copy of the deficient globin gene (β-thalassemia), or a normal copy of the β-globin or γ-globin gene (SCD), potentially resulting in normalization of the phenotype, independently of the histocompatibility barriers. However, so far, gene transfer efficiency is far from being 100% in HSCs and toxicity remains an important issue, highlighting the need to both optimize cellular therapies and make appropriate planning for applications in gene therapy.

As discussed previously, there is an excessive loss of red blood cells (RBCs) in β-thalassemia and sickle cell disease. Therefore, my hypothesis was that this erythroid cell loss would confer selective survival advantage for normal erythroid cells after BMT and consequently, a partial replacement rather than a total replacement of the recipient's bone marrow (BM) would be sufficient to obtain long-term correction of the phenotype. Transplantation of normal BM cells which contain normal HSCs would result in the formation of a normal erythroid lineage that would be selectively amplified and eventually predominate over the defective erythroid lineage. A state of mixed chimerism rather than full chimerism would have therapeutic effect. However, the minimal percentage of normal bone marrow cells sufficient to significantly correct the phenotype, was unknown when I initiated this project. The degree of partial myelosuppression required to allow reproducible engraftment with therapeutic levels of normal donor chimerism, was also unknown. Occasional cases of BMT in human clinics where a mixed chimerism allowed phenotype correction (Kapelushnik *et al.*, 1995; Walters *et al.*, 1996; Andreani *et al.*, 2000) motivated my quest.
In the same light, studies in mouse models have shown that β-thalassemic and sickle mice have more stem and progenitor cells compared to normal mice (Beauchemin *et al.*, 2004; Blouin *et al.*, 1999), which would increase the chances of targeting stem cells in the context of gene therapy.

The major goal of this thesis was to determine the minimal replacement level of the recipient's BM necessary to achieve significant correction of the disease phenotype, and consequently establish the appropriate conditions of minimal myelosuppression and cell transfer strategies that would allow to reproducibly attaining that level of BM replacement. To achieve my goal, I had to select the appropriate mouse models, generate chimeric mice, and perform the appropriate phenotype characterization.

1. Selection of the mouse models

For this study to be conveniently representative for both β-thalassemia and SCD therapeutic settings, I needed to use appropriate mouse models reproducing the severe

human phenotype for each disease. Furthermore, although β-thalassemia and SCD are both due to anomalies affecting β-globin, each has a different basis. It was therefore important to explore the potential therapeutic strategies for each disease separately, and not in the context of coupled β-thalassemia/SCD states. Consequently, I decided to use a model for each disease and perform the same type of study on them separately.

On the basis of what preceded, I selected the Hbb^0/Hbb^S model because it was well characterized and reproduces very closely the human β-thalassemia *intermedia* in the heterozygous state (Chapter I, section I.5.1), and the SAD-1 model because it's the model that reproduces the human SCD the most closely (Chapter 1, section I.5.2.1).

The Hbb^0/Hbb^S and the SAD-1 models have been bred in our lab onto C57BL/6J background for over 21 generations and are therefore considered syngeneic. During this study, I have performed on non-transplanted non-irradiated mice of the selected models all the characterization I was performing on the chimeric mice, with the C57BL/6J as wild type control. The results of this characterization (Appendixes II and III) represented external controls used to validate the phenotypes observed with the chimeric mice at 0% and 100% (internal controls).

2. Generation of chimeric mice

In order to determine the minimal percentage of normal cells that allows phenotype correction, the best method is to generate chimeric mice with different percentages of normal versus pathologic cells, and analyze the degree of phenotype amelioration of each. Therefore, with total bone marrow (BM) cells as donor graft, I used the competitive repopulation assay (CRA) because it allows obtaining mice with desired percentages of normal white blood cells (WBCs). The percentage of normal WBCs detectable in peripheral blood of transplanted mice was not always exactly the same as the percentage I injected in the donor graft (Appendix VI), as has been previously

described for CRA by David Harrison (Harrisson *et al.*, 1993). While this could be due to simple probability as Harrison suggested, it could also result from potential differences in the homing and seeding capabilities of stem cells from both cell populations, or from the differences in the percentage of stem cells within bone marrow cells of the two donor mouse strains. Therefore, I analyzed the percentage of chimerism in peripheral blood of recipient mice systematically, starting 2 months after transplantation. I also arranged the mice in groups of chimerism based on the percentage of normal WBCs detected in their peripheral blood, and which was constant overtime. Performing CRA with purified hematopoietic stem cells (HSCs) instead of total bone marrow cells would help shed some light on potential differences in homing and seeding capabilities of normal C57BL/6J versus hemi-βthal or SAD HSCs.

The characterization of the transplant-recipient mice included the analysis of donor chimerism levels in peripheral blood, RBC parameters (morphological and physiological), erythropoiesis, and the histology of various organs and the overall survival of chimeric mice.

3. Selective advantage for normal erythroid cells

The CRA allowed generating two series of hematological chimeric mice (hemi-β-thal and SAD). The percentage of normal white blood cells (WBCs) in peripheral blood ranged from 0 to 100%. There was a ~ 2- to 2.5-fold amplification of normal RBCs, consistently prevailing until the 39% chimeric cohort and then decreased linearly with progressive normal donor chimerism. This confirmed my hypothesis that normal erythroid cells do have a selective advantage over their thalassemic and sickle counterparts. This level of amplification was comparable for both diseases. This finding suggests that is likely that a relatively low percentage of normal WBCs would be sufficient for phenotype amelioration, and prompted the initiation of various testings for the phenotype, beginning with RBC parameters.

4. Protective effect of normal RBCs toward thalassemic RBCs in peripheral blood

A chimerism of normal donor cells between 19 and 24% significantly improved the typical thalassemic phenotype. The analysis of the complete blood count (CBC) showed significant amelioration of the RBC parameters and correction of anemia. Within that range of donor cell chimerism, there was ~40 and 50% normal RBCs, respectively. One point I needed to investigate is whether the phenotype correction is due solely to having less thalassemic RBCs, or whether there is an active contribution of normal RBCs. To this end, I analyzed the percentage of fragmented RBCs and hypochromic RBCs. The percentage of fragmented cells has significantly decreased by ~2.6- to 2.8-fold (Chapter III, Table S2), which is more than the expected ~1.5- to 2-fold (50%) based on the chimerism alone (in the range 19-24%). This implies that the presence of normal RBCs is preventing accumulation of damage in thalassemic RBCs. This explains at least in part, why ~50% normal RBCs can confer a phenotype correction as significant as the one obtained. Based on this, I hypothesized that with such a decrease in fragmentation there should be a decrease in the amount of hypochromic cells by more than the expected 1.5- to 2-fold.

The hypochromic cells result from less hemoglobin per RBC as they are released from the BM, and from further loss of hemoglobin due to RBC fragmentation. Their percentage has decreased by 2.6-fold and 5-fold with 19 and 24% normal donor chimerism, respectively. It could be inferred that the decreased fragmentation of RBCs resulted in less loss of hemoglobin in peripheral blood, and therefore, less hypochromic cells than expected.

Taken together, these two parameters lead to the assumption that a substantial increase in the efficiency of oxygen distribution to tissues would result. This could be due, both to the presence of ~50% normal RBCs, and to the fact that the remaining ~50% thalassemic RBCs are accumulating less damage and loosing less hemoglobin and therefore their intrinsic ability to transport oxygen has been improved. This

finding correlated with the increase in the level of hemoglobin per blood volume and per RBC and increase in RBC half-life, and with the normal lifespan of the mice. Since the increase in RBC half-life (less RBC turnover) would result in less iron release, and since a better oxygen distribution is likely to result in improvement of histology parameters, the preceding results prompted the analysis of the histological parameters in various organs

5. Factors contributing to the improvement of pathology in hemi-βthal chimeric mice

In addition to oxygen distribution, iron accumulation in tissues also affects the pathology. Usually the excessive loss of erythroid cells (Gardenghi *et al.*, 2007; Garrick *et al.*, 1989; Celada, 1982) and increased iron absorption in the intestine (Harrisson, 2002) lead to iron deposition in tissues. In the chimeric mice, the increased RBC survival, decreased fragmentation and improved erythropoiesis efficiency led to less iron being released. On the other hand, the expected improvement of oxygen distribution and the observed correction of anemia is likely to have resulted in decreased iron absorption in the intestine. Together, these factors resulted in less iron deposition and therefore and less oxidative stress. Analysis of the iron absorption kinetics in chimeric mice would shed the light on the changes with the levels of normal WBC chimerism.

Furthermore, the renal focal sclerosis and hyperplasia of mesangial cells were mitigated with 24% normal WBCs, so was the hyperplasia of liver Kuppfer cells. The spleen to body weight ratio has returned to normal range with only 19% normal WBC chimerism. With this level of chimerism there is ~40% normal and 60% thalassemic RBCs. This suggests that as long as the percentage of abnormal/thalassemic RBCs is no more than ~60%, the spleen is able to process the damaged ones among them without being overloaded, reflecting a strong RBC clearance capacity for the spleen.

Altogether, these findings indicate a global decrease in the stress, and that is likely to result in global improvement of organ function. This was confirmed by the finding that mice with ~19-24% normal WBCs and above survived to a normal lifespan. On the basis of what preceded, it became interesting to determine whether the partial replacement of the recipient's BM could result in full correction of some disease parameters.

6. Full correction of many thalassemic disease parameters with very low percentage of normal WBCs

The examination of the various disease parameters in transplanted mice has revealed that some parameters are fully corrected with less than 19% normal chimerism. For example, in the spleen, the number of CFU-S_{12} and nucleated cell, which reflect the intensity of splenic erythropoiesis, have both returned to normal range with only 10% normal WBC chimerism (and therefore ~20% normal RBCs, and ~80% thalassemic RBCs) (Chapter III, Table 2). This result suggests that the bone marrow is able to contribute erythropoiesis without stimulation of the spleen above the baseline observed in wild type, as long as the percentage of abnormal RBCs does not exceed 80%, which reflects the strong ability of a bone marrow with little normal cells to compensate for the loss of RBCs, as confirmed by the improvement of anemia with low percentage of chimerism. This finding confirms once more the therapeutic potential of the 19-24% normal WBC chimerism, and eliminates any doubt regarding the ability of little normal cells in a diseased BM to reconstitute normal function.

7. Validation of the therapeutic effect of 19-24% normal WBCs on the thalassemic phenotype

Altogether, my results explain why the range of 19 to 24% normal WBC chimerism was selected as the therapeutic threshold/range: it is sufficient to have significant amelioration of all disease parameters, but also because many parameters are already

fully corrected by a WBC chimerism of 19%. A percentage of normal RBCs of 40-50% is preventing the accumulation of damage in the remaining thalassemic RBCs, explaining how a mixed chimerism could allow long-term significant correction of the thalassemic phenotype. Important improvement of pathology was detected in mice that were between 12 months to over 20 months old, indicating that pathology correction was stable over time. Putting this study in the context of thalassemic mice receiving normal cells, these results suggest that shortly after the transplantation, the disease-induced damage would stop accumulating, and many aspects would be reversed. This resulted from a partial but significant improvement of the erythropoietic efficiency, which also contributed to the raise of the RBC number in peripheral blood, and abrogated the anemia. These results provide valuable clinical impact, giving a lot of hope for thalassemic patients with secondary decline of organ functions, for whom a BMT replacing only part of their BM could result in a global rescue. It also suggests that other globin-related diseases, such as SCD, could potentially benefit from similar BMTs.

8. Correction of SCD with low percentage of normal cell chimerism similarly to β-thalassemia

Parallel to the study on hemi-βthal mice, I determined the therapeutic threshold for SCD (26-31% normal WBCs; 50-60% normal RBCs) which reflected a selective advantage for the normal RBC population, similarly to my findings with β-thalassemia. Systematic analysis was similarly conducted for SCD and showed correction of the disease parameters with low levels of normal chimerism.

As observed with β-thalassemia, some disease parameters were fully corrected with low percentage of chimerism. For example, the intensity of splenic erythropoiesis reached normal values with only 5% normal WBC chimerism (Chapter IV, Table 2). Regarding pathology, splenomegaly was reversed and spleen to body weight ratio reached the normal range with only 21% normal WBCs. Spleen architecture was also

improved with clearly recognizable white and red pulp structures with 26% normal WBCs. The surface area of glomeruli reached the normal range with surprisingly low levels of ~5-11% normal WBC chimerism. Reversion of many pathological aspects, including iron deposit in spleen and liver, partial vessel obstruction in lungs and liver, RBC sequestration in the spleen, and thickness of alveoli in the lungs, has been noticeably observed starting 16-21% normal chimerism, and substantially with 26-31% chimerism.

Iron deposits are not observed regularly in tissues of SAD mice and the amount accumulated varies among individual SAD mice (Trudel *et al.*, 1994). This is similar to what has been described in human patients (Rao *et al.*, 1984; Washington & Boggs, 1975). However, in the spleen, and to a lesser extent, the liver, iron accumulates and therefore, follow-up of any change in its pattern with the level of chimerism would be informative. Interestingly, iron deposits in spleens of SAD chimeric mice dropped quickly and reached levels very close to normal with only 16% normal WBCs. In the liver, iron deposits was less important but also dropped to normal range with ~16% normal WBCs. This constitutes further evidence to the ability of ~26-31% normal WBC to confer significant correction of the SAD phenotype and therefore constitute the therapeutic thershold for SCD. Within this range, most parameters were significantly improved, many pathological features were fully corrected, and mice survived for a normal lifespan. Furthermore, variability among individual mice in response to the presence of normal cells was minimal. While 21% normal WBCs allowed significant correction of many parameters, variability among individual mice was elevated for some, and improved survival was not constant. 26% chimerism constitutes the transition point to a stage where phenotype correction is steady and more pronounced, histological features are better, and survival is constantly improved. Selecting 26-31% as a therapeutic "range" for SCD instead of the 26% value provides a secure zone for clinical application where any unexpected variability between individuals in response to normal cells will still allow adequate phenotype correction, instead of falling within a less therapeutic chimerism.

These results imply that there is a functional improvement in many critical organs of SAD chimeric mice with low percentage of normal WBCs, especially due to less vaso-occlusions allowing better tissue oxygenation, and therefore explain why SCD could also be corrected with a low level of normal donor cell chimerism, which allowed mice to survive to normal lifespan, although not all parameters are fully corrected with partial chimerism.

9. Full correction of all disease parameters is not required for the treatment of β-thalassemia *intermedia* and SCD

Overall, the level of phenotype correction in response to various levels of normal cells presented many similarities between β-thalassemia and SCD. This result was surprising, because the two diseases have different basis and pathophysiologies. However, given that the animal models used in this study both reproduce severe forms of the respective diseases, the similarities in some aspects of the development of anemia and the resulting ischemia/hypoxia might have allowed them to respond similarly to the presence of normal cells. This would have important clinical implications for the treatment of mixed sickle-thalassemia.

It is important to note that this study demonstrates that a significant lifelong correction of the disease, allowing normal lifespan, is possible in the presence of ~25% normal WBC chimerism, i.e. ~50% normal RBC chimerism. Although many disease parameters were fully corrected at this level of chimerism, many other parameters were partially, but significantly, ameliorated. Consequently, with ~25% normal WBCs, there is a significant, but not full, correction of the disease, which is expected since the remaining ~50% RBCs are defective. This proves that a full correction is not required to allow recovering normal organ functions and normal lifespan, therefore highlighting the strength and efficiency of the partial replacement of the diseased BM. In the light of what preceded, a previous report suggested that 100% normal hemoglobin chimerism should be sought to allow full correction of all the SCD parameters, in particular, spleen and bone marrow parameters (Kean *et al.*,

2003). My study went beyond that idea. Although comparison between the current study and the previous report should be delicate given the differences in the mouse models and backgrounds used (SAD versus BERK; Chapter I, Section I.5.2), the current study demonstrates that although full correction of all parameters is observed only with 100% normal RBC chimerism (~50% normal WBCs), efficient therapy, does not, however, require full correction of all disease parameters, and that both hematological (including spleen and bone marrow parameters) and non-hematological organs were significantly ameliorated with only ~50% normal RBCs (i.e. 25% normal WBCs).

Furthermore, although it might be inferred from the results of the current study that correcting a coupled β-thalassemia/SCD phenotype could be possible with a partial replacement of the BM, the large number of various thalassemia/SCD combinations renders concluding in this regards speculative. Determining the minimal percentage of normal WBCs that could correct the phenotype of a hemi-βthal/SAD model could give a precise answer to this issue.

A 25% normal WBCs is a moderate level of replacement and it was reasonable to assume that it could be achieved with BMT following a partial myelosuppression in SAD and hemi-βthal mice.

10. Therapeutic conditions with minimal myelosuppression and cellular requirements

After determining the therapeutic threshold for both β-thalassemia and SCD, I decided to establish the optimal therapeutic conditions that could allow replacement of ~25% of recipient BM with a normal one, after only partial myelosuppression. This was motivated by previous studies showing the possibility of engraftment in normal mice with little myeloablation (Chapter I, section I.3.4). Therefore, I decided

to explore BMTs with minimal myelosuppression with doses of irradiation of 1Gy and 2Gy as well as non-myeloablative strategies.

My ultimate goal at this level was to work on optimizing conditions that would allow reproducibility of engraftment, i.e. engraftment in 100% of recipients, and a therapeutic level of donor chimerism within the recipient's peripheral blood, with minimal requirements of cells and myelosuppression. To this end, I have set and progressively modulated five different transplantation-related parameters: dose of irradiation, number of BM cells to be transferred, number of cell doses, timing of cell transfer after irradiation, and the route of cell transfer. Various combinations of these parameters resulted in many different transplantation strategies that allowed the optimization of the BMT conditions.

10.1 BMT with partial myelosuppression and single IV injection of donor cells

Previous studies in our lab have shown that 28h and 52h following 1Gy and 2Gy irradiation of mice, respectively, the number of bone marrow cells per femur is a little lower than the preceeding day and the following days (unpublished observations), suggesting some cell elinination due to irradiation, and therefore, a possible availability of more space within the BM. I therefore hypothesized that 28h after 1Gy and 52h after 2Gy would be more favorable time for engraftment than 4h after irradiation, where the BM would be able to accommodate more donor cells than 4h after irradiation. Accordingly, BMTs were carried out where donor cells were transferred either 4 hours after irradiation (hemi-βthal and SAD mice), or at 28 and 52 hours after irradiation (SAD mice).

With one cell dose administered 4h after irradiation, the level of donor chimerism in peripheral blood of engrafted recipients was equivalent for both β-thalassemia and SCD. Contrary to my expectations, the number of mice that engrafted suggested that the best timing for cell transfer was around 4 hours after irradiation. One reason might be that the recipient bone marrow regenerating one to two days after low

irradiation dose limits the engraftment of donor cells. 1Gy conditioning showed almost equivalent efficiency to 2Gy especially for SCD, which is concordant with what has been previously suggested for the effect of low dose of irradiation (Stewart *et al.*, 1998).

In engrafted recipients, the level of donor chimerism was, for most mice, within the therapeutic range, or higher. The phenotype analyses performed on those mice demonstrated correction of disease parameters similarly to what was observed with similar levels of chimerism after lethal irradiation. Overall, these results demonstrated that a partial but therapeutic replacement of bone marrow in the hemi-βthal and SAD mouse models could be obtained by BMT following a minimal myelosuppression. On the other hand, 100% engraftment efficiency was not observed after a single injection of cells. Further optimization was required, and I had three options: 1-Increase the dose of irradiation; 2-Increase the number of donor cells, and 3-Change the cell transfer strategy. Options 1 and 2 would render clinical extrapolation more complex because of the high amounts of cells that would be required, and the myelosuppression dose would be increasing. Option three seemed the most favorable, and I proceeded into optimizing the cell transfer modalities.

10.2 The importance of multiple injections of donor cells

Relatively large amounts of cells injected to recipients in one dose were not always more efficient in engrafting than lower numbers of cells (e.g., 80 versus 60 million cells injected 4h after 1Gy, Chapter IV, Table 5A). Therefore, I hypothesized that there might be a maximal limit to the number of cells that the bone marrow could accommodate within unit time, and decided to perform BMTs with multiple/fractionated donor cell injections (2, 3, or 4) to verify whether the outcome would improve.

When cells were transfered in 2 doses 4h and 28h after 1Gy or 2Gy, engraftment was superior to that obtained with one injection, and up to 100% of mice engrafted in some groups, both hemi-βthal and SAD. Transfer of cells in 3 or 4 doses allowed

100% engraftment efficiency with all tested combinations. Taken together, these results confirmed my hypothesis and suggested that injecting high amounts of cells in one dose results in less efficient engraftment, while splitting the same cell amount in 2 or more doses maximizes the number of cells that would seed the bone marrow and therefore results in higher efficiency of engraftment. This was concordant with reports of BMTs in human clinics where more than one cell injection proved efficient (Basara *et al.*, 1998; Moreau *et al.*, 1996; Banerjee *et al.*, 2005; Byrne *et al.*, 2001). This was also the first study to demonstrate reproducible engraftment in 100% of transplanted mice with therapeutic levels of chimerism after partial myelosuppression, while previous reports presented occasional non-reproducible engraftment with equivalent or higher doses of irradiation (Bradeley *et al.*, 2002; Barker *et al.*, 1994).

These results demonstrated that moderate amounts of cells can allow reproducible engraftment in minimally myelosuppressed hemi-βthal and SAD recipients, provided the cells are administered in the right timing. The cell transfer strategy played an important role in the engraftment efficiency, in particular the multiple cell transfer. Furthermore, by comparing all the transplantation strategies explored, it now became clear that the first 24-28 hours after the irradiation constitute a "window of opportunity" for optimal engraftment. Further studies with four or more injections would clarify whether lower amounts of cells could also allow reproducible engraftment within this window.

These encouraging results prompted the investigation of the fully non-myeloablative BMT regimens to determine whether the non-myeloablative BMTs could allow high engraftment efficiency and therapeutic levels of donor chimerism with reasonable amounts of cells, and consequently conclude whether strategies of clinical values could be obtained without myelosuppression

10.3 High engraftment efficiency after BMT with no myelosuppression and IV injection of donor cells

Using non-myelosuppressed SAD mice as recipients, I tested BMTs with different amounts of total nucleated bone marrow cells and number of cell doses (1, 2 and 3). With one cell injection, the number of mice that engrafted was higher than in partially myelosuppressed mice (that received equal amounts of cells), and up to 100% of mice engrafted in one group (Chapter V, Table 1). This suggests that homing is impaired after irradiation and that the blood-bone marrow barrier (BBMB) and therefore the appropriate adhesions required for homing are better preserved without prior conditioning. This conclusion is concordant with the studies indicating that irradiation has detrimental effects on homing (Jisong *et al.*, 1999; Szylvassi *et al.*, 1999; Cerny *et al.*, 2002) and disturbs the integrity of the BBMB (Shirota & Tavassoli, 1992). Noticeably, when compared to non-irradiated recipients, more mice engrafted after 2Gy than 1Gy. This implies that although irradiation decreases homing/seeding efficiency, higher doses of mild irradiation reduces the competitive ability of recipient's stem cells more than the lower doses, allowing donor graft to expand strong enough to engraft and repopulate the peripheral blood of recipients.

Having more mice engrafting after non-myelosuppressive regimen compared to minimal myelosuppression could also be explained in part by the findings of Shirota and Tavassoli (Shirota & Tavassoli, 1992) which is the entry of cells from the bloodstream into the bone marrow microenvironment after irradiation, due to the disruption of the BBMB. The authors explained that this entry will somehow fill any empty space within the marrow thus preventing appropriate homing of transplanted cells, which constitute the minority of circulating cells. In contrast, without prior irradiation, only cells capable of actively crossing the BBMB would reach the hematopoietic microenvironment, therefore maximizing the chances of successful engraftment.

However, two and three donor cell injections into non myelosuppressed recipient mice was not superior to one cell injection, contrary to what was observed in partially myelosuppressed mice. One possible explanation is the fact that irradiation, by damaging the BBMB would result in entry into the bone marrow of only a very small

number of stem cells per unit time. Therefore dividing the cell dose in many injections maximizes the cell delivery to the bone marrow. In contrast, without prior irradiation, the BBMB is intact and splitting the cell dose would not make a major difference. However, myelosuppression seems to have a beneficial effect on repopulation by donor cells: by weakening recipient's stem cells, it helps providing expansion advantage to donor cells, without which donor cells would not expand enough to repopulate peripheral blood with in high proportion. Overcoming this limitation without irradiation would require substantial amounts of donor cells.

In comparison to control BMTs where recipients were wild-type, engraftment efficiency and levels of chimerism were similar, indicating that without any myelosuppression there does not seem to be a particular advantage for the total normal donor graft cells (Appendix V). This suggests that, with the strategies developed so far, some myelosuppression will still be required to obtain clinically relevant levels of normal cell chimerism after infusion of reasonable amounts of donor cells.

The fact that while no more than 20% of IV injected HSCs will reach the recipient BM in non-myeloablated mouse (Cui *et al.*, 1999), intra-bone marrow (IBM) BMT allows higher seeding efficiency (Yahata *et al.*, 2003; Wang *et al.*, 2003; Mazurier *et al.*, 2003, 2004; Nakamura *et al.*, 2004), prompted the exploitation of the IBM BMT without myelosuppression

10.4 Reproducible engraftment after BMT with IBM injection of donor cells

Direct injection of donor cells in the bone resulted in all mice engrafting in all but one group (Chapter V, Table 2). This result was expected, because injecting the cells directly into the bone would spare the need for homing, making engraftment more efficient. However, the level of donor WBC chimerism in peripheral blood was very low, ranging from ~2 to ~6% (Chapter V, Table 2). This suggests that donor cells might not have been all able to efficiently contribute to the repopulation of the host,

for many possible reasons. Disruption of the bone marrow microenvironment might have occured during injection and limited the seeding. In addition, leakage from the site of injection would have resulted in loss of a certain amount of donor cells. Finally, the number of cells injected per bone might have exceeded the ability of the BM microenvironment to accommodate donor cells. Distributing the cells in three doses did not change the outcome (Chapter V, Table 3). Although comparison between the IV and IBM is not feasible in this context due to differences related to the cells transplanted, taken together, these results suggest that introducing all the cells in one or two bones is less efficient than distributing them to almost all the bone marrow sites as happens after intra-venous injection. In unpublished data, I have shown by tracking experiments that donor cells injected in one femur leave the injected bone via circulation, and home to bone marrow in other bones, as has also been shown by others (Zhong *et al.*, 2002; McKenzie *et al.*, 2006). The results of this thesis suggest that such process is not sufficient to allow a high level of donor chimerism in non-myelosuppressed recipients.

Alltogether, the non-myeloablative strategies seem very promising by allowing 100% engraftment efficiency in particular, the IBM strategies. Whether the low level of donor chimerism could potentially be improved when similar trials are performed on larger mammals remains to be determined. Although further studies might prove otherwise, my results highlighted the requirements for very large amounts of cells to attain therapeutic levels of chimerism in peripheral blood. One could speculate that so far, some myelosuppression will still be required to achieve therapeutic levels of donor chimerism with reasonable amounts of donor cells.

The results discussed so far indicated that ~25% normal BM cells is sufficient for phenotype correction and that it could be reached with BMT following partial myelosuppression. Putting these findings in the context of gene therapy, these results imply that ~25% "corrected" HSC by gene transfer would potentially result in therapeutic effect. While gene therapy is appealing, there are many steps to be optimized before trying to establish conditions allowing to achieve the ~25%

successfully transduced HSC. That include testing the ability of the gene therapy vector to reproducibly transduce HSCs, and allow good *in vivo* expression of the transgene in hematopoietic cells. My results encouraged me to test these two points for a novel lentiviral vector.

11. A novel globin lentiviral vector promising for future applications in gene therapy trials

As mentioned in the introduction, previous studies of gene therapy assays in mice have obtained a variable percentage of expressing cells after using often large amounts of infectious units of retroviral or lentiviral vectors which led to multiple integrations per cell. Although variable degrees of phenotype improvement were observed, the clinical application of those strategies would be difficult, in particular due to the requirements for high amounts of viral vector which would make the clinical applications unpractical given the very large industrial amounts required, therefore limiting its applications to very few patients. On this basis, I decided to determine whether low amounts of infectious units (low MOIs) of a lentiviral vector could transduce HSCs with efficiencies high enough to be potentially of curative value for SCD, and conclude on the appropriate adjustments that could be brought to transductions with low MOI, for the same goal. Since sustained and efficient expression is also important in globin gene therapy settings, a globin vector with optimized regulatory elements would be preferable.

The analysis of the composition of the globin vectors that were previously tested in gene therapy trials in mice for β-thalasemia (May *et al.*, 2000, 2002; Persons *et al.*, 2003; Imren *et al.*, 2002; Puthenveetil *et al.*, 2004), and SCD (Pawliuk *et al.*, 2001; Levasseur *et al*, 2003), as well as a novel vector (Buzina *et al, submitted*), the BGT158 self-inactivating (SIN) lentiviral vector carrying the human γ-globin, which has many advantages, in particular the presence of valuable regulatory elements, made me select the latter for my study.

BGT158 carries the human γ-globin gene, under the control of the β-globin promoter and 3'enhancer, which leads to the production of HbF, a potent anti-sickling hemoglobin. HbF rather than HbA is preferred when planning gene therapy assays for SCD. It also includes the β-globin intron 2 containing the matrix attachment region (MAR) of the immunoglobin μ-chain, (Buzina *et al.*, *submitted*), carries the HS3 of the β-globin locus control region (LCR) necessary for strong expression from globin promoters, and the Oct-1 binding site that plays important role in chromatin opening activity of HS3 (Bharadwaji *et al.*, 2003; Rubin *et al.*, 2000). In addition, BGT158 contains the chicken HS4 (cHS4) insulator, which has been shown by many that it protects transgenes against silencing (Emery *et al.*, 2002; Puthenveetil *et al.*, 2004).

The BGT158 has been thoroughly characterized *in vitro* (Buzina *et al*, *submitted*) and the expression efficiency and specificity of the globin construct in transgenic mice was also characterized (Buzina *et al.*, *submitted*). Based on all what preceded, BGT158 was the most appropriate for my study, especially that its efficiency in transducing hematopoietic cells and maintaining long term expression *in vivo* after transplantation of transduced cells into lethally irradiated mice, was yet to be tested. Since my study consisted on testing the novel globin vector, I decided to test, in parallel, the control vector, cHS4-EGFP. EGFP transgene is under the control of a ubiquitous promoter (Buzina *et al., Submitted*), allowing a comparative analysis.

I carried out a systematic transduction optimization using the control vector with MOI ranging from 0.5 to 20. The results demonstrated that all transductions resulted in cells expressing the transgene when MOIs of 8 and above were used, although the percentage of expressing cells was variable. Long-term analysis in mice transplanted with transduced BM cells showed that EGFP and γ-globin were detected in red blood cells (RBCs) in peripheral blood for over 5-6 months after BMT. This result suggested that long-term repopulating stem cells have been transduced, and that transgene expression is maintained over time. The flow cytometry profile obtained for mice transplanted with cells that were transduced with MOI 15 and 20, showed two populations of cells with clearly distinct inensities, especially clear for EGFP

(Chapter VI, Figure 5). These results could have many explanations: including the possibility that loss of cHS4 from some cells resulting in the lower intensity/silencing (Dr James Ellis, personal communication). While the percentage of positive ("expressing"") RBCs was up to 39% with EGFP and 34% with γ-globin after ~1 month after BMT (Chapter VI, Table 2), it decreased overtime to stabilize at up to 10% by day 60-90 after the transplantation (Chapter VI, Figure 4), an event that correlated with the disappearance of the RBC population with lower EGFP intensity (data not shown).

Furthermore, in analyzed mice, the amount of γ-globin per cell (Chapter VI, Results section, last paragraph) was within the range that our lab has previously determined to be required for inhibiting HbSAD polymerization (Blouin *et al.*, 2000). Therefore, this vector seems adequate for in vivo testings, and could therefore be used for future experimentations to determine its efficiency as a candidate vector for globin gene therapy. Future optimization of transduction conditions with low MOIs would help determine the highest level of expressing cells that could be maintained at low MOI. The use of selection and amplification methods of transduced HSCs without affecting their ``stemness`` would also help achieve that goal with low MOI. On the other hand, the low transduction efficiency of murine HSCs might be due to species-specific effect, as lentiviral vectors are known to infect human cells at a much higher efficiency than murine cells (Nguyen *et al.*, 2002).

12. Potential modifications to optimize lentiviral gene transfer with low MOI

For globin diseases, long-term expression is required, and therefore an integrating vector is needed. The BGT158 globin vector is a lentiviral vector, and has proven its ability to produce variable amounts of γ-globin per RBC. There remains to find appropriate conditions that would allow low MOIs to reproducibly result in high transduction efficiency.

As I mentioned earlier, the ideal situation is achieving high percentage of transduced with minimal vector requirements. Theoretically, if ~25% HSCs are transduced, if the resulting erythroid cells are expressing at adequate level per cell, then after transplantation, we should expect the formation of ~50% corrected RBCs in peripheral blood, due to the selective advantage for normal/corrected erythroid cells. Previous studies have shown this to be possible with high MOIs (Rivella *et al.*, 2003; Imren *et al.*, 2002; Persons *et al.*, 2003).

a-Cytokine mix

The developing fact that HSC is a heterogenous group of various subpopulations of stem (Chapter I, section I.1.2) suggests that it might be possible that each subpopulation has some different properties, and therefore react differently to cytokines. Isolating different subpopulations of HSCs and testing their responses to various cytokines would help obtaining a more suitable cytokine mix that would allow optimal transduction with minimal change in the stem cell phenotype, i.e., differentiation or loss of the self-renewal or the homing capabilities. Whether particular cytokines can change the permissivity of stem cells to transduction remains an interesting question.

Importantly, and in the light of what preceded, many reports have indicated that stem cells need to be in the G1 phase of the cell cycle to be permissive for lentiviral transductions (Uchida *et al.*, 1998; Korin and Zack, 1998; Zack *et al.*, 1990, 1992). Since most HSCs are quiescent cells, it might be important to find the appropriate combination of cytokines that would allow stem cells to temporarily and reversibly exit G0 into G1 without altering their stemness.

b-Oxygenation

Some reports have indicated that low oxygen tension favors the maintenance of HSCs in the "stem" state (Nilsson *et al.*, 1997, 2001; Lord, 1992; Cipolleschi *et al.*, 1993). Current transduction protocols incubate cells in atmospheric oxygen. Lowering the oxygen concentration might better preserve stem cells during transduction, therefore

increasing the number of long-term repopulating HSCs remaining at the end of transduction time. Whether such conditions could allow HSCs to be more permissive to transduction remains to be determined. Testing transductions under various oxygen concentrations would shed some light on this issue.

c-Transductions duration and number

Previous studies have reported very variable transductions time, from 5-8 hours (Imren *et al.*, 2002, 2004; Rivella *et al.*, 2003) to almost a day (Persons *et al.*, 2003), and obtained comparable efficiencies. Longer transduction times might influence the stem cell properties and favor differentiation, which would decrease the final number of transduced cells in the "stem" state. Testing variable transduction times with low MOI would clarify this point. The use of two successive transductions has also been reported (Puthenveetil *et al.*, 2004). Testing this strategy with the current vector will clarify its impact.

d-Concentration of the vector in the transduction mix

Although the planning of HSC transductions is often based on the use of particular MOI, the final concentration of the vector in the transduction mix might play an important role in the efficiency of transduction. Higher vector concentrations would result in increased interactions between the viral particles and the cells, therefore leading to higher transduction efficiencies, an effect that is expected to vary with MOI. Exploring various viral vector concentrations with the same MOIs would be helpful.

13. RBC half-life as a cornerstone in the therapy of anemias

RBC half-life was reduced in the hemi-βthal and SAD-1 mouse models, and was rescued by various levels of normal chimerism. RBC half-life was also reduced in irradiated mice versus the non-irradiated counterparts. After performing a systemic analysis of this effect (Appendix I) I was able to confirm that irradiation leads to

reduction of RBC half-life as a long-term effect. This was much more pronounced on thalassemic and sickle RBCs than on the wild-type RBCs where the reduction in RBC half-life was relatively mild. However, by analyzing the level of amplification of normal RBCs in chimeric SAD mice transplanted either after conditioning (Chapter IV, Figure 1, and data not shown), or without conditioning (Chapter V, Table 1), I found them to be comparable. Accordingly, irradiation did not have any detectable influence on the balance between the two RBC populations (normal and diseased) in chimeric mice, despite its unequal impact on individual populations, and therefore, did not impact the outcome. However, understanding the implications of this effect might be helpful for clinical settings. Also this information would help determining how new factors would inadvertently increase the hematopoietic stress by increasing RBC turnover, and eventually helps finding ways to avoid such consequences in the context of anemia therapeutics.

In addition, RBC half-life decreased with age: older mice have a shorter RBC half-life than the younger ones (Appendix I). This could explain at least in part the unexplained anemia that develops with age. Previous reports attempting to find an etiology for the unexplained anemias in elderly presented many possibilities, including reduced pluripotent hematopoietic stem cell reserve, decreased production of hematopoietic growth factors, reduced sensitivity of stem cells and progenitors to growth factors, marrow microenvironment abnormalities, androgen deficiency, unrecognized chronic kidney disease, undiagnosed myelodysplasia, or early stage ACD (anemia of chronic diseases) (Balducci & Hardy., 1998; Balducci, 2003). However, there remained cases where none of these possibilities applied. There were recent reports showing that the level of erythropoietin is reduced in many elderly without any detectable chronic or acute pathology that could be causative, and this was associated with an otherwise unexplained anemia (Ferrucci *et al.*, 2007). Whether the reductions in both RBC half-life and in erythropoietin levels are related remains an interesting project.

While this study provided no indication that these two factors could impact the chimeric balance of the RBC populations after BMT following myelosuppression, understanding the underlying mechanisms might prove useful for the therapeutics of anemias.

14. Clinical extrapolation of the current study

Ultimately, the current study would allow standardization of human BMTs for β-thalassemia and SCD to allow efficient applications for virtually all patients. That is, allow older patients (>55-60 years old) and patients with co-morbidities, who cannot tolerate strong myeloablation, to benefit from the cellular therapy (and eventually gene therapy), and ameliorate both their quality of life and lifespan. The results of this study cannot, however, be extrapolated directly to the human clinic.

I have established conditions that could be used for setting preclinical basis of cellular therapies for β-thalassemia *intermedia* and SCD. Despite the absence gene therapy clinical trials for these diseases so far, for reasons of safety, efficiency of gene transfer, and expression, knowing the minimal percentage of cells needed to be ``corrected`` by gene transfer is a pre-requisite for efficient future gene therapy trials especially that gene transfer efficiency into human HSCs is still far from being 100%.

Furthermore, the findings presented by this thesis could be applied to both autologous and allogenic BMT. Around 25% replacement of recipient's BM after allogenic BMT, or 25% "corrected" cells by autologous BMT, should have equivalent impact, given all appropriate conditions met. However, additional variables exist in the allogenic BMT setting, where potential graft-versus-host disease and graft rejection would require additional attention.

The finding that a low partial donor chimerism and a minimal dose of myelosuppression is sufficient for the therapy of these two diseases promises many improvements at clinical level: recipients would not need a full or strong

myeloablation; therefore the conditioning-related risks of temporary full immune suppression, infections and death, would be minimal. In addition, the occurrence of side effects common after lethal irradiation such as cataract, endocrine problems, and alopecia, would be minimal too. Furthermore, patients who cannot tolerate high intensity myeloablation, either due to co-morbidities, or to advanced age, would also be safely treatable. That might not include 100% of patients, but a substantial increase in the potential patients could be expected.

The amount of cells to be transplanted would have to be extrapolated in relation to the patient's body weight and the source/purity of stem cells used, as is usually done in current BMTs. The extrapolation of the myelosuppression level could take into consideration the effect of a certain dose of irradiation versus a certain dose of chemotherapy (in relation to body weight), as both types of myeloablative agents are used in human clinics. 1Gy and 2Gy are moderate; however, the final dose in humans might be a little different.

In this study, SCD and β-thalassemia *intermedia* were investigated. When extrapolating to human clinics, both β-thalassemia *intermedia* and *major* could be targeted. However, in β-thalassemia *major*, ineffective erythropoiesis is more severe, and therefore, normal RBCs would have a stronger selective advantage over thalassemic RBCs. Whether this would require a lower or higher percentage of donor cells to correct the disease remains to be investigated.

Furthermore, although I aimed to correct two specific blood diseases, it is possible that other erythrocyte-related diseases could potentially benefit from the strategies established during this study, to variable extents. Cellular therapies are becoming more and more important in clinics. Hematopoietic stem cells (HSCs) are very promising for therapeutics. Although the pure "stem" requirements are yet to be unraveled, with the available knowledge, stem cell transplantations are proving to be a very powerful tool, growing stronger overtime.

CHAPTER VIII

CONCLUSION

My studies have contributed to the advancement of the cellular therapy field for globin diseases, as well as further understanding of BMT physiology and hematopoietic stem cell (HSC) homing. It also contributed to the understanding of gene therapy planning for hemoglobinopathies. My results also confirm the importance of the mouse models that reproduce the human β-thalassemia and SCD. I have demonstrated for the first time that a partial replacement of only one fourth of the diseased recipient bone marrow (BM) with a normal counterpart is all that is needed for significant long term correction of murine β-thalassemia *intermedia* and SCD. Importantly, I proved that this replacement could be reproducibly achieved by BMT after a mild dose of myelosuppression.

I also showed that BMT without prior myelosuppression allows high efficiency engraftment. On the other hand, until further optimizations prove otherwise, a certain degree of myelosuppression is still required to achieve therapeutic levels of chimerism. In my opinion, the well known dogma that some space needs to be emptied to allow donor cells to engraft, should now read: "some space needs to be emptied to confer enough competitive advantage for donor cells to expand sufficiently and yield therapeutic levels of chimerism in peripheral blood, after infusion of a reasonable amount of donor cells", as simple engraftment could be observed without emptying space. While this "updated" dogma reflects a closer image to the requirements of successful BMT, however, it is not complete: a low myelosuppression dose does not always "empty" space, as is the case with low doses of 1Gy which cause only a very slight reduction in the number of BM cells.

In addition, I demonstrated the efficiency of a novel lentiviral vector to produce variable levels of γ-globin and allow long term expression of the transgene. These results suggest that this vector could be used for future applications in gene therapy assays of hemoglobinopathies.

Furthermore, my results have shed some light on some aspects of stem cell homing. There seem to be a maximal threshold for the number of stem cells homing to the BM

per unit time. BMT strategies with multiple injections after minimal myelosuppression had better impact on therapeutic outcome than equal amounts of cells transferred in the classical single dose method. Furthermore, my results suggested that the process of homing probably follows a pattern of saturation. My study has also supported the possibility that donor cells need to reach a certain minimal percentage within recipient BM in order to lead to detectable chimerism in peripheral blood.

In summary, this study has provided valuable contribution to the advancement of BMT therapies for β-thalassemia and sickle cell disease. It would ultimately increase the efficency of BMTs for globin diseases and expand the range of patients that could be treated.

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Appendix I- Radiation and aging reduce RBC half-life

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Abstract

Irradiation is a frequent component of conditioning regimens that precede bone marrow transplantations used to treat many diseases, including anemias like β-thalassemia and sickle cell disease (SCD). Since red blood cell physiopathology is a cornerstone in the treatment of anemias, it is important to determine whether its survival is influenced by irradiation and aging, two factors impacting clinical outcome. Using wild type mice as well as mouse models for β-thalassemia and sickle cell disease (SCD), we showed that RBC half-life decreases significantly with increasing dose of irradiation, and is up to 3.3-fold lower than in non-irradiated counterparts. Furthermore RBC half-life shows a strong and significant decrease with age, up to 2.1-fold lower in older mice compared to younger ones. We thus demonstrated for the first time significant decrease of RBC half-life after irradiation, as well as with aging of mice. These results would encourage to explore the clinical implications of the decreased RBC half-life following irradiation, and explains at least in part, the tendency to develop anemia in elderly without apparent disease.

Introduction

Bone marrow transplantations (BMTs) represent a curative treatment option for a variety of human diseases, including β-thalassemia and sickle cell disease (SCD), two hereditary anemias. Anemias are a group of diseases characterized by decreased total concentration of hemoglobin in peripheral blood, and involving anomalies at the red blood cell (RBC) level (**1**). β-thalassemia is characterized by a decreased or no production of β-globin subunit of hemoglobin, while SCD is due to an abnormal β-

globin chain leading to the formation of hemoglobin HbS that polymerizes upon deoxygenation. Both anomalies lead to ineffective RBC production and RBC destruction, and are accompanied by high degree of morbidity and mortality (**2**).

The central element in the treatment of anemias is thus the correction of (RBC) parameters and decreasing their turnover. Once a certain normal RBC population is established, better oxygen distribution and, in case of SCD, less vaso-occlusion, are established, resulting in general amelioration of recipients phenotype.

BMTs are often preceded by a conditioning regimen consisting of irradiation and/or chemotherapy (**3**). However, irradiation is known to have many side effects, such as cataract, functional disabilities and cancer (**13-18**). We thus decided to investigate whether irradiation affects RBC turnover in various mouse models including wild-type C57BL/6J, thalassemic hemi-βthal mice, and sickle SAD mice, where both transgenic mice are on C57BL/6J background. We demonstrate that increasing doses of irradiation lead to increased RBC turnover. The RBC half-life was progressively and significantly affected in thalassemic and sickle mice with increasing doses of irradiation starting 1Gy, while wild type mice showed change starting 2Gy. Furthermore, the age of mice affected RBC half-life which showed significant decrease with increasing age, in all tested mice groups.

Results

Generation of thes experimental mice

To investigate the effect of irradiation on red blood cell (RBC) half-life, C57BL/6J, SAD and hemi-βthal mice were divided each in 4 groups according to the irradiation dose received: 0Gy, 1Gy, 2Gy, 8.75Gy. To avoid interference that might be caused by transplantation, non irradiated mice, as well as mice subjected to 1Gy or 2Gy were not transplanted. However, mice receiving the lethal dose of 8.75Gy would die without bone marrow transplantation. Therefore, they were transplanted with cells obtained from donor mice of the corresponding group (C57BL/6J, SAD or hemi-βthal), leading to chimeric mice with 100% normal C57BL/6J cells, 100% SAD cells,

or 100% hemi-βthal cells, two months after transplantation (data not shown), and constituted appropriate material for the analysis of RBC half-life.

Lower RBC half-life of wild-type mice with increasing dose of irradiation

After 1Gy, RBC half-life remained unchanged, but decreased by 19% after 2Gy, and by 31.42% after 8.75Gy (**Table I.1**). Since the test was performed 2 months after irradiation, an acute effect of irradiation can be ruled out. Therefore, we suggest that the presence of long term effect of irradiation is leading to increased RBC turn-over, in a dose-dependent manner.

Table I.1 Variation of RBC half-life with increasing dose of irradiation

Irradiation Dose (Gy)	Mouse (strain)	n	RBC ½ life (days)	Age of mice (months)
0	**C57BL/6J**	**5**	**21.0** ±4.4	**4.0** ±0.0
	SAD	**3**	**21.1** ±1.0	**5.0** ±0.0
	Hemi-βthal	**5**	**13.9** ±0.7[k]	**6.2** ±1.6
1	**C57BL/6J**	**5**	**20.0** ±1.5	**4.0** ±0.0
	SAD	**2**	**15.5** ±0.2[f, b]	**4.0** ±0.0
	Hemi-βthal	**5**	**12.0** ±4.2[e]	**11.0** ±0.0
2	**C57BL/6J**	**6**	**17.0** ±3.3	**4.0** ±0.0
	SAD	**3**	**12.9** ±1.8[c, g]	**4.0** ±0.0
	Hemi-βthal	**5**	**11.3** ±3.3[h]	**10.0** ±3.7
8.75	**C57BL/6J**	**5**	**14.4** ±2.9[a]	**13.6** ±4.2
	SAD	**4**	**6.6** ±1.6[d, i]	**20.5** ±4.0
	Hemi-βthal	**2**	**4.2** ±2.4[j]	**9.0** ±0.0

t-test, irradiated v/s non-irradiated, same strain: a, p<0.03; b, p<0.007; c, p<0.005; d, p<0.00003; 1Gy, sample v/s C57BL/6J: e, p<0.01; f, p<0.002; 2Gy, sample v/s C5 7BL/6J: g, p<0.05; h, p<0.02; 8.75Gy, sample v/s C57BL/6J: i, p<0.002; j, p<0.03; Non-irradiated, hemi-βthal v/s Cd7BL/6J, k,p<0.03

More pronounced reduction of RBC half-life in sickle and thalassemic mice after irradiation

To verify whether irradiation affects thalassemic and sickle RBCs in the same intensity as wild-type RBCs, we analyzed RBC half-life in SAD (**4, 5**) and hemi-

βthal (**6**) mouse models (**Table I.1**). The decrease in half-life of SAD RBCs was 26.54% after 1Gy, 38.86% after 2Gy, and 68.72% after 8.75Gy. Hemi-βthal RBC half-life decreased by 16.66% after 1Gy, 18.7% after 2Gy, and 69.78% after 8.75Gy. These results indicate an important increase of sensitivity to radiation of sickle and thalassemic RBCs. SAD RBCs seem to be more sensitive than thalassemic RBCs to low irradiation doses, while the sensitivity to lethal irradiation dose appears to be equivalent. The dose-dependence effect of irradiation is very pronounced.

RBC half-life decreases with age

Mice used in the previous section were of comparable ages, but mostly young. We thus wondered whether older mice have the same RBC half-life, and therefore analyzed RBC half-life of non-irradiated old mice of all three strains, and compared them to that of younger non-irradiated mice analyzed in the previous section (**Table I.2**). Interestingly, RBC half-life of old (17.3 months, n=11) C57BL/6J mice was 29.18% lower than young (4.0 months, n=5) ones. SAD RBC half-life at 17 months age (n=9) was 53.08% lower than that of 4-months-old SAD mice (n=3). 15.8 months hemi-βthal mice (n=5) showed RBC half-life 41% lower than 6.2 months old (n=5) ones.

Table I.2 Variation of RBC half-life with age

Mouse (Strain)	n	Age (months)	RBC ½ life (days)
C57BL/6J	**5**	**4.0** ±0.0	**20.9** ±4.4
	11	**17.3** ±2.6	**14.8** ±2.9[a]
SAD	**3**	**5.0** ±0.0	**21.1** ±1.0
	9	**17.0** ±3.3	**9.9** ±3.6[c,e]
Hemi-βthal	**5**	**6.2** ±1.6	**13.9** ±0.7[d]
	5	**15.8** ±2.9	**8.2** ±1.3[b,f]

t-test, old v/s young, same strain: a, $p<0.04$; b, $p<0.0002$; c, $p<0.000009$; Young C57BL/6J v/s young hemi-βthal: d, $p<0.03$; old sample v/s old C57BL/6J: e, $p<0.004$; f, $p<0.000008$

DISCUSSION

Irradiation constitutes a component of numerous conditioning regimens used prior to bone marrow transplantations (BMT) to suppress the recipient immune response to a level low enough to allow engraftment of donor cells and, in case of allogenic BMT, prevent graft rejection. Although many studies have shown that recipient mice can engraft without prior myeloablation (**7, 8, 9, 10**) irradiation is still required for opening spaces in recipient bone marrow facilitating engraftment and allowing donor cells to expand to a clinically relevant proportion. Therefore, and until appropriate non-myeloablative strategies with therapeutic outcome become available, a certain degree of myelosuppression would still be required. Consequently, the impact of various components of conditioning regimens on disease parameters should be assessed to help getting better control over the therapeutic outcome. We investigated this subject in the light of anemias, in particular the effect of gamma irradiation on the red blood cell (RBC) survival, a cornerstone in the cure of anemias.

We have selected mouse models that are very representative of two human diseases: the hemi-βthal (**6**) mouse replicating the human thalassemia intermedia, and SAD mouse (**4, 5**), replicating the human sickle cell disease.

We showed that RBC half-life of wild-type, thalassemic, and sickle mice, decrease to varying degrees after total body irradiation (TBI) (**Table I.1**). Wild-type mice appear to be the least affected, and their RBCs half-life is significantly shortened only after lethal dose of irradiation. In contrast, SAD RBCs are the most sensitive and their half-life decreases significantly after only 1Gy TBI (**Table I.1**). Thalassemic RBCs sensitivity to irradiation seems intermediate at low irradiation doses, but becomes equivalent to that of SAD RBCs after a lethal irradiation dose. The effect of radiation has therefore presented a dose-dependence, with higher doses resulting in a more pronounced reduction in RBC half-life.

A significant effect of aging on RBC turnover was observed with older mice having lower RBC half-life. Assuming that the decline in RBC half-life is progressive, then on average, RBC half-life seems to decrease by 0.45 day/month in wild-type, 0.59 day/month in thalassemic mice, and 0.93 day/month in SAD mice. However, whether this decrease is linear remains to be determined. Interestingly, compared to the younger counterparts, RBC half-life of aged SAD mice is 2.13 times lower in non-irradiated mice (n=9) (**Table I.2**), and 2.67 time lower in irradiated ones (n=8) (data not shown), suggesting that the sensitivity to irradiation seems to increase with age. However, further investigations are needed to confirm this hypothesis.

Altogether, these results have demonstrated for the first time, that radiation and aging decrease RBC survival. This observation supports the exploitation of the corresponding clinical implications Investigating the mechanisms behind these observations would be of clinical benefit.

Materials and Methods

Mouse strains

The sickle cell mouse model (SAD-1 mouse) was generated by coinjecting into fertilized eggs the βSAD and the human α-globin genes, each with the locus control region (LCR) (**4**). SAD-1 mice were genotyped as previously described (**4**). The hemizygous β-thalassemic mice (hemi-βthal) were generated by deletion of the murine β^{major} and β^{minor} globin genes and closely reproduce the human β-thalassemia intermedia (**6**). Hemi-βthal mice were genotyped as previously described (**6**). Both mouse lines were bred onto C57BL/6J-*Gpi1*^{b}/*Gpi1*^{b} background for >21 generations, therefore considered syngeneic. Congenic C57BL/6-*Gpi1*^{a}/*Gpi1*^{a} (Glucose phosphate isomerase isotype 1a) mice carry a hematopoietic cell marker and were obtained from Dr J. E. Barker (the Jackson labs, Bar Harbor, ME, USA). All mice were maintained in a specific pathogen free environment and experimental procedures were conducted in compliance with the guidelines of the Canadian Council on Animal Care (CCAC)

Irradiation

Mice were exposed to two different low doses of irradiation: 1G, 2Gy and a lethal dose of 875Gy (JL Shephered & Associates, Mark I-68A-1 Research irradiator, San Francisco, CA, USA), as previously described (**12**).

Generation of Chimeric Mice

Nucleated bone marrow (BM) cells were harvested from donor animals using IMDM (Iscove's Modified Dulbelco Medium, GIBCO, Grand Island, NY, USA). 2 x 10^6 cells were IV injected to lethally irradiated recipient mice in lateral tail vein, 4h after irradiation, as previously described (Chapters 3 and 4, materials and methods).

Red Blood Cells (RBC) half-life determination

The RBC half-life was determined using a nonradioactive protocol which consisted on biotinylation of the entire RBC cohort and monitoring for RBC replacement as previously described (**12**).

Statistical Analysis

Values were expressed as mean ± standard deviation. Two samples student T-test was used to determine statistical analysis with Microsoft excel software, and Fisher test with SigmaStat 3.1.

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Appendix II. Characterization of non-transplanted Hemi-βthal mice with the respective C57BL/6J controls

Table II.1 Complete blood count of hemi-βthal mice

		Mature Erythrocytes					Reticulocytes			
Mice	n	RBC[f] (10^6/µl)	Hb[h] (gédl)	MCV[e] (fl)	MCH[g] (pg)	%hypo[c] (HC<22g/dl)	H[b]	M	L[a]	%[d]
Hemi-βthal	**18**	**6.1** ±0.7	**6.2** ±0.2	**39.8** ±2.5	**10.0** ±0.3	**26.5** ±10.3	**31.1** ±14.0	**33.1** ±7.8	**35.5** ±7.0	**14.7** ±3.5
C57BL/6J	**18**	**10.1** ±0.8	**15.0** ±0.9	**51.3** ±2.5	**14.9** ±0.7	**0.6** ±0.4	**9.4** ±4.1	**39.4** ±4.2	**51.1** ±6.9	**3.5** ±0.7

Abbreviations: RBC, red blood cell; Hb, hemoglobin; MCV, ,mean cell volume; MCH, mean hemoglobin concentration; hypo, hypochromic cells; H, high; M, medium; L, low; HC, hemoglobin concentration;
t-test, C57BL/6J versus hemi-βthal; a, $p<0.004$; b, $p<0.0003$; c, $p<1.5 \times 10^{-7}$; d, $p<1.9 \times 10^{-9}$; e, $p<1.3 \times 10^{-5}$; f, $p<4 \times 10^{-17}$; g, $p<6 \times 10^{-19}$; h, $p<1.4 \times 10^{-25}$

Table II.2 Quantification of RBCs morphological abnormalities and half-life in hemi-βthal mice

		RBC morphology			RBC turnover	
Mice	n	Target Cells[a]	Fragmented Cells[b]	Heinz Bodies[c]	n	Half-life (days)
Hemi-βthal	**9**	**7.6** ±3.6	**8.1** ±2.5	**47.2** ±7.7	**5**	**8.2**±1.3
C57BL/6J	**6**	**2.6** ±3.0	**0**	**0**	**11**	**14.8**±2.9

Abbreviations: RBC, red blood cell;
t-test, C57BL/6J versus hemi-βthal; a, $p<0.05$; b, $p<10^{-5}$; c, $p<10^{-7}$

Appendix II. Characterization of non-transplanted Hemi-βthal mice with the respective C57BL/6J controls

Table II.3 Erythropoiesis of hemi-βthal mice

Chimeric Mice (% normal WBC)	n	Proerythroblasts (% of total cells)		Basophilic Erythroblasts (% of total cells)		Late basophilic / Polychromatophilic erythroblasts (% of total cells)		Orthochromatophilic erythroblasts (% of total cells)	
		Bone marrow[a]	Spleen[e]	Bone marrow[d]	Spleen[d]	Bone marrow	Spleen[d]	Bone marrow[c]	Spleen[b]
Hemi-βthal	**8**	**5.7** ±2.3	**2.8** ±1.8	**40.9** ±6.4	**40.6** ±13.5	**3.5** ±1.1	**6.2** ±2.2	**6.6** ±2.9	**15.6** ±4.9
C57BL/6J	**7**	**7.0** ±3.0	**1.9** ±1.4	**20.8** ±1.1	**2.7** ±1.3	**6.8** ±0.8	**1.7** ±1.1	**21.7** ±4.1	**41.0** ±14.9

Abbreviations: WBC, white blood cell;
t-test, C57BL/6J versus hemi-βthal; a, $p<0.004$; b, $p<0.002$; c, $p<1.3 \times 10^{-5}$; d, $p<7 \times 10^{-8}$; e, $p<1.6 \times 10^{-11}$

Table II.4 Quantification of cellularity and multipotent cells of hemi-βthal mice

Mice	N	Femur (10^7 nucleated cells)	Spleen[c] (10^7 nucleated cells)	Femur[a] (10^2 CFU-S_{12})	Spleen[b] (10^2 CFU-S_{12})
Hemi-βthal	**6**	**4.3** ±0.7	**58.0** ±16.0	**22.3** ±6.4	**39.9** ±12.5
C57BL/6J	**4**	**5.2** ±1.1	**14.0** ±3.6	**12.9** ±6.0	**3.7** ±1.5

Abbreviations: CFU-S12, colony forming unit in spleen at day 12; l;
t-test, C57BL/6J versus hemi-βthal; a, $p<0.04$; b, $p<0.0008$; c, $p<0.0007$

Appendix II. Characterization of non-transplanted Hemi-βthal mice with the respective C57BL/6J controls

Table II.5 Pathologic assessment of hemi-βthal mice

				Iron deposits			
Mice	**n**	**Spleen wt/ body wt %[d]**	**n**	**Spleen[c]**	**Kidney*[a]**	**Heart**	**Liver[b]**
Hemi-βthal	**12**	**1.74** ±0.67	**6**	**10.8** ±3.8	**1.7** ±0.9	**0.7** ±0.5	**2.2** ±1.4
C57BL/6J	**22**	**0.28** ±0.09	**9**	**1.0** ±0.9	**0.3** ±0.2	**0.1** ±0.1	**0.2** ±0.02

Abbreviations: wt, weight; *, iron quatified in the cortex;
t-test, C57BL/6J versus hemi-βthal; a, $p<0.04$; b, $p<0.02$; c, $p<0.002$; d, $p<0.0001$

Table II.6 Survival of hemi-βthal mice

Mice	**n**	**Lifespan** (months)
Hemi-βthal	**24**	**13.4** ±5.7
C57BL/6J	**theo**	**22-24**

Appendix III. Characterization of non-transplanted SAD mice with the respective C57BL/6J controls

Table III.1 Complete blood count of SAD mice

Mice	n	RBC[a] ($10^6/\mu l$)	Hb (g/dl)	MCV[d] (fl)	CHCM[c] (g/dl)	% Hyper[b] (HC >35g/dl)
SAD	**35**	**11.0** ±0.5	**15.4** ±0.8	**44.5** ±1.7	**30.8** ±1.0	**1.1** ±0.6
C57BL/6J	**39**	**10.4** ±0.8	**15.1** ±1.0	**50.5** ±2.6	**27.7** ±1.3	**0.05** ±0.06

Abbreviations: RBC, red blood cell; Hb, hemoglobin; MCV, ,mean cell volume; CHCM, cell hemoglobin concentration mean; hyper, hyperchromic cells;
t-test, C57BL/6J versus SAD; a, $p<0.008$; b, $p<2 \times 10^{-11}$; c, $p<3 \times 10^{-17}$; d, $p<5 \times 10^{-18}$

Table III.2 Quantification of RBCs half-life in SAD mice

Mice	n	Half-life (days)
SAD	**9**	**9.9** ±3.6
C57BL/6J	**11**	**14.8** ±2.9

Appendix III. Characterization of non-transplanted SAD mice with the respective C57BL/6J controls

Table III.3 Erythropoiesis of SAD mice

Chimeric Mice (% normal WBC)	n	Proerythroblasts (% of total cells)		Basophilic Erythroblasts (% of total cells)		Late basophilic / Polychromatophilic erythroblasts (% of total cells)		Orthochromatophilic erythroblasts (% of total cells)	
		Bone marrow	Spleen	Bone marrow[b]	Spleen[d]	Bone marrow[a]	Spleen[d]	Bone marrow[e]	Spleen[c]
SAD	**10**	**7.6** ±4.4	**1.9** ±1.2	**24.0** ±3.7	**28.5** ±12.3	**6.0** ±2.2	**15.0** ±7.4	**9.0** ±3.5	**34.7** ±11.6
C57BL/6J	**13**	**7.0** ±2.2	**2.7** ±1.7	**20.6** ±5.1	**4.3** ±2.9	**4.5** ±1.5	**3.3** ±1.2	**18.5** ±4.5	**53.1** ±7.5

Abbreviations: WBC, white blood cell;
t-test, C57BL/6J versus SAD; a, $p<0.004$; b, $p<0.03$; c, $p<0.0003$; d, $p<3 \times 10^{-5}$; e, $p<8 \times 10^{-7}$

Table III.4 Quantification of cellularity and multipotent cells of SAD mice

Mice	N	Femur (10^7 nucleated cells)	Spleen (10^7 nucleated cells)	Femur[a] (10^2 CFU-S_{12})	Spleen[b] (10^2 CFU-S_{12})
SAD	7	**48.1** ±13.3	**26.4** ±9.4	**36.8** ±24.1	**21.8** ±11.7
C57BL/6J	7	**41.7** ±8.1	**17.2** ±8.7	**12.3** ±7.2	**6.6** ±2.4

Abbreviations: CFU-S12, colony forming unit in spleen at day 12; l;
t-test, C57BL/6J versus SAD; a, $p<0.04$; b, $p<0.02$

Appendix III. Characterization of non-transplanted SAD mice with the respective C57BL/6J controls

Table III.5 Pathologic assessment of SAD mice

		Spleen			Kidney Glomeruli Area ($x10^2\mu m^2$)		
Mice	**n**	**Spleen wt/ Body wt %**[c]	**n**	**Iron deposit**[d]	**n**	**Outer cortex**[b]	**Inner cortex**[a]
SAD	**17**	**0.47** ±0.15	**4**	**3.2** ±0.7	**3**	**30.3** ±0.9	**65.9** ±13.2
C57BL/6J	**28**	**0.29** ±0.11	**4**	**0.7** ±0.4	**5**	**22.9** ±3.3	**38.8** ±9.6

Abbreviations: wt, weight;
t-test, C57BL/6J versus SADl; a, p<0.03; b, p<0.008; c, p<0.005; d, p<0.003

Table III.6 Survival of SAD mice

Mice	n	**Lifespan** (months)
SAD	276	15.8±5.3
C57BL/6J	theo	22-24

Appendix IV. Engraftment efficiencies with mini-transplants

Table IV.1 Engraftment efficiency in partially myelosuppressed and fully non-myeloablated transplanted SAD mice

Partial Myeloablation, intravenous cell injection -1Gy, 1 injection 4hrs after irradiation 4×10^7, 31.57% (6/19) 6×10^7, 54.14 (4/7) 8×10^7, 50.0% (4/8) -1Gy, 1 injection 28hrs after irradiation 4×10^7, 35.29% (6/17) 6×10^7, 100% (10/10) 8×10^7, 40.0% (4/10) -1Gy, 2 injections 4h & 28h after irradiation 6×10^7, 14.28% (1/7) -1Gy, 3 injections 4, 16 & 18hrs after irradiation 6×10^7, 100% (7/7) -1Gy, 4 injections, 4, 16, 22, 28hrs after irradiation 6×10^7, 100% (7/7) -2Gy, 1 injection 4hrs after irradiation 2×10^7, 19.04% (4/21) 4×10^7, 41.66% (5/12) 5×10^7, 62.5% (5/8) -2Gy, 1 injection 52hrs after irradiation 2×10^7, 53.84% (7/13) 5×10^7, 37.5% (3/8) -2Gy, 2 injections 4h & 24h after irradiation 4×10^7, 85.71% (6/7) -2Gy, 3 injections 4, 16 & 28hrs after irradiation 4×10^7, 100% (5/5) -2Gy, 4 injections, 4, 16, 22, 28hrs after irradiation 4×10^7, 100% (6/6)
No Myeloablation,intravenous cell Injection -4×10^7, 1 injection, 53.33% (8/15) -8×10^7, 1 injection, 91.66% (11/12) 2 injections, 53.84% (7/13) -12×10^7, 1 injection, 100% (8/8) 2 injections, 70% (7/10) 3 injections, 81.81% (9/11)
No Myeloablation, intra-bone cell Injection, BM cells from 5FU treated C57BL/6J*Gpi1*^{a} One cell injection -1×10^6, 80% (8/10) -2×10^6, 100% (10/10) -3×10^6, 100% (4/4) -4×10^6, 100% (5/5) -6×10^6, 100% (5/5) -8×10^6, 100% (5/5) -10×10^6, 100% (7/7)
No Myeloablation, intra-bone cell Injection, BM cells from 5FU treated C57BL/6J*Gpi1*^{a} Three cell injections -1×10^6, 85.71% (6/7) -2×10^6, 85.71% (6/7) -2×10^6 & prior bleeding, 85.71% (6/7)

Appendix IV. Engraftment efficiencies with mini-transplants

Table IV.2 Engraftment efficiency in partially myelosuppressed transplanted hemi-βthal mice

Partial irradiation, intravenous cell injection
-1Gy, 1 injection 4h after irradiation
4×10^7, 0% (0/6)
6×10^7, 42.8% (3/7)
-1Gy, 2 injections 4h & 28h after irradiation
4×10^7, 42.85% (3/7)
6×10^7, 100% (6/6)
-2Gy, 1 injection 4h after irradiation
2×10^7, 50% (4/8)
4×10^7, 85.71% (6/7)
-2Gy, 2 injections 4h & 28h after irradiation
2×10^7, 80% (4/5)
4×10^7, 100% (5/5)

Appendix V. Donor chimerism in wild-type recipients

Table V.1 Engraftment and chimerism in wild-type recipients

Donor cells	Route	Irr (Gy)	Cells x10^6	n (total)	n (engr)	% Chimerism	
						WBC	RBC
Bone marrow	I.V.	1	60	7	5	77.3 ±42.2	79.6 ±43.5
	I.V.	2	40	7	5	76.5 ±39.4	81.7 ±38.6
Bonw marrow	I.V.	0	60	5	4	14.8 ±5.0	24.7 ±7.3

Abbreviations: I.V., intravenous; WBC, white blood cells; RBC, red blood cells; n(tot), total number of mice transplanted; n(engr), number of transplanted mice that engrafted.

Appendix VI. Production of chimeric mice by Competitive Repopulation Assay (CRA)

Table VI.1 Infused versus detected percentage of normal WBC in hemi-βthal chimeras

Infused % WBCs	**n**	**Detected** % WBCs
0	**8**	**0** ±0.0
7.5	**8**	**15.3** ±24.1
10	**13**	**15.1** ±12.0
15	**15**	**30.2** ±29.9
20	**14**	**44.3** ±32.3
25	**14**	**41.7** ±28.2
30	**12**	**47.4** ±26.6
50	**9**	**64.8** ±23.2
75	**5**	**75.5** ±25.8
100	**5**	**100** ±0.0

Abbreviations: WBCs, white blood cells; Infused %, percentage of normal WBCs in the graft infused to recipients; Detected %, percentage of normal WBCs detected in peripheral blood of recipients after transplantation;

Appendix VI. Production of chimeric mice by Competitive Repopulation Assay (CRA)

Table VI.2 Infused versus detected percentage of normal WBC in SAD chimeras

Infused % WBCs	**n**	**Detected** % WBCs
0	**16**	**0** ±0.0
10	**9**	**8.9** ±6.2
12	**8**	**12.5** ±14.8
15	**15**	**11.1** ±9.5
18	**16**	**16.9** ±13.2
20	**17**	**24.9** ±25.1
23	**15**	**24.1** ±16.8
25	**14**	**30.8** ±21.8
30	**6**	**25.7** ±6.2
35	**10**	**22.0** ±15.3
50	**11**	**51.7** ±19.4
80	**2**	**80.3** ±7.6
100	**10**	**100** ±0.0

Abbreviations: WBCs, white blood cells; Infused %, percentage of normal WBCs in the graft infused to recipients; Detected %, percentage of normal WBCs detected in peripheral blood of recipients after transplantation

Appendix VII. Co-Authors Agreement