EX VIVO EXPANSION OF HEMATOPOIETIC STEM CELLS FOR USE IN NONMYELOABLATIVE TRANSPLANTATION

by

SILVIA BAKOVIC

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ABSTRACT

Hematopoietic stem cell transplantation (HSCT) is used to treat a wide range of hematologic and non-hematologic disorders. Recently, interest has grown in the potential of autologous HSCT coupled to gene therapy for the treatment of genetic blood disorders as a way of avoiding the severe immunologic reactions associated with allogeneic HSCT. However, the remaining risks in using myeloablative conditioning regimens to allow relatively small numbers of transplanted HSCs to be transplanted greatly limit the applicability of this approach. Nonmyeloablative regimens would be an appealing alternative but necessitate the generation of large numbers of genetically corrected HSCs to achieve therapeutic levels of chimerism. In this thesis I have explored the potential of forced overexpression of homeobox genes as a strategy to obtain the degree of HSC expansion required. In a first series of experiments, I found that *HOXB4* and *NUPHOX* transduced and expanded HSCs maintain the ability of fresh HSCs to produce sustained, high level, polyclonal, lympho-myeloid chimerism when transplanted into mice given 2-2.5 Gy. I then tested the therapeutic efficacy of *ex vivo* expanded HSCs in nonmyeloablated mice with severe β-thalassemia caused by the homozygous deletion of the β-major globin gene (β-MDD). The results of these experiments showed that this approach could produce a dramatic improvement in the hematocrit, hemoglobin and RBC morphology and ultimately the cure of the thalassemic phenotype that was not achievable in recipients of equivalent numbers of unmanipulated BM cells or of cells transplanted immediately after transduction. Again, the cured mice displayed a sustained, high level of polyclonal chimerism. Together these data provide "proof of principle" of the curative potential of *ex vivo* expanded

HSCs in a preclinical model of β-thalassemia treated with nonmyeloablative conditioning.

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ABBREVIATIONS

5-FU	5-fluorouracil
BG	benzylguanine
BM	bone marrow
β-MDD	β-major double deletion
BMT	bone marrow transplantation
CB	cord blood
CFC	colony-forming cell
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CRU	competitive repopulating unit
DLA	dog leukocyte antigens
DHFR	dihydrofolate reductase
DMEM	Dulbecco's modified Eagles medium
Epo	erythropoietin
FACS	fluorescence-activated cell sorting
FL	flt3-ligand
G-CSF	granulocyte colony-stimulating factor
GFP	green fluorescent protein
GM-CSF	granulocyte/macrophage colony-stimulating factor
GVH	graft-versus-host
GVHD	GVH disease
GVT	graft-versus-tumor
Hb	Hemoglobin
hd	homeodomain
Hox	clustered homeobox gene
HSCs	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
HVG	host-versus-graft
Ig	immunoglobulin
IGF-2	insulin-like growth factor-2

IL	interleukin
IRES	internal ribosomal entry site
MDR1	multi-drug resistance 1
MGMT	methylguanine methyltransferase
mHA	minior histocompatibility antigens
MHC	major histocompatibility complex
MSCV	murine stem cell virus
MTX	methotrexate
MUD	matched unrelated donor
NUP98	nucleoporin-98
PB	peripheral blood
PBSC	peripheral blood stem cells
PE	phycoerythrin
Pep3B	C57Bl6/Ly-Pep3b
PGP	p-glycoprotein
PI	propidium iodide
PT	post transplantation
RBC	red blood cell
Rh-123	rhodamine-123
SCD	sickle cell disease
SCF	stem cell factor (also called Steel Factor)
SCID	severe combined immunodeficiency
SD	standard deviation
TBI	total body irradiation
TFs	transcription factors
TPO	thrombopoietin
UCB	umbilical cord blood
WBC	white blood cells
WT	wild type

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To my parents

CHAPTER 1 INTRODUCTION

1.1 Thesis overview

The work presented in this thesis is directed at the goal of improving the safety and efficacy of hematopoietic stem cell transplantation for treating malignant and non-malignant blood disorders. Currently, the transplantation of bone marrow (BM) or other sources of normal hematopoietic stem cells (HSCs) is the only therapeutic option for achieving cures for many disorders affecting the hematopoietic system. These include both hematopoietic malignancies and inherited genetic blood disorders. The curative potential of hematopoietic stem cell transplants relies on their ability to reestablish a normal functioning hematopoietic system in affected patients. This is accomplished by the proliferation of a very primitive population of multipotent hematopoietic cells present within the BM, the HSCs, which are capable to reconstitute a new HSC population as well as the other derivative components of the hematopoietic system as a whole. Despite the curative potential of HSCT, this modality is associated with significant morbidity and mortality due to the intense patient conditioning regimens used, as well as poorly understood problems of delayed myeloid recovery or immune reactions caused by disparities between the host and the graft. Moreover, in many instances, a source of donor cells with an adequate number of HSCs or appropriately matched to the recipient may not be available. One promising strategy to overcome many of these problems is to develop methods for significant expansion of the number of HSCs prior to transplantation. This would, in principle, enable the use of HSC sources that may be limiting in the number of HSCs they can provide, as for example, might be the case after a specific clinical genetic manipulation strategy or if the use of cord blood HSCs

for treating adult patients were planned. The availability of increased numbers of HSCs for transplant could also make it feasible to use less toxic, nonmyeloablative conditioning regimens if sufficiently large numbers of donor HSCs could be generated to "out-compete" the residual host HSCs, still present in the host. This latter possibility is particularly attractive in the context of transplant based treatments for genetic blood disorders where nonmyeloablative conditioning and transplantation of normal matched donor HSCs or genetically modified patient HSCs (autologous transplantation) could be very effective.

The work described in this thesis was aimed at addressing these challenges in a mouse model. The major goal was to develop and test strategies to expand HSCs *in vitro* for clinical applications that could lead to a safe and useful extension of HSC-based therapies, such as HSCT and gene therapy of genetic blood disorders. The following sections provide a background on key aspects of the biology and regulation of HSCs with particular emphasis on possible approaches to enhance their self-renewal as well as a survey of evolving approaches to improve the safety and broaden the clinical applications of HSC therapies.

1.2 Hematopoiesis and the importance of HSCs

1.2.1 Developmental organization of hematopoiesis

Hematopoiesis is the process by which mature blood cells are produced from immature multipotent stem cells. This developmental process is called monophyletic, which means that a single stem cell, the HSC, can give rise to all the mature blood cell types in the body. The monophyletic theory of hematopoiesis states that multipotent

HSCs multiply to produce more multipotent HSCs, thus ensuring that their numbers are maintained at steady and adequate numbers throughout life. Upon cell division, HSCs can self-renew and maintain their multipotency by symmetric cell division or undergo asymmetric cell division. In the first case, both daughter cells are essentially functionally equivalent to the cell that generated them. In the latter case, one cell, remains multipotent and the other one is fated to terminally differentiate. Alternatively, both daughter cells lose multipotency and begin to differentiate.

The generation of multiple hematopoietic precursors and lineages occurs through a series of steps that are referred to as intermediate progenitors. Current evidence supports a "roadmap" of hematopoietic lineage development as schematically depicted in Figure 1.1. Major bifurcations include the choice between lymphoid versus myeloid development and subsequent restrictions to major lineages. Example of these early developmental stages are the common lymphoid progenitors (CLPs), which can generate only B, T and NK cells, and common myeloid progenitors (CMPs), which can generate only red cells, platelets, granulocytes and monocytes (Akashi et al., 2000; Kondo et al., 1997). However, recent evidence suggests that the HSC compartment is more heterogeneous than previously thought and may be composed of distinct subsets of cells that are multipotent but have different pre-determined propensities to differentiate into various types of myeloid versus lymphoid cells (Adolfsson et al., 2005) Dykstra et al. submitted). The production of terminally differentiated cells that cannot proliferate anymore and die after a period of time that ranges from hours (for neutrophils) to decades (for some lymphocytes) (Smith, 2003) marks the end of this developmental process.

The proliferative capacity of HSCs is such that a relatively small number of these cells can produce billions of new cells daily to replace hematopoietic cells lost to normal

cell turnover processes as well as to illness or trauma. HSCs can be transplantable, proliferate to regenerate multilineage hematopoiesis, and can robustly sustain the hematopoietic system (Smith and Storms, 2000). In the mouse, a single HSC can reconstitute the entire hematopoietic system for the natural lifespan of the animal (Osawa et al., 1996)

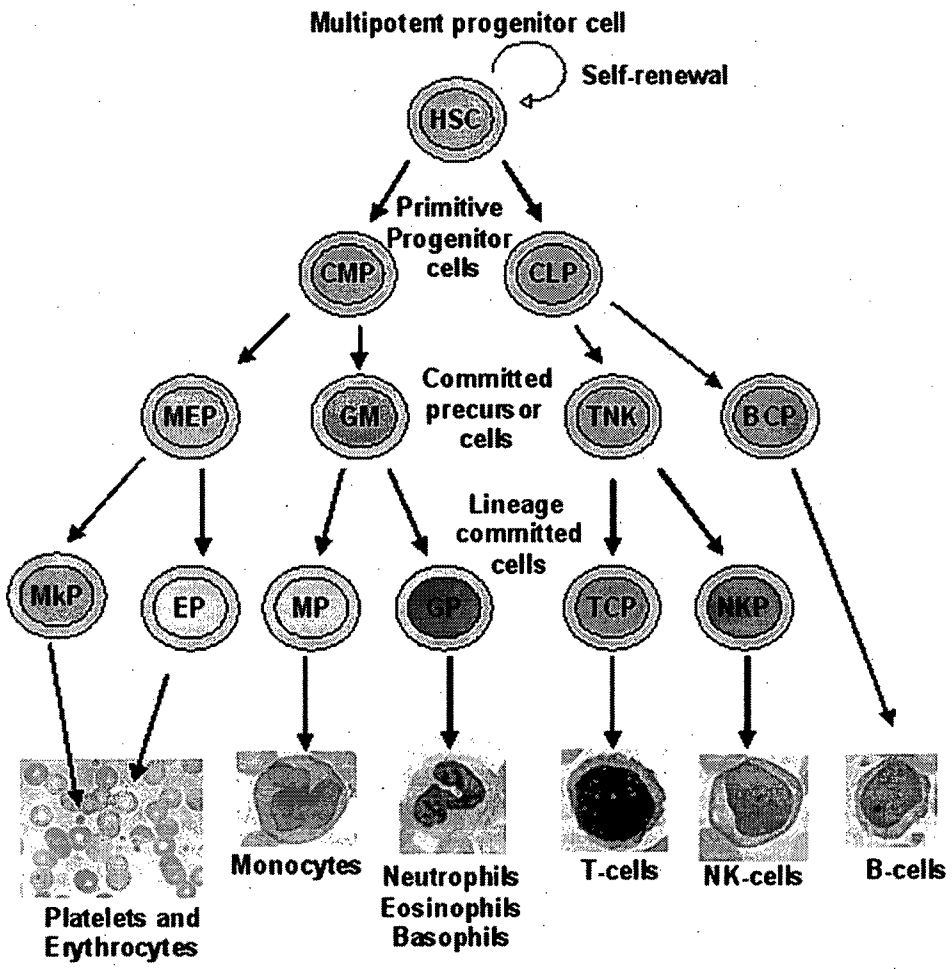


Figure 1.1 Schematic representation of hematopoietic cell development. Adapted from (Kaushansky, 2006). CLP, common lymphoid progenitor; CMP common myeloid progenitor; MEP, megakaryocyte/erythrocyte precursor; GM, granulocyte/macrophage precursor; TNK, T cells and natural killer cells precursor; BCP, B-cell precursor; Mk, megakaryocytes.

Growth and development of HSCs into terminally differentiated cells is governed by a complex interplay between HSCs and progenitors and various environmental

factors. These include cytokines, chemokines, extracellular matrix components, other molecules presented on the surface of hematopoietic and non hematopoietic cells.

1.2.2 Proof of the existence of HSCs

The drive to understand the foundation of the hematopoietic system came in response to the clinical need for cells capable of rescuing the hematopoietic system of individuals suffering from radiation accidents. These studies began more than 50 years ago with the observation by Jacobson *et al.* (Jacobson et al., 1949) that mice receiving lethal doses of radiation were able to survive when they had their spleen shielded which was followed by the report by Lorenz *et al.* (Lorenz et al., 1951) that lethally irradiated mice could also be protected by an infusion of spleen or marrow cells. Subsequently it was shown by Ford *et al.* (Ford et al., 1956) that lethally irradiated mice protected by infused marrow had their hematopoietic tissue colonized by cytogenetically distinguishable cells derived from the donor.

In 1961, Till and McCulloch described the formation of colonies in the spleen of lethally irradiated animals 10 days after they had been infused with a relatively small number of marrow cells (Till and Mc Culloch, 1961). The cells from which these colonies arose were called colony-forming units spleen (CFU-S). Because these CFU-S were shown to possess extensive proliferation capacity and be capable of multilineage differentiation and self-renewal, they were thought to be HSCs (Till et al., 1964). To test the long-term performance of these cells, Hodgson *et al.* tested the regeneration of CFU-S in serial transplants (Hodgson and Bradley, 1979). From these studies, they inferred that CFU-S might only be capable of short-term repopulation and that there existed a more primitive population of cells to account for the long term

repopulating function (Ploemacher and Brons, 1989). In addition, although CFU-S colonies often contain multiple cell types, the presence of lymphoid cells could not be reproducibly demonstrated, leading to the hypothesis that they represent a cell with myeloid-resticted differentiation potential (Schofield, 1978).

Cell separation techniques were instrumental in leading to the discovery of a fraction of BM cells that was highly depleted of CFU-S but still capable of sustaining long-term hematopoiesis *in vivo* (Jones et al., 1989; Jones et al., 1990). Further studies using physically separated cell types confirmed that CFU-S have limited short-term repopulating capacity and are distinct from more primitive cells capable of multilineage repopulation for extended (> 2 months) period of times (Morrison and Weissman, 1994; Szilvassy et al., 1989; van der Loo et al., 1994)

Definitive evidence of the existence of multipotent HSCs with lifelong lympho-myeloid repopulating activity came from clonal tracking studies. These were initially based on the use of radiation-induced chromosomal markers and later random genomic integration sites of retroviral vectors. This allowed unique marking of individual HSCs with a genetic signature and visualization of the progeny derived from them. Furthermore, it allowed defining the relationship of various cells in the hematopoietic hierarchy (Dick et al., 1985). Proviral marking studies also proved powerful to distinguish cells with different repopulating potential, detecting those capable of completely repopulating all lineages in the hematopoietic system and those capable of repopulating only certain lineages (Lemischka et al., 1986). More recently, the features of HSCs have been provided by tracking the progeny generated in recipients of highly purified single cells. These studies definitely showed that a single cell was capable of substantial reconsitution of all elements of the hematopoietic system of lethally irradiated mice for extensive periods of time (Osawa et al., 1996).

1.2.3 Phenotypic and functional characterization of HSCs

HSCs from multiple sources can be identified and quantified by specific functional assays that detect their long term repopulating ability *in vivo* (Szilvassy et al., 2002). They can also be phenotypically distinguished from other cell types by a unique profile of expression of specific cell surface molecules and dye exclusion properties, although most of these are not stable with HSC activation status (Eaves, 2002).

In mice there are two main assays used to detect and quantify HSC activity. The first was developed by Harrison (Harrison, 1980) and it measures the competitive repopulating activity of a test population by competing it against an equal number of cells from a genetically distinct (but immunologically compatible) population containing a large number of HSCs (to minimize the variance). The test population is then shown to be similar, more competitive or less competitive than the control population based on the proportional contribution to the RBCs and/or WBCs several months later. These values can also be used to derive quantitative differences in HSC frequencies if it is assumed that the average repopulating activity of each HSC in the test population is the same as in the control population (Harrison et al., 1993). This can prove a possible limitation where this assumption can be called into question (e.g. when using genetically modified cells).

A second widely used assay for quantitating HSCs is based on use of limit dilution principles in combination with competitive repopulation. The limit dilution assay initially described by Szilvassy *et al.* (Szilvassy et al., 1990), was performed by transplanting limiting numbers of male "test" cells of unknown HSC content into lethally irradiated syngeneic female recipients together with a radioprotective dose of compromised competitor cells ($1\text{-}2X10^5$). Hematopoietic tissues of recipient mice were

analyzed by Southern blot 5 or more weeks post transplantation and had to contain ≥5% cells of male origin in order to be considered reconstituted by a primitive cell. The dilution dose of the test cells that was able to give long-term lymphomyeloid repopulation in this competitive setting in 63% of the transplanted mice was assumed on the basis of Poisson statistics to contain at least one so called competitive repopulating unit (CRU). In recent years congenic mice that differ at the Ly5 locus have been introduced to distinguish between donor and competitor cells and this has allowed for the more sensitive and ready detection of donor cells in various tissue suspensions (BM, Thymus, spleen and PB) by staining with antibodies conjugated with fluorescent markers and analysis by flow cytometry. With this method of detection, the current criteria for establishing the repopulation from a CRU requires that ≥1% of donor derived cells are detected in recipients in both myeloid and lymphoid lineages at 16 or more weeks post-transplant. Identical proviral integrations found in lymphoid and myeloid lineages in mice transplanted at limit dilution, confirmed that these cells were derived from the same original HSC. Another version of this assay makes use of sublethally irradiated W^{41}/W^{41} congenic recipients. These mice are compromised in their HSC content due to a mutation in the c-Kit receptor gene. The sublethal radiation spares enough endogenous cells to allow the recipients to survive in case the test sample does not contain any long-term repopulating cells (Antonchuk et al., 2002; Miller and Eaves, 1997).

In normal adult mouse BM, murine HSCs are small cells with minimal cytoplasm. They also express high levels of the multidrug resistance proteins (PGP and BCRP1/ABCG2) (Jones et al., 1996; Uchida et al., 2004). These cells tend not to express surface markers seen on mature hematopoietic cells (lineage markers) but can

express low levels of the Thy-1 surface protein and relatively high levels of the Sca-1 surface marker (Spangrude et al., 1995; Weissman, 2000). When single cells from the $Thy1^{low}$ $Sca1^{+}$ Lin- population were injected intravenously, 1 out of 20 engrafted but the majority of clones gave rise to transient reconstitution and only a quarter gave rise to long-term repopulation (Morrison et al., 1995b). Furthermore, the $Thy1^{low}$ $Sca1^{+}$ Lin- population was divided according to the retention of the fluorescent dye Rhodamine 123 (Rh-123) and it was shown that only the $Rh\text{-}123^{low}$ subset of the $Thy1^{low}$ $Sca1^{+}$ Lin- population was multipotential (Spangrude and Johnson, 1990), with a minimum of 1 in 40 $Sca1^{+}$ Lin- $Rh\text{-}123^{low}$ cells or 5 $Thy1^{low}$ $Sca1^{+}$ Lin- $Rh\text{-}123^{low}$ capable to engraft lethally irradiated recipients for up to 19 weeks and 24 weeks, respectively (Li and Johnson, 1992; Spangrude et al., 1995). More recently Hoechst 33342 dye was used to purify HSCs and stem cell activity was demonstrated in the population of cells that has the highest efflux capacity of this dye (Goodell et al., 1996). This subset of cells in conjunction with $Rh\text{-}123^{low}$ Lin- cells was determined to be highly enriched for HSCs, with a HSC frequency of 1 in 2.5-3 cells (Uchida et al., 2003).

The two most recent strategies make use of SLAM (Signaling Lymphocyte Activation Molecule) (Kiel et al., 2005) and of EPCR (Endothelial Protein C Receptor) (Balazs et al., 2006) expression to purify HSCs. SLAM proteins such as CD150, CD48 and CD244 are adhesion and signaling receptors that are not essential for HSC function but are now proving to be very powerful for distinquishing a diversity of primitive hematopoietic cells (Wagers, 2005). Almost half of the cells (45%) that express CD150 but do not express CD48 and the megakaryocytic marker CD41 are capable of long-term multilineage reconstitution of lethally irradiated recipients (Kiel et al., 2005). While a number of purificiation strategies have been effective for identifying adult HSCs, unfortunately many of the markers have proven not to be stable in actively

cycling (Zhang and Lodish, 2005) and/or in HSCs at other stages of ontogeny (fetal HSCs) (Morrison et al., 1995a; Rebel et al., 1996) Interestingly, SLAM markers have been recently demonstrated to be stable and the SLAM purification strategy is therefore the only strategy that is so far consistent in the identification of HSCs and progenitors in adult BM and fetal liver (Kim et al., 2006).

There have also been major advances in enrichment and phenotyping of human HSCs with the development of powerful transplantation methods based on use of immune compromised mice and fetal sheep (McCune, 1996; Zanjani et al., 1994). Some understanding of human HSCs have also come from the transplantation of purified populations of autologous cells back into large animals, such as non-human primates (Berenson et al., 1988). Phenotypically human HSCs and progenitors have been reported to be small quiescent cells that express high levels of the surface glycoprotein CD34 and do not express the differentiation marker CD38 (Bhatia et al., 1997b). In addition, they do not express lineage commitment markers and express low levels of Thy-1 (Smith, 2003) and can be purified in fetal human BM (Baum et al., 1992), adult BM (Murray et al., 1994) and mobilized peripheral blood (Murray et al., 1995) as CD34+ Thy-1+ Lin- population. Recent studies indicate that some HSCs and progenitors do not express CD34 (Bhatia et al., 1998). As found for murine HSCs, human HSCs are enriched in a population of cells that have low retention of the dye Rh-123 (McKenzie et al., 2007). As for murine HSCs, these phenotypic properties of human HSCs are not stable and can be modified by cell cycle progression (Dorrell et al., 2000), *ex vivo* culture (Danet et al., 2001) and transplantation (Dao et al., 2003). Human HSCs are also known to differ from more mature types of hematopoietic cells in their high expression of aldehyde dehydrogenase (ALDH) which allows their selective

isolation by treatment with cyclophosphamide derivatives (Hess et al., 2004; Udomsakdi et al., 1992).

1.2.4 Different sources of HSC

HSCs are found during embryogenesis in different anatomical sites such as the yolk sac, the aorta-gonad mesonephros, the placenta and the fetal liver. After birth HSCs colonize the BM (Mikkola and Orkin, 2006) and throughout adulthood they are primarily found in the BM but can also be located in extramedullary sites such as the liver and the spleen (Taniguchi et al., 1996). HSCs are also present in other tissues including PB (Wright et al., 2001), umbilical cord blood (UCB) (Holyoake et al., 1999) and placenta. In clinical practice today, three sources of cells are used for clinical HSCTs: BM harvests, growth factor-mobilized PB collections and UCB cells.

BM for transplantation is usually obtained from the donor's anterior and posterior iliac crests with the donor under spinal or general anesthesia. Over the past decade, primitive HSC mobilized into the peripheral blood have gradually replaced BM harvesting from the iliac crest as the preferred HSC source for autologous and allogeneic transplantation. Obtaining large numbers of primitive cells from mobilized PB is easier, less invasive and it allows more rapid neutrophil, platelet recovery and faster immune reconstitution (Appelbaum, 2003; Arai and Klingemann, 2003).

Cord blood, which is the blood that remains in the umbilical cord and placenta following birth, offers substantial logistic and clinical advantages, such as the relative ease of procurement and availability, the absence of risk for mothers and donors, the reduced likelihood of transmitting infections, potential reduced risk of graft versus host disease (GVHD) and less stringent criteria for donor-recipient matching and selection

(with the potential of finding donors for minority populations) (Rocha and Gluckman, 2006). Due to the limited number of HSCs that can be collected, UCB transplantation is difficult to use for treating adult patients and it has generally been limited to pediatric patients. It is likely that expanding UCB HSCs will lead to more rapid engraftment and less transplant-related complications even with highly mismatched donors. Indeed the promise of UCB but limitations in their number is a major impetus for the development of HSC expansion strategies such as described in subsequent sections.

1.3 HSCT

HSCs play a critical role in the outcome of HSCT, now increasingly used as a powerful treatment option in a range of life-threatening blood, immune system or genetic disorders. Transplantation of BM cells in humans was pioneered in the 1960's by E. Donnall Thomas whose work showed that BM cells infused intravenously could repopulate the BM of irradiated patients affected by malignant blood disorders and rescue the hematopoietic system (Thomas, 1999a; Thomas, 1999b; Thomas et al., 1959; Thomas et al., 1957). This was later explained by the presence in the infused BM of a population of human HSCs, capable of giving rise to all the differentiated cells of the hematopoietic system. Currently, around 45,000 patients each year are treated by HSCT, a number that has been increasing in the past decade. Although most of these cases have involved patients with hematological malignancies – such as lymphoma, myeloma and leukemia – there is growing interest in using HSCT to treat solid tumours and non-malignant diseases such as β-thalassemia and Sickle Cell anemia (Sorrentino, 2004).

1.3.1 HSCT and Gene therapy

There are three types of HSCT: In autologous transplants, the infused cells are taken from the patient. In syngeneic transplants, patients receive cells from an identical twin. In allogeneic transplants, the cells are from a different individual, usually an HLA-matched brother or sister, or haploidentical parent (related donors). However, increasingly, cells from matched but unrelated donors (MUDs) are being used. When allogeneic transplants are performed, the recipient requires immunosuppressive medications to minimize severe immunologic reactions between the donor and the recipient. Allogeneic BMT is often the only option for patients affected by inherited blood disorders but because of the associated morbidity, it is only performed in extreme cases. To overcome this problem, gene therapy strategies to treat and cure these disorders by correcting the patients' own cells are a very attractive option and have been intensively pursued for the last 15 years (Anderson, 1992; Mulligan, 1993). Since many of these disorders are monogenic and the mutation in the gene has been characterized, the approach is to remove HSCs from the affected individual, transfer the wild type functional gene in to the HSCs *ex vivo* with a vector and subsequently reintroduce the modified cells back into the patient (Figure 1.2).

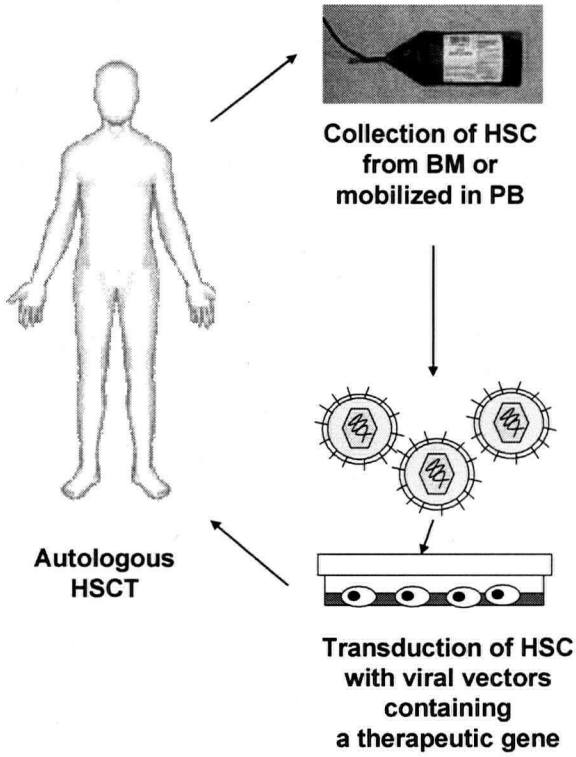


Figure 1.2 Schematic of gene-therapy protocols in humans. Harvested HSCs are cultured with fibronectin, cytokines and retroviral supernatant for one to four days. Autologously transplanted cells migrate to the marrow and initiate hematopoiesis with corrected HSCs.

Conventional allogeneic and autologous HSCT are associated with serious morbidity and even mortality due to the toxic conditioning regimens; in the case of allogeneic transplants there is also a risk of GVHD; and with autologous HSCT coupled to gene therapy there are significant problems associated with very poor recovery of corrected HSCs. Two related approaches to address and solve these issues are the use of sublethal conditioning and strategies to achieve HSC expansion.

1.3.2 Movement towards nonmyeloablative conditioning

In conventional HSCT, the hematopoietic system of the host is totally eliminated by intensive cytotoxic therapy and subsequently replaced by the infusion of donor hematopoietic cells that lead to the reconstitution of the whole hematopoietic system. The pre-transplantation conditioning is aimed at eliminating malignant cells and, in the

case of allograft, host immune cells that mediate rejection. However, in congenital blood disorders there is no need to eradicate a malignant population of cells from the BM and a minimal PB chimerism would be sufficient to ameliorate the disease phenotype.

Conventional myeloablative allogeneic BMT has relied upon administration of supralethal doses of total body irradiation (TBI) and/or cytotoxic chemotherapeutic agents. Given their intensity, myeloablative conditioning regimens have been associated with significant toxicity, which has limited their use to otherwise healthy, relatively young patients. To extend the use of allogeneic HSCT to include older patients and those with comorbid conditions, reduced intensity or truly nonmyeloablative conditioning regimens have been introduced. Nonmyeloablative regimens have been based on minimal TBI alone (2 Gy) or minimal TBI combined with different doses of Fludarabine and Busulfan. Oral administration of Busulfan has been associated with unpredictable absorption and relatively large inter- and intrapatient variability. Recently an intravenous formulation of busulfan has become available and has been shown to provide greater predictability in blood levels. However, this approach requires further dose escalation studies to determine the acceptable level of toxicity and allow sufficient engraftment of genetically modified cells (Kahl et al., 2006; Kang et al., 2006). In general, nonmyeloablative regimens have had few toxicities, have produced only mild myelosuppression, and have been associated with a low incidence of mortality, even in elderly patients and those with comorbid conditions (Baron and Storb, 2006).

These reduced intensity regimens minimize morbidity and mortality associated with HSCT-based therapies. From the outcome of growing clinical trials for hematologic malignancies treated with reduced conditioning regimens, it has emerged that they can

produce mixed to full donor chimerism. Mixed chimerism would be highly suitable for the treatment of genetic blood disorders where a minimal PB chimerism would ameliorate the disease phenotype. However, it also emerged that when nonmyeloablative conditioning regimens are used, significant chimerism and hence better outcome is achieved only when large transplant doses are used. For this reason, strategies to enrich for HSCs would have a major impact on the success of nonmyeloablative HSCT.

1.3.3 Nonmyeloablative BMT: lessons from studies of mice

Initially it was thought that intravenously injected HSCs could reconstitute the hematopoietic system only if a suitable "niche" was made available in the BM cavity (Schofield, 1978) through myeloablative treatments such as TBI. Once the space was created, donor cells could home there, start proliferating and reconstitute the hematopoietic system of the host. This concept, however, was challenged by reports of the successful transplantation of marrow into submyeloablated or even nonmyeloablated murine hosts where supposedly part or all of these niches were already occupied by endogenous HSCs.

The first report which suggested that engraftment in the absence of myeloablation was possible came from Micklem in 1968 (Micklem et al., 1968). He showed that reconstitution of up to 8.5% of the hematopoietic system with donor cells could be obtained in normal unconditioned recipients transplanted with $2X10^7$ cells three months post transplant. A similar phenomenon was reported by Brecher in 1982 when his group showed that 16-25% donor-derived hematopoiesis could be achieved in normal unconditioned recipients of $4X10^7$ BM cells per day for 5 consecutive days,

indicating that "niche availability" was not a limitiation to eliciting the repopulating activity of injected HSCs. (Brecher et al., 1982). Similar levels of repopulation (between 0 and16%) were also achieved by Saxe who transplanted 10^6-10^8 cells per mouse (Saxe et al., 1984). In recent years, Stewart *et al.* showed persistent donor-derived hematopoiesis in unconditioned recipients for up to 2 years after the transplantation of $4X10^7$ cells on each of 5 consecutive days (Stewart et al., 1993).

The amount of cells transplanted in these studies is massive, reaching the equivalent of almost half of the BM of a mouse. It is important to note that in myeloablated mice the same dose of cells and even a minimal dose of 10^4-10^5 cells would produce 60-100% donor PB chimerism. However in nonmyeloablated recipients, transplanted cells have to compete with the endogenous surviving BM cells for appropriate conditions of stimulation (Figure 1.3).

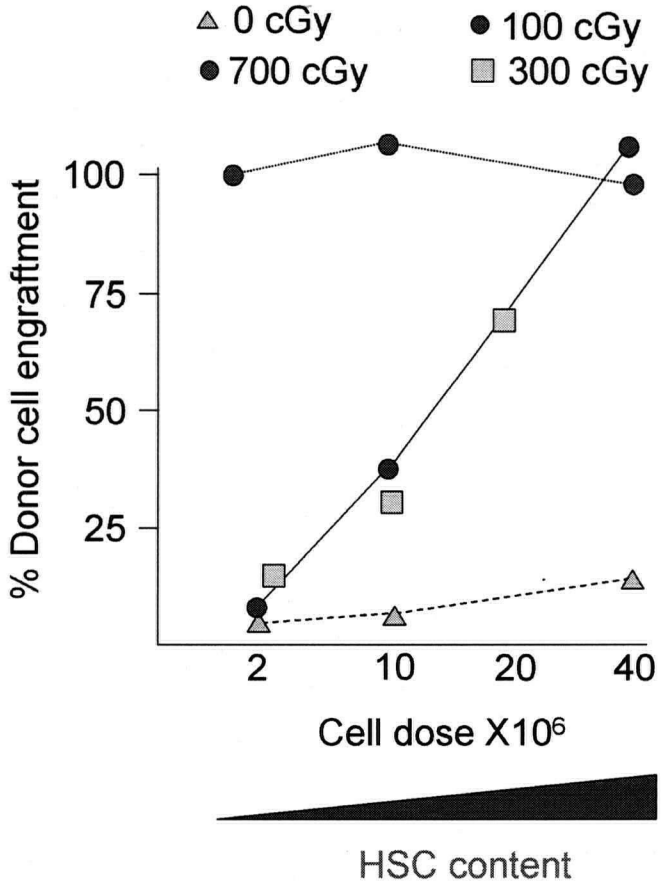


Figure 1.3 Cell dose and engraftment in minimally irradiated recipients. Marrow cells in doses of 2, 2.5, 10, 20 or 40 million cells were infused into recipients treated with 0, 1, 3 and 7 Gy, and the percentage of engraftment was determined 2 months and 6 months post transplantation. The results show that donor cell readout in hosts after transplant is related to cell dose and irradiation dose to recipient animals. Modified from (Stewart et al., 1998) and (Goebel et al., 2002).

What really determines the final chimerism in minimally ablated or nonmyeloablated mice is still a matter of debate. Recently it was shown by using parabiotic mice that HSCs can be found at constant rates in the PB at any given time (Wright et al., 2001), implying that there is a constant exodus of HSCs from the BM or extramedullary sites into the PB circulation (and *vice versa*). This finding was complemented by the demonstration that in unconditioned recipients 0.1% to 1% of HSCs exit or enter the circulation at any given time point. This finding was interpreted as indicating that this is the number of niches available for engraftment in a normal adult mouse which thus accounted for the ability of a very large number of injected

HSCs to eventually compete for blood cell output (Bhattacharya et al., 2006). In support of this hypothesis was the observation that transplanting the cells repetitively over a period of time produced higher levels of contribution than transplanting a single high dose of cells. Moreover, it turns out that in nonmyeloablated recipients the level of chimerism achieved is mathematically predicted by the ratio of the number of cells (and hence HSCs) in the host relative to the number of cells (and hence HSCs) transplanted. Thus the transplantation of $4X10^7$ cells into normal adult mice that have a BM cellularity of roughly $5.3X10^8$ cells lead to donor 7% chimerism (Quesenberry et al., 2005). Based on these studies, it appears that it is the ratio of host to donor HSCs rather than availability of niches that is critical (Colvin et al., 2004; Rao et al., 1997; Stewart et al., 1998). If this model is correct, then a treatment of 1 Gy that kills more than 85% of HSCs (Stewart et al., 2001) should increase the donor-to-host HSC ratio of a set graft and thus lead to a dramatic increase in final donor chimerism. In fact, transplantation of $4X10^7$ cells in mice that received 1 Gy as their conditioning regimen resulted in the predicted 60 to 80% chimerism.

Additional issues come to light in considering non-ablative transplants for gene therapy. In gene therapy protocols cells are usually prestimulated and then kept in cytokine cocktails to enhance viral-mediated gene insertion. This cytokine treatment leads to better rates of genetic transduction but also triggers cell cycle progression. This has been shown to be detrimental to HSC behavior because, after entering the cell cycle, these cells show loss of repopulating potential in both myeloablated and nonmyeloablated mice (Kittler et al., 1997; Peters et al., 1996). This engraftment defect is also reported for cells treated with 5-fluorouracil (5-FU), which is a drug widely used to allow for the transduction of mice, primates and human HSC. HSCs are usually stimulated into cell cycle by the intravenous injection of the drug 5-FU. This treatment

results in HSC function impairment and in defective long-term repopulating capability in nonmyeloablated hosts (Ramshaw et al., 1995). Recently Goebel *et al.* compared the reconstituting capability of fresh BM, 5-FU treated BM and 5-FU treated BM transduced with retroviral vectors in minimally conditioned mice. This study showed that the competitive repopulating activity for marrow treated with a retroviral-mediated gene transfer protocol that included 5-FU treatment was similar to 5-FU-treated BM but subsequent *ex vivo* culture resulted in decreased overall activity in 1.6 Gy-irradiated hosts as compared to transplants of fresh BM, due to the decreased HSC content found at the end of the *in vitro* culture (Goebel et al., 2002).

1.3.4 β-thalassemia and Sickle cell disease in humans

Hemoglobins are the major oxygen-carrying molecules of the body. They are packaged into RBCs in quantities sufficient to carry enough oxygen from the lungs to the tissues to meet the needs of cells for oxidative metabolism. These quantities are enormous, nearly two pounds of hemoglobin is present in the body of a reasonably sized man at any given time. Defective synthesis of the β chains of adult hemoglobin A leads to an imbalance in chain production with the accumulation of free α chains in RBC precursors and RBCs. This accumulation causes intramedullary destruction of RBC precursors and markedly ineffective erythropoiesis that results in severe hemolytic anemia.

1.3.5 β-thalassemia

The β-thalassemias and Sickle cell disease (SCD) are severe congenital anemias that result from a mutation that causes the deficient or altered synthesis of the β chain of hemoglobin. Both are commonly inherited in an autosomal recessive manner and according to the World Health Organization, approximately 180 million people are heterozygous for one of the several forms of genetic disorders of hemoglobin synthesis (Lucarelli and Clift, 2004)

β-thalassemia results from a mutation of the β-globin gene that reduces (β^+-thalassemia) or eliminates (β^0-thalassemia) β-globin chain synthesis and hence compromises hemoglobin production. Nearly 200 different mutations have been described in patients with β-thalassemia and related disorders. The majority are point mutations in the β-globin gene and occasionally deletions. β-thalassemia is the most common monogenic disorder worldwide, with an estimated 365,000 affected infants born each year.

The clinical β-thalassemia phenotypes are subdivided into three broad categories. One extreme is β-thalassemia major (also known as Cooley's anemia), where individuals have severe anemia and are dependent on RBC transfusions for survival. The opposite extreme is β-thalassemia minor, where individuals are asymptomatic. Any phenotype that falls in between is classified as β-thalassemia intermedia (Urbinati et al., 2006). Infants with thalassemia major who receive no treatment die in early infancy from congestive heart failure or other complications of severe anemia.

1.3.6 Sickle cell disease

In SCD, a single nucleotide substitution in the β-globin gene results in the substitution of valine for glutamic acid on the surface of the variant β-globin chain. The resulting hemoglobin polymerizes in the RBCs when deoxygenated and the RBCs assume the shape of a sickle, become dehydrated and rigid, and adhere to the vascular endothelium. This polymerization of SCD RBCs causes accelerated RBC destruction, erythroid hyperplasia and vaso-occlusion. Vaso-occlusion leads to the damage of many organs, eventually causing long-term disabilities (Sadelain, 2006). The carrier states of the thalassemia syndromes and SCD are protective against malarial infections and thus the highest prevalence of these diseases is seen in the Mediterranean areas, Asia, the Middle East and Africa (Urbinati et al., 2006).

1.3.7 Conventional management of β-thalassemia and SCD

For both β-thalassemia and SCD, transfusion therapy is life-saving and aims to correct the anemia, suppress the massive ineffective erythropoiesis, and inhibit increased gastrointestinal absorption of iron. However, transfusion therapy leads to iron overload, which is lethal if untreated. The prevention and treatment of iron overload are the major goals of current patient management.

Currently the only available curative treatment for these hemoglobinopathies is allogeneic HSCT. Over 2000 patients have received HSCT for β-thalassemia major worldwide and outcome has proven to be highly dependent on the health status of the recipient at the time of transplantation. In relatively healthy recipients, the probability of cure and survival was 87%, but in patients with more advanced disease, the mortality rate was as high as 47% (Gaziev and Lucarelli, 2005). These figures appeared to be

even worse when matched unrelated donor BM was used. Due to the high morbidity and mortality associated with highly toxic myeloablative regimens, HSCT has been so far a suitable option for only a small proportion of affected patients.

Fewer patients with SCD (~200) have been treated with HSCT (Gaziev and Lucarelli, 2005). The mortality rate after the procedure has been reported to be 8-10% and the significant toxicity of myeloablative BMT has restricted its application to patients who have already experienced severe and irreversible complications. However, one report of the outcome of HSCT of asymptomatic patients has suggested that better rates of overall cure and survival (93%) may be obtainable (Vermylen et al., 1998). Late complications such as growth disturbances, endocrine complications and infertility are issues to ponder when considering conventional HSCT for the treatment of hemoglobinopathies.

Nonmyeloablative HSCT is a safer option due to the minimal toxicity of preparative regimens and is being considered for the treatment of a wider variety of patients, although the possibility of inadequate chimerism leading to lack of cures and recurrence of sickle cell crises remains a concern.

1.3.8 Genetic treatment of β-thalassemia

Allogeneic BMT is curative but not devoid of complications. Safe transplantation requires the identification of histocompatible donors to minimize the risks of graft rejection and GVHD. In the absence of a suitable donor, the genetic correction of autologous HSCs represents a highly attractive alternative to achieve a cure. This approach at once resolves the search for a donor and eliminates the risks of GVHD and graft rejection associated with allogeneic HSCT. In order to successfully treat β-

thalassemia using gene therapy, it is necessary to permanently transfer one or more copies of the β-globin gene into HSCs, with appropriate timing and levels of expression of the transduced gene exclusively in the RBC precursors they generate. Achieving therapeutic β-globin expression has represented a tremendous obstacle for over a decade, but recent studies indicate that therapeutic levels of hemoglobin synthesis can be achieved in the progeny of virally transduced HSCs under myeloablative conditioning (Sadelain, 2006). With a lentiviral vector carrying extended sequences of the locus control region and a β-globin gene, May *et al.* demonstrated the production of the transduced β-globin gene in 17 to 24% of the total hemoglobin tetramers in red cells derived from transduced HSCs. This level of β-globin production was sufficient to ameliorate the anemia and the red cell morphology of mice affected by β-thalassemia that were transplanted with β-globin transduced HSCs (May et al., 2000). Similarly, in a transgenic mouse model of SCD, Pawliuk *et al.* demonstrated, after lentiviral transduction of HSCs, the production of a β-globin variant that prevents sickling in 99% of circulating red blood cells, contributing to up to 52% of the total hemoglobin tetramers. This high level of β-globin production led to subsequent prompt correction of the hematological parameters of the SCD affected mice (Pawliuk et al., 2001). Additionally, Imren *et al.* demonstrated, with an optimized lentiviral vector, the transduction of virtually all of the HSCs in the graft. This led to the sustained contribution of the transduced β-globin to ~32% of total β-globin chains in ~95% of the red blood cells with subsequent correction of the thalassemic phenotype (Imren et al., 2002). Since in myeloablative conditions, a therapeutic effect can be achieved even if only a minority of HSCs are genetically modified, the remaining challenge is to achieve

high-level and permanent hematopoietic reconstitution by genetically modified cells when a nonmyeloablative less toxic regimen is used.

1.3.9 A murine model of β-thalassemia major

This model was described by Skow *et al.* in 1983 and is characterized at the molecular level by a spontaneous 3.7Kb deletion encompassing the entire β^{major} locus including 5' and 3' sequences (Goldberg et al., 1986; Skow et al., 1983). β-globin genes in mice are encoded by a multigene cluster located on chromosome 7. There are four functional genes in the mouse β-globin gene cluster: *bh1*, an early embryonic globin gene expressed primarily in yolk sac-derived cells from 9.5 to 12.5 days of gestation; *y*, a late embryonic globin gene expressed primarily in fetal liver-derived cells from 11.5 to 16.5 days of gestation; *b1* (β^{major}) and *b2* (β^{minor}), adult globin genes first expressed at 9.5 days of gestation in yolk sac-derived cells, then expressed in RBCs produced in the fetal liver and spleen and thereafter in the BM. In the mouse, the β^{major} gene is responsible for 80% of β-globin in adult RBCs and β^{minor} for the remaining 20% (Shehee et al., 1993).

In the model of β-thalassemia described by Skow, most mice homozygous for the β^{major} gene deletion survive to adulthood and reproduce but they are smaller at birth than their littermates and exhibit a hypochromic (reduced RBC hemoglobin), microcytic (reduced RBC size) anemia with severe anisocytosis (irregular size), poikilocytosis (irregular shape), reticulocytosis and inclusion bodies in a high proportion of circulating RBCs. All of these features are comparable to those seen in the human β-thalassemias and make this an attractive model for studies of curative strategies (Imren et al., 2002).

1.4 Approaches to achieving HSC selection and expansion

Retroviral transduction protocols are widely used for gene therapy applications but major drawbacks of this promising approach are the rapid loss of HSCs *in vitro* and the recovery of minimally corrected HSCs at the end of the manipulation. Therefore, in this field there is a compelling need to find ways to preserve or increase HSC function during and after their *ex vivo* manipulation to allow the application of such treatments to be usefully extended to patients given a safer, nonmyeloablative preparative regimen. In order to accomplish this, much effort has been aimed at elucidating the mechanisms involved in the regulation of HSC self-renewal in order to be able to manipulate the fate of these cells *in vitro* with preservation of their subsequent *in vivo* functional activity. These include various strategies to enable the transplanted HSCs to outcompete the large numbers of surviving endogenous HSCs in nonmyeloablated recipients by expansion of the transduced HSCs before transplantation or by enabling their selective growth after transplantation, as summarized below.

HSCs are able to perpetuate themselves through self-renewal divisions. Understanding the mechanisms behind the decision of a HSC to self-renew or differentiate might allow for the manipulation of these pathways to preserve the stemness of HSCs after cell division and ultimately for HSC expansion.

The fate of HSCs appears to be the consequence of a complex and still poorly understood interplay between intrinsic and extrinsic regulators. These regulators influence the behavior of HSCs by ultimately triggering processes like proliferation, survival, apoptosis, self-renewal or differentiation. All of these processes are executed and regulated by a complex network of gene products that interact both intrinsically within the HSCs and between the HSCs and various cytokines, extracellular matrix

components and molecules released by or expressed by surrounding cells in their environment. In the next paragraphs, some of the extrinsic and intrinsic regulators that have been identified so far, will be described. In addition, strategies to expand or selectively promote the self-renewal and therefore expansion of genetically modified HSCs for gene therapy purposes will also be summarized.

1.4.1 HSCs *in vivo* selection strategies

Approaches to achieve the *in vivo* selection of transduced HSCs expressing a therapeutic gene rely on the selective elimination of untransduced cells by specific drugs and subsequent selective survival and growth of transduced cells that co-express a therapeutic gene and a drug-resistance gene. A number of genes encoding drug-resistance molecules are currently under investigation; such as the human dehydrofolate reductase (*DHFR*), multi-drug resistance 1 (*MDR1/ABCB1*), and methylguanine metyltransferase (MGMT) drug-resistance genes.

DHFR was first tested for its ability to protect BM cells *in vivo*. HSCs transduced with *DHFR* variants become resistant to antifolate agents, such as methotrexate and trimetrexate (Li et al., 1994; Spencer et al., 1996), and can be positively selected both *in vitro* and *in vivo*. DHFR naturally functions to generate tetrahydrofolate, which is necessary for the *de novo* synthesis of pyrimidines and purines. Antifolate drugs act by binding tightly to the active site of the enzyme, thereby inhibiting *de novo* nucleotide biosynthesis. Single amino acid substitutions in the enzyme active site can result in decreased binding efficiencies for antifolate drugs, thereby preserving the ability of the enzyme to generate sufficient tetrahydrofolate (Sorrentino, 2002).

MDR1/ABCB1 encodes p-glycoprotein (PGP), a membrane pump that effluxes a variety of small molecues in an ATP-dependent fashion. Overexpression of PGP confers resistance to a variety of chemotherapeutic agents including vinblastine, colchicine, doxorubicin and paclitaxel. Co-expression of *MDR1* with a therapeutic gene allows for enrichment of genetically modified cells and provides a strategy for increasing the number of corrected cells since the untransduced cells would be killed by the drug treatment. Large-animal studies and clinical trials using MDR1 as a selectable marker demonstrated overall disappointing results. Among the problems observed were excessive toxicity, low initial marking and moderate and/or transient selection after drug administration (Neff et al., 2006).

MGMT is a gene that encodes a DNA repair enzyme that protects cells from DNA-damaging agents, such as nitrosourea and DNA-methylating agents. These drugs are potent stem cell toxins that cause severe and progressive BM suppression by inducing apoptosis. The mechanism of action of these drugs is to modify cellular DNA through the addition of alkyl adducts at the O^6-position of guanine. The MGMT protein repairs these adducts by directly removing the alkyl residue. The co-expression of a therapeutic gene and *MGMT* variants allows selective resistance to alkylating agents and benzylguanine (Chinnasamy et al., 1998; Davis et al., 2000; Hickson et al., 1998; Persons et al., 2003; Sawai et al., 2001). The variant *MGMT* is the most powerful selective drug resistance gene defined so far, and it provides a survival advantage to the transduced HSCs and their extensive selection, even when limited numbers of transgene-carrying cells are transplanted in recipients given a nonmyeloablative conditioning regimen (Budak-Alpdogan et al., 2005). Using MGMT selection, long-term maintenance of 50% of the total blood cell output from transduced cells could be attained in large animal models (Trobridge et al., 2005).

Drug resistance genes are the only proven system for *in vivo* selection of transduced HSCs but these strategies also raise concerns about the high toxicity of the drug treatments required for adequate selection. Furthermore recent discovery of molecules that are able to influence HSC self-renewal *in vitro* offer another promising approach to achieve HSC expansion pre-transplantation and avoid the toxicity associated with strategies that require *in vivo* drug selection.

1.4.2 Extrinsic regulators of HSC self-renewal

Numerous cytokines have been identified as important extracellular regulators of hematopoiesis. These include steel factor (SF, also known as stem cell factor [SCF]), Flt3-ligand (FL), Thrombopoietin (TPO) and cytokines from the family that signal through gp130. The combination of interleukin-11 (IL-11), SF, and FL, has been found to stimulate self-renewal of adult mouse HSCs to produce an amplification *in vitro* of 3 to 4 fold (Audet et al., 2001; Miller and Eaves, 1997). IL-6 in concert with FL, SF, IL-3, and G-CSF has been shown to support the expansion *in vitro* of human cord blood HSC (Bhatia et al., 1997a; Conneally et al., 1997). In gene therapy applications, cytokines that are capable of maintaining stem cell function in culture during retrovirus-mediated gene transfer are IL-3 and IL-6 for murine HSCs (Bodine et al., 1989) and IL-3, IL-6, TPO, SCF, FL and G-CSF for large animal HSCs (Trobridge et al., 2005).

Nakauchi *et al.* quantitatively assessed the self-renewal capacity of HSCs at a clonal level *in vitro* by examining the effect of various cytokines on purified CD34- cKit+ Sca1+ Lin- cells from adult mice marrow and which represent ~20% pure HSCs. Among the cytokines examined, SCF and TPO were the minimum cytokines to most efficiently induce self-renewal cell divisions. In contrast, SCF + IL-3, SCF + IL-6, and

SCF + IL-11 + FL appeared to be less effective (Nakauchi et al., 2001). On the other hand, Uchida *et al.* using purified Lin-Rho-SP+ cells, showed that the combination of SCF + IL-11 + FL was superior to TPO+SCF in maintaining HSC activity *in vitro* (Uchida et al., 2003).

More recently Lodish's group identified additional factors that, together with other growth factors, were found to sustain the *ex vivo* expansion of long term repopulating cells (8 to 30 fold). These factors are the insulin-like growth factor 2 (IGF-2) (Zhang and Lodish, 2004) and angiopoietin-like proteins (Zhang et al., 2006a).

In addition, the extracellular matrix contains various ligands that activate receptors implicated in a wide range of developmental processes and these have also been found to be important for the regulation of HSC self-renewal. The pathways activated by these soluble, but matrix-bound ligands or by transmembrane ligands present on adjacent cells, include the Notch pathway, the Wnt pathway and the Sonic Hedgehog pathway (Akala and Clarke, 2006; Nakano, 2003).

Hints of the involvement of Notch signaling in hematopoiesis came from overexpression of the Notch1 receptor. This study showed that immortalized HSCs capable of differentiating into both lymphoid and myeloid cells after transplantation could be produced with the overexpression of an active form of the Notch1 receptor (Varnum-Finney et al., 2000). The involvement of the Wnt pathway in hematopoiesis was confirmed by various studies with Wnt ligands. One study that linked the effect of a Wnt ligand with primitive human cell self-renewal, made use of Wnt5a. The administration of Wnt5a to mice was shown to induce the increased repopulation of human HSCs in NOD/SCID mice and lead to primitive hematopoietic development of human blood stem cells *in vivo* (Murdoch et al., 2003). A study from the same group also demonstrated the involvement of the Sonic Hedgehog pathway in hematopoiesis.

By addition of soluble forms of Sonic Hedgehog to cultures of human cord blood cells, they showed an increase in the number of cells with multipotent repopulating capacity in NOD/SCID mice (Bhardwaj et al., 2001). Similarly to nonhematopoietic systems where different signaling pathways have been shown to cross-talk, in the hematopoietic system as well multiple interactions between different pathways exist (Duncan et al., 2005) and they could be responsible, together with cytokine signaling, for the fine tuning of HSCs self-renewal.

Use of growth factors for stimulation of HSC self-renewal is a promising approach to maintain or increase HSCs *in vitro* but in order to achieve greater expansion, much attention has recently been directed at harnessing intrinsic regulators that could influence HSC fate. These are described in the following section.

1.4.3 Intrinsic regulators of HSC self-renewal

The expression and repression of specific genes are believed to ultimately determine the developmental fate of stem cells. Genetic programs establish the HSC pool early on during development and subsequently mediate decisions between proliferation and quiescence, self-renewal and differentiation, and specific lineage restriction events. This way the numbers and types of blood cells produced is balanced throughout a lifetime.

Many of the genes involved in hematopoiesis and HSC regulation were first discovered because of their involvement in leukemic transformation. Using genetic approaches, such as loss-of-function or gain-of-function mouse models and conditional gene targeting strategies, their role was further confirmed in normal HSC development and lineage differentiation (Teitell and Mikkola, 2006). A major proportion of these

genes encode transcription factors (TFs). TFs are sequence-specific DNA-binding proteins that regulate gene expression and hence control proliferation and many differentiation processes like embryogenesis, organogenesis and also hematopoiesis, due to their ability to activate and coordinate expression of lineage-specific genes. Some of these TFs appear to be required for the establishment of HSCs early in development, others for the maintenance of HSCs throughout life and others for the amplification and self-renewal of HSCs. For example, SCL/tal1 (Stem Cell Leukemia) is a TF that was initially identified by its association with human mixed lineage leukemias and then later shown to have a vital role in the establishment of HSCs during embryonic development. This TF is necessary for the specification of HSC fate in early embryos (Shivdasani et al., 1995) but is dispensable for HSC engraftment, self-renewal and multi lineage differentiation in the adult (Mikkola et al., 2003).

1.4.3.1 Homeobox transcription factors

A major class of TFs that come in to play in the adult is the **homeobox family** of TFs encoded by *HOX* genes. HOX proteins are an evolutionary conserved family characterized by a 60 amino acid sequence that specifies a helix-turn-helix DNA-binding domain called the homeodomain. Regions outside the homeobox in the variable domain provide binding specificity to homeoproteins for specific DNA sequences through cooperative binding to other regulatory proteins. Mammalian *Hox* genes are organized in four genomic clusters (A, B, C and D) that are localized on four different chromosomes (7, 17, 12 and 2, respectively in humans) (Figure 1.4). During embryogenesis, they exhibit a site and time-specific pattern of expression along the anterior-posterior axis of the embryo that correlates with their relative chromosomal

position, meaning that the expression order is colinear with the 3'-5' organization of *Hox* genes on the chromosomes (van Oostveen et al., 1999).

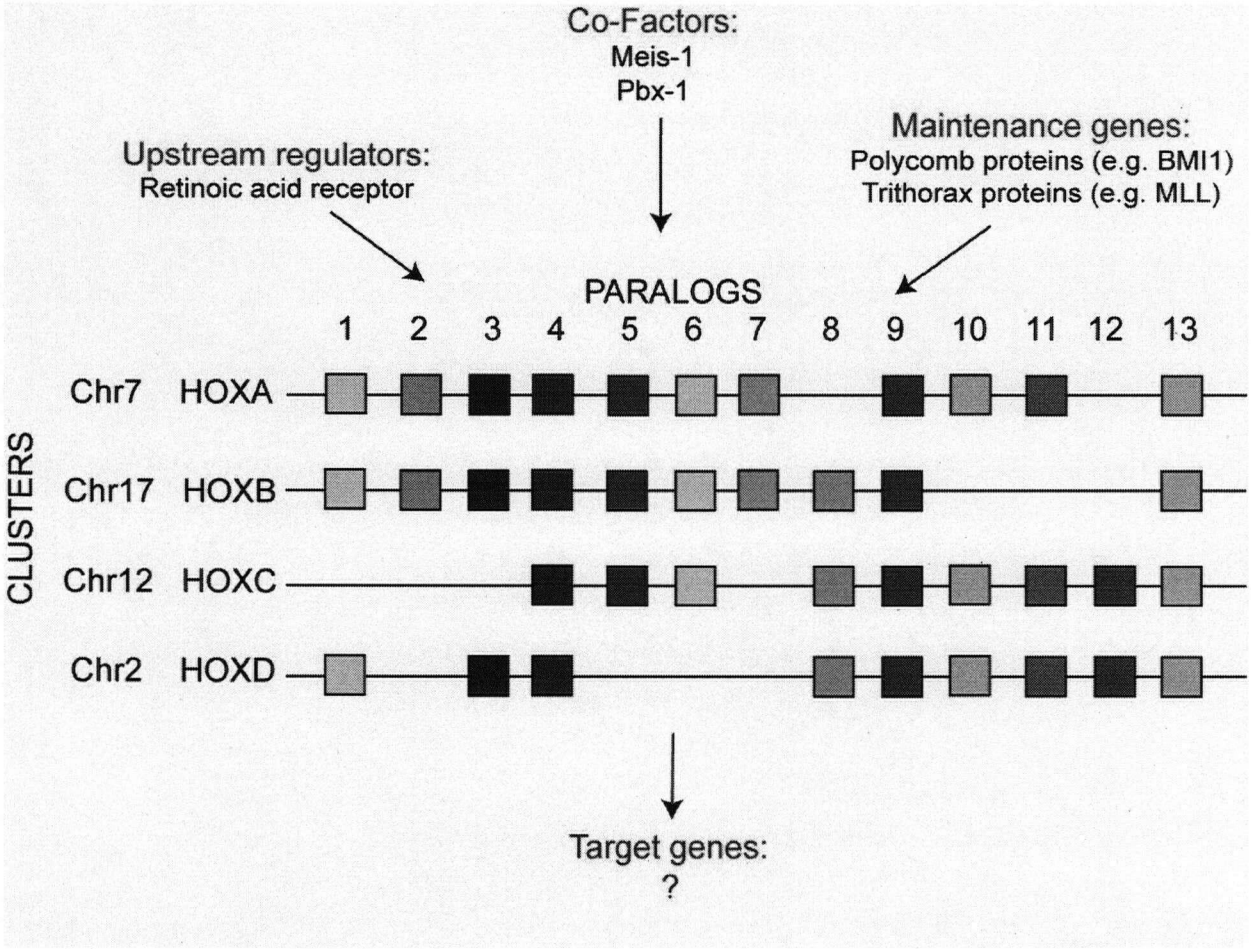


Figure 1.4 Schematic representations of human *HOX* gene clusters and the factors that regulate their expression or interact with their protein products.

HOX TFs were first implicated in regulating hematopoiesis as a result of studies describing their expression in human and murine hematopoietic cell lines and by evidence of their involvement and aberrant expression in hematological malignancies (Lawrence and Largman, 1992). In the hematopoietic system, *Hox* gene expression was first reported by Adams and co-workers (Kongsuwan et al., 1988), who showed the presence of several Hox transcripts in hematopoietic cells with some being specific to

particular subsets of hematopoietic cells. *HOX* gene expression was also assessed in primary human BM cells and it was found that the majority of A and B cluster genes were preferentially expressed in primitive subpopulations (Sauvageau et al., 2004). However, knockout studies of *Hox* genes of the B cluster including combined deletion of Hoxb1 through Hoxb9, have not revealed major hematopoietic defects (Bjornsson et al., 2003) (Brun et al., 2004) likely because of redundancies amongst Hox family members (Bijl et al., 2006). In contrast, overexpression studies of many HOX genes, including *HOXB3*, *HOXB4*, *Hoxa9* and *HOXA10* have shown major effects on HSC self-renewal and differentiation, demonstrating the possibility of a substantial involvement in regulating hematopoietic cell development and homeostasis (Sauvageau et al., 1995; Sauvageau et al., 1997; Thorsteinsdottir et al., 2002; Thorsteinsdottir et al., 1997).

1.4.3.2 Additional HSC regulators

Other related genes (see Figure 1.4 mentioned above) that have emerged as important HSC regulators are *Cdx4* (Davidson et al., 2003), *Mll* (Ernst et al., 2004a) and HOX cofactors such as *Meis1* (Hisa et al., 2004) and *Pbx1* (DiMartino et al., 2001). The knockouts of these genes showed profound hematological abnormalities. Cdx4 is also a TF, while MLL belongs to the Trithorax-group of chromatin regulators that are involved in gene activation. Both these proteins activate the expression of different *Hox* genes that have a role in the proliferation of leukemic as well as normal hematopoietic cells (Ernst et al., 2004b; Wang et al., 2005). Not surprisingly, TFs that appear to be major players in regulating HSC specification and self-renewal have been extensively

investigated as potential candidates for enhancing HSC self-renewal in overexpression studies.

HSC self-renewal and maintenance can also be accomplished by the transcriptional repression of genes. Polycomb group proteins are a family of TFs responsible for chromatin remodeling and maintenance of gene silencing. Some of the proteins that are part of the Polycomb Repression Complex 1, such as Bmi1, Mel18 and Rae28 have been implicated in the regulation of HSC self-renewal and lineage restriction (Akala and Clarke, 2006). Studies of ***BMI-1*** have shown that loss of expression of this gene leads to a decrease in the number of HSCs present in the adult and, in competitive repopulation experiments, these cells also fail to sustain lymphomyeloid hematopoiesis long term (Park et al., 2003). This major effect on HSCs has been studied also in leukemic cells where the loss of expression of *BMI-1* leads to compromised survival of leukemic stem and progenitor cells. Eventually these *BMI*-deficient cells undergo proliferation arrest, differentiation and apoptosis (Lessard and Sauvageau, 2003). A direct link between *BMI-1* and HSC self-renewal came from overexpression studies in purified HSCs (Iwama et al., 2004). Analysis of the potential of daughter cells derived from single *BMI-1*-overexpressing HSCs showed that this promoted HSC self-renewal and enhanced their ability to execute symmetrical divisions resulting in a 3-fold increase in clonogenic progenitors with high proliferative potential compared to control; however, the magnitude of HSC expansion after 10 days *in vitro* was not assessed.

In addition to genes involved in transcriptional activation or repression, genes that regulate apoptosis and cell cycle progression have also been found to influence HSC survival and self-renewal. For example, transgenic mice overexpressing ***BCL-2***, a gene encoding an anti-apoptotic protein, have been shown to have 2.4 fold increase in

HSC numbers *in vivo* (measured by phenotype) and their marrow cells display a higher competitive repopulation ability in lethally conditioned mice as compared to marrow cells from wild type mice. However, this study did not discriminate as to whether the greater competitiveness was simply due to an increase in the number of HSCs transplanted from the BCL-2 transgenic BM or whether the BCL-2 overexpressing HSCs also were able to generate more differentiated cells (Domen et al., 2000).

An example of a cell cycle regulator found to play an important role in the maintenance and self-renewal of HSCs is **p21**. P21 is a cell cycle inihibitor and its suppressed expression leads to a 2-fold increase in HSC numbers in the BM of otherwise unperturbed adult mice by promoting HSCs to continue to enter the cell cycle and execute symmetric divisions resulting in their initial accumulation. However, this deregulation of quiescence is associated with early exhaustion of their self-renewal activity, as demonstrated by their inability to self-renew and reconstitute serially transplanted animals (Cheng et al., 2000).

These genes represent just a few of the many genes now shown to be involved in the establishment, maintenance and regulation of HSCs. Nevertheless, how these interact remains largely unknown as does the likely involvement of other genes, proteins and epigenetic changes.

1.4.4 HSC *in vitro* expansion strategies

HOXB4 is one of the TFs that has emerged as having a potent ability to stimulate the self-renewal and expansion of HSCs *in vitro*. **HOXB4** is a 361 amino acid human protein in which only 9 amino acids are divergent from the mouse protein and none of these occur in the homeodomain. HOXB4 is expressed in primitive

hematopoietic cells of both species and is downregulated in their more mature progenitors and differentiated blood cells (Pineault et al., 2002; Sauvageau et al., 1994). Initial studies that led to the discovery of an effect of HOXB4 on HSC activity made use of retroviral vectors to genetically engineer the overexpression of this gene in adult murine BM cells. When cells that had been transduced with a control vector were transplanted into lethally irradiated mice, HSC numbers in the BM regenerated to only 5-10% of normal levels (a 20-fold expansion), whereas cells that had been transduced with a retroviral vector encoding *HOXB4* reconstituted the HSC compartment to the size present in normal mice; i.e., a total expansion of 1000-fold and ~50 times more than that obtained from the control HSCs. In addition, proviral integration analysis of the cells regenerated in secondary transplants of the *HOXB4*-transduced cells showed that enhanced self-renewal was the mechanism responsible for their increased amplification *in vivo* (Sauvageau et al., 1995; Thorsteinsdottir et al., 1999).

Further studies demonstrated that HOXB4 could also induce the expansion of mouse HSCs *in vitro*. Usually, when BM cells are cultured in the presence of cytokines, HSCs are rapidly lost. However, when mouse HSCs were transduced with *HOXB4* and then cultured for 10 days, there was a net 40-fold increase in the number of HSCs detectable which were by that time ~1,000-fold higher than the number of HSCs detectable in cultures of HSCs that had been transduced with a control vector. This was measured by a limit dilution competitive repopulation assay (Antonchuk et al., 2002).

In attempts to understand the mechanism of action of HOXB4 and possibly further increase the expansion of HSCs *in vitro*, it was observed that cells engineered to overexpress HOXB4 become 20-fold more competitive if PBX1, a HOX co-factor and homeobox containing protein, was knocked down (Krosl et al., 2003b). Similarly,

abolition of p21 expression significantly enhanced the regenerative activity of *HOXB4*-transduced BM cells (Miyake et al., 2006).

Overexpression studies using human cord blood (CB) cells and nonhuman primate BM cells have also demonstrated an effect of HOXB4 on primitive populations capable of long-term repopulation in transplantation assays, although the magnitude of the expansion was much less than has been demonstrated for murine HSCs. In the experiments with human CB cells, CRUs assayed in a NOD/SCID xenograft model were shown to be amplified 4-fold (Buske et al., 2002). In non-human primates co-transplanted with equal numbers of HOXB4 and control cells, a 5-fold greater contribution of the HOXB4 overexpressing cells compared to the control cells was seen at 6 months post-transplant (Zhang et al., 2006b).

Subsequent studies showed that the effects obtained using retroviral vectors to raise intracellular levels of HOXB4 could also be obtained using a HOXB4 fusion protein that contained a plasma-membrane permeabilization sequence to facilitate cell entry (Krosl et al., 2003a). Similarly, human cells cultured on stromal cells engineered to secrete HOXB4 were found to display enhanced HSC activity (Amsellem et al., 2003).

Homeobox TFs are known to act during development in gradients resulting in different quantities eliciting different effects. In the last few years, several studies have shown that this is likely also the case for HOXB4 effects on hematopoietic cells (Klump et al., 2005). An extremely high concentration of HOXB4 was shown to lead to enhanced regeneration of human CD34+ cells in the BM of NOD/SCID mice but also to severely impair their lymphomyeloid differentiation *in vivo* (Schiedlmeier et al., 2003). On the other hand, a study that used adenoviral vectors to achieve the transient enforced expression of HOXB4 in primitive populations of CB cells did not observe

increased proliferation of primitive progenitors but instead it showed a significant increase in myeloid differentiation *in vitro* (Brun et al., 2003). Recently, another study demonstrated that very high overexpression of HOXB4 inhibited the differentiation of primary murine BM cells *in vitro* by reducing the rate of commitment (Milsom et al., 2005). Another study comparing the types and extent of lineage outputs obtained *in vivo* from embryonic stem cell-derived hematopoietic cells and adult hematopoietic cells overexpressing HOXB4 demonstrated that myeloid cell production was increased and T lymphoid development was suppressed over a wide range of expression levels but that very high HOXB4 expression interfered with RBC output (Pilat et al., 2005). Altogether, these studies point to the idea that the fate of HOXB4-transduced hematopoietic cells may depend both on the absolute level of HOXB4 expression obtained and on the stage of differentiation of the cells transduced. To take advantage of the effects of increasing HOXB4 levels in HSCs for clinical applications, it will clearly be critical to more fully understand how these may affect hematopoietic cell growth and differentiation and design strategies that retain HSC expansion effects without impairing the normal output of any mature blood cells.

Recent studies have suggested that the ability to induce a significant expansion of HSCs may extend to other *HOX* genes, which have been identified for their role in leukemogenesis. For example, ***HOXA9***, which has been implicated in acute myeloid leukemia, is preferentially expressed in primitive cells. In addition, Hoxa9$^{-/-}$ mice have impaired HSCs behaviour and numbers (Lawrence et al., 2005; Sauvageau et al., 1994) suggesting that this gene is an important regulator of primitive hematopoietic cells. Not surprisingly, overexpression of Hoxa9 in murine BM cells leads to a 15-fold increase in HSC frequency *in vivo* in secondary transplantation assays (Thorsteinsdottir et al., 2002).

NUP98-HOX fusion proteins have also emerged as potent HSC expanding factors. The NUP98 protein is a component of the nuclear pore complex, involved in transport of RNA and protein across the nuclear membrane (Slape and Aplan, 2004). All NUP98-HOX fusions reported to date that have been found in patients affected by leukemia include the N-terminus of NUP98, which contains a region of multiple phenylalanine-glycine repeats that may act as a transcriptional co-activator through binding to CBP/p300 (Kasper et al., 1999) and the C-terminus of the *HOX* gene product, including the intact homeodomain and a variable portion of the flanking amino acids (Lam and Aplan, 2001) (Figure 1.5).

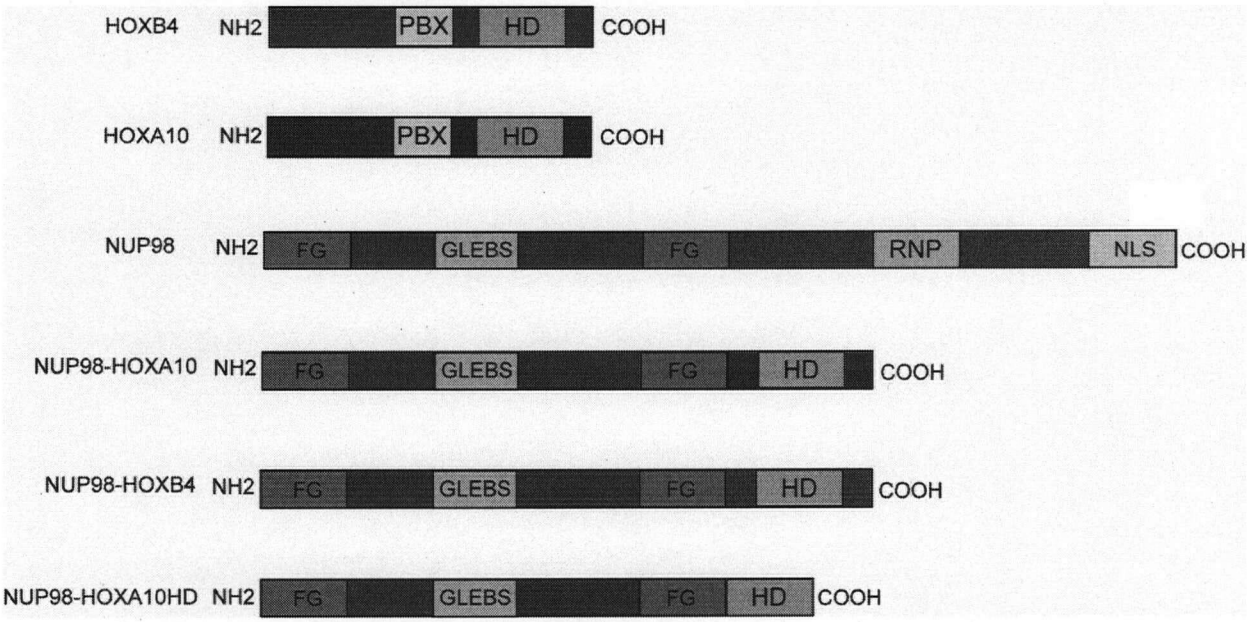


Figure 1.5 Schematic representations of HOX and NUP98-HOX fusion proteins. PBX is the PBX co-factor binding domain, HD is the Homeodomain, FG is a sequence of repeated Glycine and Phenylalanine, GLEBS is a binding site for RAE1 which is an mRNA-specific carrier, NLS is a nuclear localization signal, RNP is a binding domain that likely facilitates transport of mRNA through the nuclear pore.

In a previous study from our laboratory (Pineault et al., 2004), the effects of an engineered fusion of NUP98 with HOXB4 or HOXA10 were reported. BM cells transduced with retroviral vectors encoding either of these fusion proteins were found to produce a markedly higher number of CFU-S after a few days of culture. Furthermore, overexpression of the *NUP98-HOXA10* fusion gene appeared to block terminal differentiation leading to a sustained output of cells with a "primitive" phenotype. In light of these data, Ohta *et al.* studied the effect of the overexpression of these fusion genes on HSC expansion after a more prolonged period of culture. The average magnitude of the HSC expansion achieved by overexpression of the *NUP98-HOXB4* fusion gene was ~300 fold while using the *NUP98-HOXA10* fusion it was possible to achieve an expansion of HSC numbers of more than 2000 fold. Mice transplanted with the latter cells ultimately develop leukemia after a long latency suggesting that other hits are required for this leukemogenic effect. An additional important finding of this study was the ability of the *HOXA10* homeodomain denuded of all adjacent sequences to mimic the effects of the complete exon 2 of *HOXA10* when incorporated into a fusion protein with the same NUP98 sequences (Ohta et al., 2007). Interestingly, mice transplanted with cells overexpressing this fusion protein didn't develop leukemia. This is the first time that such high levels of expansion of functionally verified HSCs have ever been achieved *in vitro*.

NUP98-HOX fusion proteins have a strong effect on the proliferation and self-renewal of primitive populations of the blood and they have differential intrinsic potential to cause overt leukemia. Additionally, some of these fusion proteins in order to become leukemogenic require the interaction with co-factors (e.g. meis1). Due to their major impact on early populations of the blood they are good candidates to test and exploit for *ex vivo* HSC expansion.

1.5 β-thalassemia and SCD as a model to test the potential of HSC expansion and nonmyeloablative conditioning

An important application of *in vitro* HSC expansion strategies is for gene-therapy, where the *HOXB4* gene or *NUP98-HOX* fusion genes might be considered for their ability to produce a selective expansion of genetically corrected HSCs. As already noted, an important limitation of current gene-therapy protocols is the relatively inefficient transduction of human HSCs achievable, which in many cases would result in an inadequate proportion of transduced blood cells producing inadequate levels of the therapeutic protein to achieve a curative effect. If the HSCs were transduced with vectors carrying a therapeutic gene and an additional gene such as *HOXB4* or a *NUP98-HOX* fusion gene, then preferential expansion of the subpopulation of genetically modified HSCs might occur, leading to therapeutic levels of "corrected" cells. In theory, such a strategy could be applied to any genetic disease in which corrected HSCs would not per se have acquired a survival advantage, for example, as is the case in SCD and thalassemia (Sorrentino, 2004).

From previous studies of different mouse models of β-thalassemia or SCD, it has been found that the corrected RBCs have a survival advantage compared to the original RBCs, but this phenomenon does not extend to any growth or survival difference at the level of the HSCs. In a transplantation model of β-thalassemia it was observed that despite the amplification of the genetically normal erythroid component and the improvement of the anemic phenotype with 10 to 20% engraftment of genetically normal HSCs, 50% HSCs engraftment is necessary to bring the Hemoglobin (Hb) concentration in the PB within a normal range (Persons et al., 2001).

In gene therapy applications, the efficacy of the treatment would depend also on the level of expression of the transduced gene and on the gene transfer efficiency. In the same study, using thalassemic mice crossed with transgenic mice expressing different amounts of a γ-globin gene (the fetal counterpart of the β-globin gene), Persons *et al.* (Persons et al., 2001) showed that clinical improvement of a mild form of thalassemia could be achieved if 20% of the HSCs contained a therapeutic gene that, in the erythroid lineage, was expressed at 15% or more of the level of the normal α- or β-globin genes. In a mouse model of BM transplantation for SCD, it was similarly shown that a 25% normal myeloid chimerism resulted in more than 90% normal Hb in the blood, but to cure the anemia, levels of chimerism as high as 70% were necessary (Iannone et al., 2001). Moreover, it was necessary to achieve a level of chimerism in the HSC compartment of at least 50% (which leads to 100% replacement of sickle cells in the PB) to correct all sickle-mediated organ pathologies (Kean et al., 2003). This implies that mixed chimerism can ameliorate SCD but the complete elimination of the abnormal Hb from the blood is only possible with a very high HSC contribution. HSC expansion strategies might thus be critical to achieve the required level of HSC chimerism to obtain cures in patients with these genetic diseases. The same strategies might also allow the establishment of sufficient chimerism in patients conditioned with nonmyeloablative regimens.

1.6 Thesis objectives

HSCT is the only curative option for people affected by malignant and non-malignant blood diseases. When this procedure was developed, it was thought that a myeloablative treatment was necessary to create space in the BM of the recipient and allow the transplanted cells to engraft. However, myeloablative regimens carry a

significant risk of severe morbidity and mortality that restrict the use of the procedure. To reduce these problems, less intensive preparative regimens, which are associated with minimal toxicities, are now being investigated clinically. Recent clinical trials corroborated by experimental data in mouse models suggest that the HSC content of the graft may be critical for success in this setting.

Accordingly, the first aim of my thesis was to ascertain if strategies to expand HSCs *ex vivo* would affect and improve the outcome of nonmyeloablative HSCT and to determine if the quality and quantity of the expanded HSCs would be beneficial in this setting. To this end, *HOXB4* and *NUP98-HOX* fusion genes were tested for their ability to promote sufficient HSC expansion *ex vivo* to achieve high levels of chimerism in nonmyeloablated recipients.

The second aim was to determine if the same type of HSCs expansion strategies would be useful for the treatment and therapy of a murine model of β-thalassemia using a nonmyeloablative conditioning regimen to treat the recipient. In order to do this, *HOXB4* and *NUP98-HOX*-expanded HSCs were transplanted into nonmyeloablated β-thalassemic mice and their hematological parameters were then monitored to follow the effect on their disease status long term.

CHAPTER 2 MATERIAL AND METHODS

2.1 Retroviral vectors

The retroviral constructs used in this project are Murine Stem Cell Virus (MSCV) based oncoretroviral vectors. The MSCV 2.1 (Hawley et al., 1994) vector was first modified by replacing the PGK-neo cassette with a sequence containing the internal ribosomal entry site (IRES) sequence derived from the encephalomyocarditis virus and the gene for enhanced green fluorescent protein (GFP). This MSCV IRES GFP vector (GFP vector) served as a control and backbone for cloning of a *HOXB4* cDNA upstream of the IRES, to create MSCV HOXB4 IRES GFP (HOXB4-GFP vector). In the same way, retroviral vectors containing the fusion gene of NUP98 and the second exon of *HOXB4* or *HOXA10* or the homeodomain coding sequence of *HOXA10* were created. The cDNA construction of these fusion genes was previously described (Pineault et al., 2004).

2.2 Generation of retrovirus

Production of helper-free retrovirus was carried out by standard procedures (Pawliuk et al., 1994), using virus-containing supernatants from transfected amphotropic Phoenix packaging cells (Kinsella and Nolan, 1996) to infect the ecotropic packaging cell line $GP^{+}E86$ (Markowitz et al., 1988). The retroviral titers of the GFP and HOXB4-GFP producer cells were 1×10^{6}/mL and 4×10^{5}/mL respectively, as assessed by transfer of GFP expression to NIH-3T3 cells. Absence of helper-virus generation in the GFP and HOXB4-GFP producer cells was verified by failure to serially transfer virus-conferring GFP expression to NIH-3T3 cells.

2.3 Mice

Parental strain mouse breeders were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and subsequently bred and maintained at the British Columbia Cancer Research Centre animal facility. They were housed in microisolator units and provided with sterilized food, water, and bedding. Irradiated animals were additionally provided with acidified water (pH 3.0). Strains used as bone marrow transplant donors were either C57Bl6/Ly-Pep3b (Pep3b) or the F1 hybrid of (C57Bl/6Ly-Pep3b × C3H/HeJ) ([PepC3] $_{F1}$). Recipients were C57Bl/6, C57Bl/6J$^{W41/W41}$ (W^{41}), the F1 hybrid of (C57Bl/6J × C3H/HeJ) ($[B6C3]_{F1}$) and mice affected by β-thalassemia (β-MDD) (C57BL/6 Hbb^{th-1}/Hbb^{th-1}). The identity of homozygous β-MDD mice was confirmed by isoelectric focusing analysis of RBC lysates to detect characteristic single, slow-migrating Hb tetramers consisting of two murine α and two murine β minor globin chains and by restriction digestion analysis on genomic DNA followed by Southern blot.

Donor and recipient strains are phenotypically distinguishable on the basis of allelic differences at the Ly5 locus: donor Pep3b are Ly5.1 homozygous and donor [PepC3] $_{F1}$ are Ly5.1/5.2 heterozygous, whereas recipient C57Bl/6 and $[B6C3]_{F1}$ are Ly5.2 homozygous.

2.4 Transduction of primary murine bone marrow cells, in vitro culture of hematopoietic cells and transplantation

Primary mouse bone marrow cells were transduced as previously described (Kalberer et al., 2000; Sauvageau et al., 1995). Briefly, bone marrow cells were extracted from mice treated 4 days previously with 150 mg/kg 5-fluorouracil (Faulding) and cultured for 48 hours in Dulbecco's modified eagle's medium (DMEM)

supplemented with 15% fetal bovine serum (FBS), 10 ng/mL hIL-6, 6 ng/mL mIL-3, and 100 ng/mL mSCF. Media, serum and growth factors were purchased from StemCell Technologies (Vancouver, BC, Canada). The cells were then harvested and co-cultured with irradiated (40 Gy x-ray) $GP^{+}E86$ viral producer cells for 48 hours in the same medium with the addition of 5 μg/mL protamine sulfate (Sigma, Oakville, ON, Canada). Loosely adherent and nonadherent cells were recovered from the co-cultures and incubated for an additional 7 or 10 days under the same conditions. Cells were split whenever cultures reached confluency. Retrovirally transduced bone marrow cells were monitored based on GFP expression using a FACSCalibur (Becton-Dickinson, Mississauga, ON, Canada). Total cell numbers were evaluated using a hematocytometer. 7-10 days after transduction, normal recipients were prepared by treatment with 2.5-2 Gy of whole body irradiation using a ^{137}Cs or an X ray irradiator. β-MDD recipients were treated with 2 Gy. Irradiated normal mice or β-MDD mice were transplanted with proportions of the culture within 12 to 24 hours after irradiation by tail vein injection.

2.5 In vivo repopulation

Peripheral blood cell progeny of transduced cells were tracked at various intervals posttransplant by expression of GFP and Ly5.1. One hundred μL of blood was extracted from the tail vein, and the erythrocytes were lysed with ammonium chloride (StemCell Technologies). Leukocyte samples suspended in Phosphate Buffer Saline solution (PBS, StemCell Technologies) with 2% FBS were incubated sequentially on ice with biotinylated anti-Ly5.1 together with phycoerythrin (PE) – labled anti B220 or anti CD4 and anti CD8, or anti Gr1 (Ly6G) and anti Mac1 (CD11b). Samples were subsequently stained with APC-labeled streptavidin (SA) (Becton Dickinson). All

samples were washed with PBS and 1 μg/mL propidium iodide (PI; Sigma) prior to analysis on FACScalibur (Becton-Dickinson) flow cytometry machines. Expression of Ly5.1 identified donor-derived cells, and expression of GFP identified retrovirally transduced cells. At the time of animal sacrifice, BM samples were analyzed by flow cytometry in the same manner.

2.5 CFC assay, in vitro expansion of myeloid colonies and clonality analysis

To generate clonal myeloid cell populations, GFP+ BM cells were plated at low density in methylcellulose medium (M3434, StemCell Technologies) containing 3 U/mL erythropoietin, 50 ng/mL mSF, 10 ng/mL hIL-6, and 10 ng/mL mIL-3. Well isolated myeloid colonies were plucked 10 days later and transferred into liquid cultures and expanded for 7-14 days in DMEM, supplemented with 15% FBS (6250 STI), mIL-3 (6 ng/ml), hIL-6 (10 ng/ml), mSCF (100 ng/ml), in order to provide sufficient genomic DNA for clonality analysis. Genomic DNA was isolated using DNAzol reagent (Invitrogen, Burlington ON, Canada) per the manufacturer's recommendations. Southern blot analysis was performed as previously described (Sauvageau et al., 1994). Unique proviral integrations were identified by digestion of DNA with HindIII, which cleaves once within the provirus. Digested DNA was then separated in 1% agarose gel by electrophoresis and transferred to zeta-probe membranes (Bio-Rad, Mississauga, ON, Canada) (Sambrook et al., 1989). Membranes were probed with α^{32}P-dCTP-labeled GFP sequence.

2.6 CRU assay

HSCs were detected and enumerated using a limit-dilution transplantation-based assay for cells with competitive, long-term, lympho-myeloid repopulation function. The

basic procedure (Szilvassy et al., 1990), and a modification employing sublethally irradiated W^{41}/W^{41} recipients (4.5 Gy ^{137}Cs γ-radiation) as a source of endogenous competitor cells (Miller and Eaves, 1997), have been described in detail previously. Briefly, irradiated W^{41}/W^{41} recipients were injected with 10^2 to 2×10^5 cells, and the blood obtained by tail vein bleeding of these mice was analyzed by flow cytometry a minimum of 12 weeks posttransplant for evidence of lympho-myeloid repopulation. Mice that had greater than 1% donor-derived (GFP^+) cells in both lymphoid (SSC^{low}, FSC^{low}) and myeloid (SSC^{hi}, FSC^{hi}) subpopulations were considered to be repopulated with transduced cells. Discrimination by flow cytometry of myeloid and lymphoid cells was confirmed using cell-surface staining to detect lineage-specific markers (Gr-1, Mac-1, B220, CD4 and CD8). CRU frequencies in the test bone marrow sample were calculated by applying Poisson statistics to the proportion of negative recipients at different dilutions using Limit Dilution Analysis (StemCell Technologies) software.

2.7 Hematologic parameters

Blood from the tail vein was used to analyze red blood cell (RBC) indices and reticulocyte counts using the Sysmex SE 9500 system (Sysmex Corp of America, Long Grove, IL). Blood smears were stained with methylene blue for manual reticulocyte counts to validate the Sysmex reticulocyte counts in the majority of cases and these numbers correlated well. Blood smears were also stained with Wright-Giemsa using an automatic stainer. Smears were reviewed blinded by 2 independent hematologists.

2.8 Identification of retroviral integration sites

This protocol was adapted from the method previously reported by (Riley et al., 1990). Genomic DNA (1μg) from myeloid colonies expanded *in vitro* (described in

section 2.5 above) was digested with *PstI* and the fragments were ligated overnight at room temperature to a double stranded bubble linker (*PstI* linker Top 5'-CTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGCTGTCCTCTCCTTCCTG CA- 3' and *PstI* linker bottom 5'-AAGGAGAGGACGCTGTCTGTCGAAGGGTAAGGAACGGACGAGAGAAGGGAGAG-3'). The Bubble linker contains a 29-nucleotide non-homologous sequence (underlined) that prevents binding of the linker primer in the absence of minus strand generated by the GFP- specific primer corresponding to the GFP sequence within the MSCV retroviral vector. Nested PCR was performed on one tenth of the ligation products. PCR-A used a linker specific primer (Vectorette primer 224 5'-CGAATCGTAACCGTTCGTACGAGAATCGCT-3') and a GFP-specific primer (GFP-A 5'-ACTTCAAGATCCGCCACAAC-3') under the following conditions: one cycle of 94°C for 2 minutes, 30 cycles of :94°C for 30 seconds, 65°C for 30 seconds, 72°C for 2 minutes, and 1 cycle of 72°C for 2 minutes. A 1µl aliquot of PCR-A reaction product (one twenty-fifth) was used as a template for the second nested PCR (PCR-B) using Nested Linker Primer B (5'-TACGAGAATCGCTGTCCTCTCCTT-3') and a GFP-specific primer (GFP-C 5'-ACATGGTCCTGCTGGAGTTC-3') under the same conditions described for PCR-A. The products of PCR-B were separated by gel electrophoresis on a 1.5% Agarose/TAE gel. Individual bands > 550 base pairs were excised and purified using the Qiaex II Gel Extraction Kit (QIAGEN) then subcloned into the PCR2.1 vector using the PCR2.1 TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's protocol. The TOPO ligation product was transformed with 25µl TOP10 Chemically Competent Cells. DNA was extracted from Ampicillin resistant colonies by standard procedures and digested with *KpnI* and *EcoRI* to select for LTR containing clones. Positive clones were sequenced with an LTR-specific primer (5'-

TCCGATAGACTGCGTCGC-3'). Sequence results that contained both the *PstI* linker sequence and the MSCV 3'LTR were believed to contain captured genomic DNA sequence which was further analysed by BLAT Search using the UCSC Mouse Database (http://www.genome.ucsc.edu/ assembly of March 2006). The same assay was performed using *AstI* instead of *PstI*.

2.9 Western blot analysis

Protein extracts from primary BM cells or spleen cells from mice treated with 2 Gy and transplanted with HOXB4 or NUP98-HOXA10hd expanded cells were analysed by western blot using the 1l2 anti-HoxB4 monoclonal antibody (Gould et al., 1997) or the L205 anti-NUP98 polyclonal antibody (Cell Signaling Technology #2288, (Fontoura et al., 1999)) respectively. As a control, protein extracts from primary BM cells from Pep3B mice or from cell lines (PG13HOXB4 or 293T) were used. Protein extracts were electrophoresed on NUPAGE 4-12% Bis-Tris gel (Invitrogen cat no. NP0321BOX) and blotted to PVDF membranes (Millipore Immobilon-P transfer membrane). For evaluation of HOXB4, blots were probed with a 1:250 dilution of hybridoma supernatant in TBS, 3% BSA, 0.001% Tween-20 and visualized with HRP-conjugated donkey-anti-rat secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). For NUP98-HOXA10hd testing, blots were probed with a 1:500 dilution of antibody supernatant in 5% skim milk powder and TBS and visualized with HRP-conjugated goat-anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). Protein expression was detected with Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Boston MA).

CHAPTER 3 ENHANCED REPOPULATION OF SUBLETHALLY CONDITIONED MICE USING *EX VIVO* EXPANDED *HOXB4*-TRANSDUCED HEMATOPOIETIC STEM CELLS

The material in this chapter has been prepared for publication with the title "High Level Polyclonal Reconstitution of Nonmyeloablated Mice with expanded *HOXB4*-transduced Hematopoietic Stem Cells" and is co-authored by H. Ohta, who contributed with the transduction of the cells and performed some of the CRU assays, S. Imren, who contributed with the analysis and diagnosis of the thalassemic mice and with the preparation of the manuscript, B. Cavilla, who provided the data on the cell dose response relationship in nonmyeloablated mice, C.J. Eaves and R.K. Humphries, who contributed with designing the experiments, with the critical analysis of the data and with the preparation of the manuscript.

3.1 Introduction

HSCT is the only cure for some genetic disorders such as SCD and thalassemia, but the morbidity and mortality associated with conventional myeloablative preparative regimens has limited its use to only a minority of the affected patients (Kean et al., 2003; Roberts et al., 2005). Reduced intensity regimens prior to BMT could minimize morbidity and mortality associated with stem cell transplantation-based therapies but require large transplant doses to achieve significant chimerism. Clinical trials have shown that patients with hemoglobinopathies who can achieve stable 20-30% mixed chimerism after transplantation, lead a normal life without any further blood transfusions however reaching this level of chimerism in nonmyeloablative conditions has been challenging. Several studies in animal models have shown that with no pre-conditioning or under nonmyeloablative conditioning extremely high transplant doses of cells are required in order to achieve detectable chimerism (Brecher et al., 1982; Kittler et al., 1997; Micklem et al., 1968; Saxe et al., 1984; Stewart et al., 1993; Stewart et al., 1998; Wu and Keating, 1993). This problem is further exacerbated in gene therapy settings of autologous BMT where there is massive loss of HSC resulting from several manipulations needed for gene transfer of a therapeutic gene. Thus the number of corrected HSCs at the end of *ex vivo* manipulations is generally not sufficient to out-compete the remaining host HSCs in nonmyeloablative settings. One approach to overcome the requirements of massive transplant doses is to confer a selectable advantage to HSCs. This has been achieved by genes/factors that could confer on HSCs a growth advantage or would allow for their *in vivo* selection. A number of genes encoding drug-resistance molecules are currently under investigation, such as the human dehydrofolate reductase (DHFR), multi-drug resistance 1 (MDR1), and

methylguanine metyltransferase (MGMT) drug-resistance genes (Allay et al., 1998; Chinnasamy et al., 1998; Davis et al., 2000; Hickson et al., 1998; Li et al., 1994; Persons et al., 2003; Sawai et al., 2001; Spencer et al., 1996). A side effect to this strategy is the high toxicity of the selective drugs. An alternative and perhaps complementary approach is to use methods to expand HSC before transplantation. Until recently this option was difficult to explore since methods for substantial HSC expansion *ex vivo* were not available. Based on the ability of forced human HOXB4 expression to increase HSCs (40-fold) within 10 days *in vitro* (Antonchuk et al., 2002), we have investigated the applicability of using HOXB4 expanded HSCs in recipients given reduced intensity preparative regimens. Retroviral transduction and use of 5-fluorouracil (5-FU) treated BM have been shown to adversely affect engraftment in unconditioned recipients. Goebel *et al.* showed that the competitive repopulating activity for marrow treated with a retroviral-mediated gene transfer protocol that included 5-FU treatment and subsequent *ex vivo* culture resulted in decreased overall engraftment in 1.6 Gy-irradiated hosts compared to fresh marrow (Goebel et al., 2002). Since our protocol include retroviral transduction of cells and treatment with 5-FU we therefore investigated whether these HOXB4 *ex vivo* expanded HSC are still as competitive as fresh BM cells and are achieved in sufficient numbers to be useful in a nonmyeloablative BMT. In particular, we monitored the capacity of these HSCs to engraft, to give rise to normal lymphomyeloid progeny and to sustain long-term hematopoiesis in nonmyeloablated recipients.

3.2 Results

3.2.1 Donor chimerism and transplant cell dose in recipients given nonmyeloablative conditioning and unmanipulated BM cells

To establish the baseline for comparison to *ex vivo* expanded HSC, we first assessed the relationship between transplantation dose with fresh BM cells and chimerism achieved in recipients given 2 Gy TBI. As shown in Figure 3.1, at least 5 X 10^5 cells are needed to achieve chimerism above 3%. With increasing transplant doses, we observed a strong correlation between the level of donor chimerism and transplant cell dose achieving a maximum of 55% with the transplantation dose of 1 X 10^8 cells, which roughly is equivalent to the whole BM of a mouse (Figure 3.1).

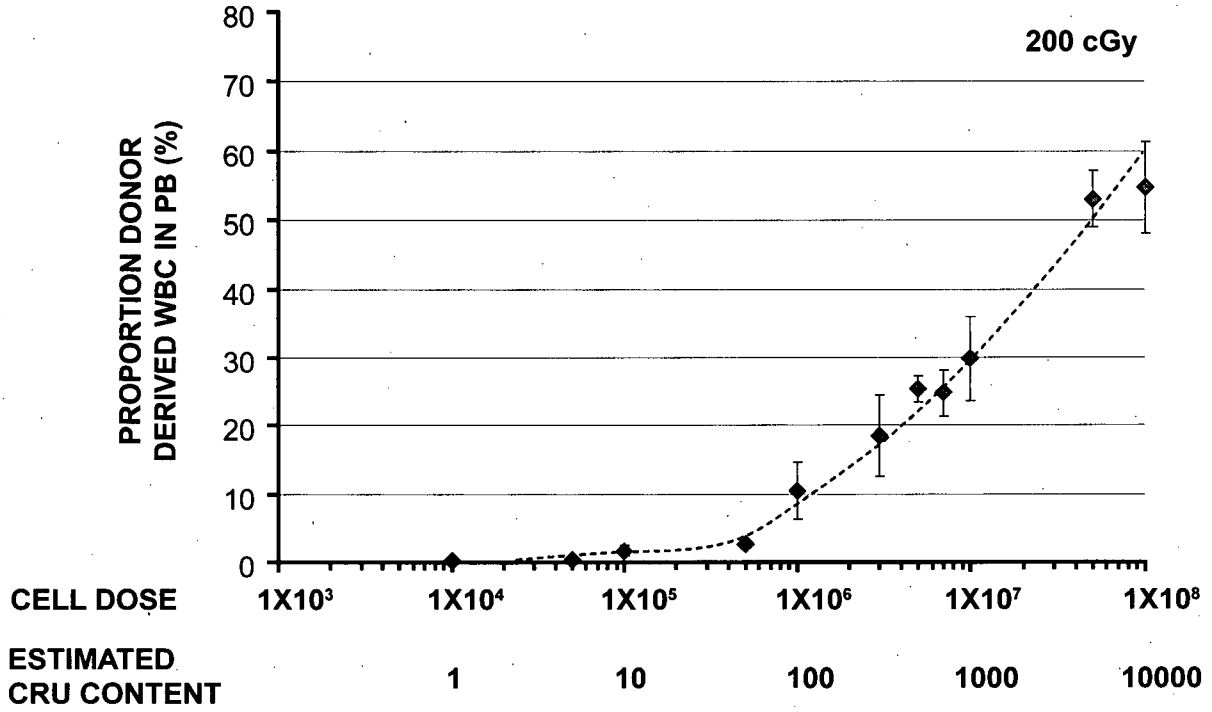


Figure 3.1 Cell dose response of average BM engraftment in minimally ablated hosts. BM was harvested from 18 donors and was transplanted into 31 recipients over the dose range 1 X 10^4 to 1 X 10^8 cells. Three mice were transplanted at each cell dose, with the exception of 5 X 10^4 and 1 X 10^8 where only two mice were transplanted. Recipient mice were given 2 Gy of preparative radiation prior to transplantation. Engraftment levels were assessed 2 months after transplantation on the basis of WBC chimerism. Each point is the average of three mice and error bars represent the standard deviation of the mean. In cases where error bars are not apparent, they are smaller than the size of the marker. The data shown in this graph was generated by Ben Cavilla.

3.2.2 Transplantation of *in vitro* expanded *HOXB4*-transduced HSCs following nonmeyloabative conditioning yields sustained, high level donor chimerism

We then tested whether HOXB4 could be used to stimulate *ex vivo* expansion of HSCs that could be obtained in sufficient numbers and with retention of sufficient repopulating ability to achieve robust levels of chimerism under nonablative conditions.

BM cells from mice treated with 5-FU 4 days previously were prestimulated for 2 days, transduced with *GFP* alone or *HOXB4* and *GFP* for 2 days and then cultured for an additional 7 days. Transduction efficiencies as assessed at the end of the culture period were essentially identical for both arms (35% and 25% for GFP versus HOXB4 arms respectively). The ability to contribute to chimerism was compared for *GFP*- versus *HOXB4*-transduced cells in mice transplanted immediately after transduction and after the further 7 day culture period to allow for HSC expansion. As shown in Figure 3.2A, in mice conditioned with 2.5 Gy, equivalent levels of chimerism were obtained when equal numbers of *HOXB4*- or control *GFP*-transduced cells (progeny of 260,000 starting cells or ~ 50 CRU) were transplanted immediately after termination of the infection period (chimerism of 13% ± 7% for HOXB4 and 13% ± 6% for GFP). This was consistent with the anticipated HSC yield and the absence of an *in vivo* competitive advantage of *HOXB4*-transduced cells in sublethally conditioned mice. When transplants were carried out 7 days after infection, chimerism levels for control *GFP*-transduced cells were diminished, reaching stable levels of only 6% ± 2% consistent with a further decline in HSC numbers with culture. In contrast, *HOXB4*-transduced cells transplanted 7 days after transduction yielded dramatically higher levels of chimerism (48% ±10%) than achieved with cells transplanted immediately after infection consistent with at least an 80-fold expansion in HSC number *in vitro* and retention of repopulating potential (Figure 3.2B). This HSC expansion quantification was extrapolated from the dose response curve of unmanipulated cells in nonmyeloablated mice shown in Figure 3.1. Importantly, chimerism achieved with HOXB4 expanded HSC was lymphomyeloid (Figure 3.2C). Consistent with significant *ex vivo* HSC expansion and retention of robust repopulating function, substantial levels of chimerism (8% ±3% at 7 months post transplantation) were also achieved with even

a 10-fold lower transplant dose of cultured *HOXB4*-transduced cell (26,000 starting cell equivalents or~ 5 starting HSCs). In contrast, with this low dose of cells essentially no chimerism was detected for mice transplanted with *GFP*- transduced cells (Figure 3.2B).

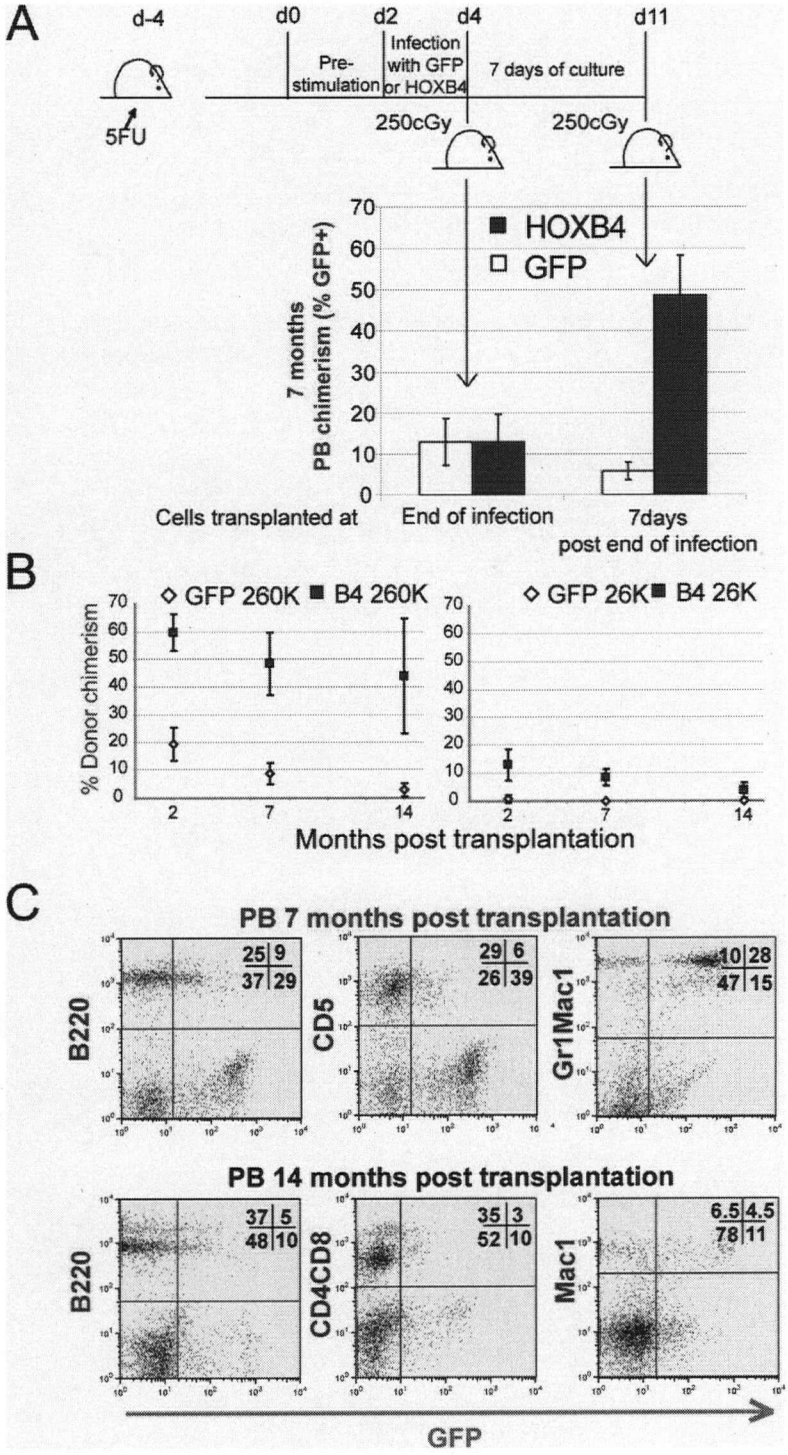


Figure 3.2 (A) Experimental design and comparison of chimerism for transduced cells transplanted before or after extended *ex vivo* culture. 5-FU BM was harvested and prestimulated for 2 days in a cocktail of cytokines containing 100 ng/ml mSF, 10 ng/mL hIL-6, 6 ng/mL mIL-3. BM cells were then transduced for 2 days with oncoretroviral vectors (MSCV-IRES-GFP and MSCV-HOXB4-IRES-GFP) and then either transplanted in to mice immediately after infection or after an additional 7 days in culture. Recipient mice were conditioned with 2.5 Gy. Chimerism was assessed by measuring GFP+ cells in the WBC at 7 months post transplantation by flow cytometry analysis. Mice were transplanted with the progeny of 260,000 original 5-FU BM cells.
(B) Kinetics of engraftment in mice transplanted with transduced cells after extended culture. Cells were transduced with *GFP* or *HOXB4*. Mice were transplanted with 2 doses of cells: the progeny of 26,000 or 260,000 original 5-FU cells. Chimerism was assessed by measuring the GFP+ proportion in the WBC population at 2, 7 and 14 months post transplantation by flow cytometry analysis. Each data point represents the average of 4 to 5 mice and error bars represent the standard deviation from the mean.
(C) Lineage distribution in the WBC compartment of a mouse transplanted with cells transduced with *HOXB4* and cultured for an additional 7 days. The lineage distribution was assessed at 2 months post transplantation (data not shown), at 7 and 14 months post transplantation by flow cytometry analysis. WBC were stained with antibodies that detect B lymphoid cells (anti-B220PE) T lymphoid cells (anti-CD5PE or anti-CD4CD8PE) and myeloid cells (anti-Gr1PE and/or anti-Mac1PE). The abscissa indicates the GFP fluorescence intensity and the ordinate indicates levels of detected expression for a particular cell surface lineage marker, B220 and CD5/CD4CD8 for lymphoid cells, Gr1 and Mac1 for myeloid cells.

These findings were confirmed and extended in 2 additional experiments in which mice were transplanted with expanded progeny of a lower range of starting cells, from 8,000 to 80,000 or an estimated 1-2 to 15 starting HSCs and transplanted into mice receiving reduced conditioning of 2 Gy. Figure 3.3A shows the combined data from 2 experiments. At 4 (experiment 1) and 6 months (experiment 2) post-transplant, extremely low levels of donor-derived cells were present in recipients of either dose of control *GFP*-transduced and expanded cells. In contrast, chimerism as high as 35% (mean=27%, n=5) was reached following the transplantation of the expanded progeny of 80,000 *HOXB4*-transduced cells and up to 22% with the smaller transplant dose (mean=7.6%, n=6). The level of chimerism achieved for the expanded progeny of 80,000 *HOXB4*-transduced cells is equivalent to that previously documented upon transplantation of 1 x 10^7 unmanipulated fresh BM cells and thus equivalent to a transplant dose of ~ 1,000 HSCs (see Figure 3.1). Measurement of the HSC content in the BM cultures transduced with *HOXB4* or with *GFP* by limit dilution assay for CRU confirmed that the level of expansion achieved in the HOXB4 arm ranged from 23- to 80- fold and thus resulting in an HSC content in the transplant of 360 or 1,000 HSC respectively (Figure 3.3B and Table 3.1). This data shows that the transplantation of the progeny of a minimal number of starting HSCs expanded *in vitro* lead to the achievement of significant level of chimerism in nonmyeloablated mice. This level of chimerism would never be achievable with the transplantation of the same number of HSCs before expansion or more importantly with unmanipulated HSCs.

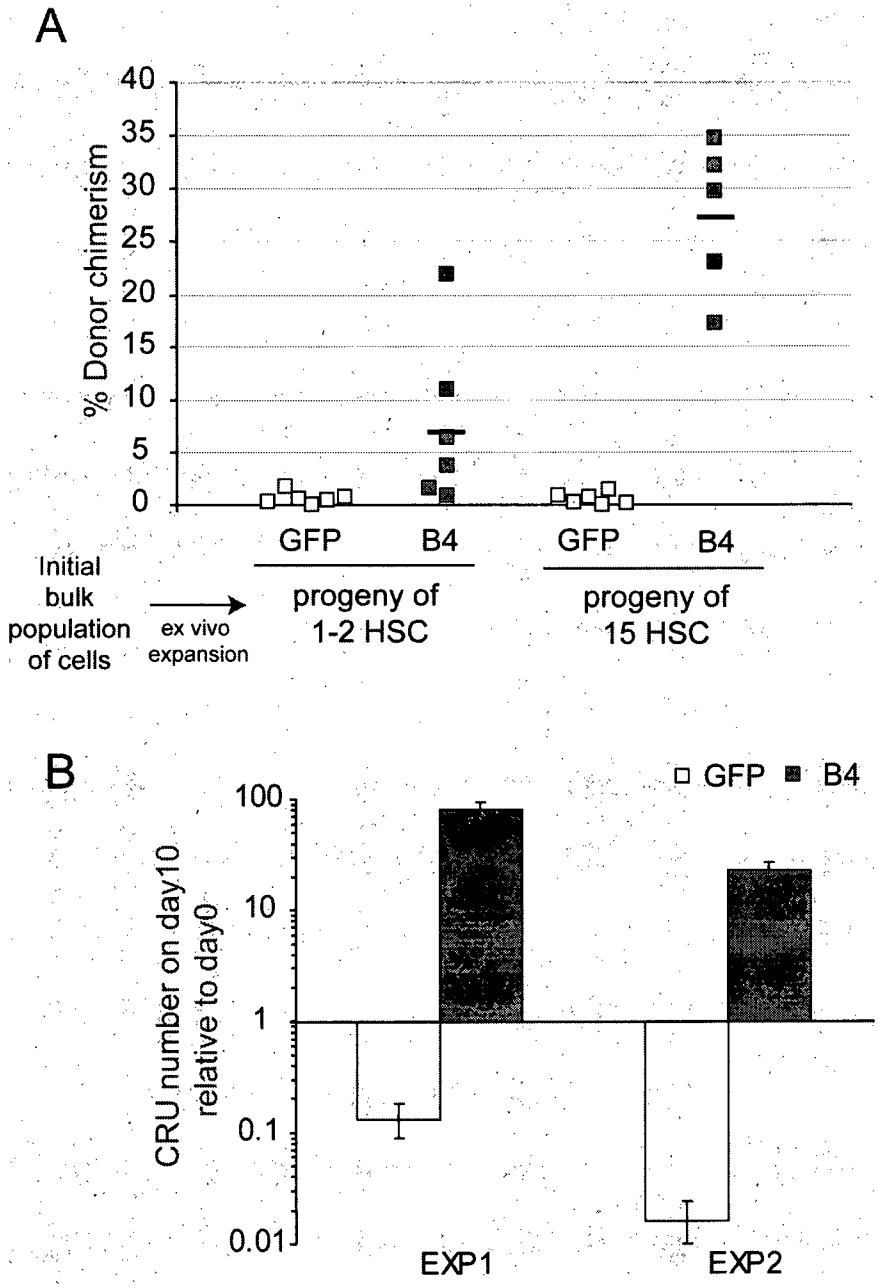


Figure 3.3 Significant chimerism achieved with HOXB4 expanded HSC.
(A) The chimerism is determined by the GFP proportion of cells in the WBC compartment. Data from one experiment was collected at 4 months post transplantation while data from the second experiment was collected at 7 months post transplantation. The transduction protocol is as described in chapter 2. At the end of the infection, cells were kept in culture for an additional 7 days and then transplanted into mice that were pre-conditioned with 2 Gy. Two doses of cells were transplanted: the progeny of 8,000 or 80,000 original 5-FU cells. Each data point corresponds to a mouse. Each arm has a total of 5 to 6 mice. The solid horizontal bar indicates the average chimerism in the different arms of the experiment.
(B) Comparison of CRU content between starting cultures (day0) and after *in vitro* culture (day10).

Table 3.1

	CRU FREQUENCY (+/- S.E.)	
	EXP1 (24 weeks PT)	**EXP2 (16 weeks PT)**
Day of harvest	**1/ 6114 (1/9739 - 1/3838)**	**1/ 5040 (1/8848 - 1/2871)**
Day 10 GFP	**1/46969 (1/103897-1/21233)**	**1/319395 (1/860000 -1/118620)**
Day 10 HOXB4	**1/76 (1/139 - 1/41)**	**1/218 (1/444 - 1/107)**
FOLD EXPANSION	**80X**	**23X**

3.2.3 Highly polyclonal donor chimerism in mice transplanted with expanded *HOXB4*-transduced HSCs following nonmeyloabative conditioning.

The high levels of chimerism achieved by transplantation of *HOXB4*-transduced cells after *in vitro* culture are consistent with significant *ex vivo* expansion of HSCs. Previous studies carried out with transplants in lethally conditioned recipients have shown that HOXB4 induced expansion of HSC *in vitro* is highly polyclonal, consistent with the ability of HOXB4 to enhance the self-renewal of a broad spectrum, perhaps all, successfully transduced HSCs. However, concerns have been raised due to the potential risk of random insertion of the retroviral vectors in sequences with oncogenic potential and subsequent malignant transformation of few transduced HSC clones. To confirm that the high level donor chimerism observed in the setting of nonmyeloablative conditioning was a result of the contribution of many *HOXB4*-transduced HSCs expanded in culture rather than clonal dominance of few transduced HSCs, we harvested BM cells from 3 primary recipients presented in Figure 3.2B, 19 months post

transplantation for clonal analysis of the individual transduced HSCs contributing to host hematopoiesis (Figure 3.4). Two of these mice (mouse a and b) were transplanted with the expanded progeny of 260,000 and the 3rd one (mouse c) with the expanded progeny of 26,000 *HOXB4*-transduced cells. To gain a measure of number of HSCs contributing to hematopoiesis at the time of analysis, DNA was isolated from CFC-derived myeloid colonies generated *ex vivo* as described in Materials and Methods. FACS-selected GFP+ bone marrow cells were used to assure the donor origin of the colonies generated. Southern blot analysis was performed on DNA from a total of 120 myeloid colonies from these 3 mice (n=36 for mouse a, 45 for mouse b and 39 for mouse c). A unique integration pattern, which corresponds to a CFC clone derived from a unique HSC, was observed in 51 out of the 120 colonies (23 of 36 colonies, 18 of 45 colonies and 10 of 39 colonies for mouse a, b and c respectively). Each integration pattern identifies the uniqueness of every HSC clone from which CFC colonies were generated and each unique pattern was found from 1 to multiple times. The summary of the data is presented in Figure 3.4. Interestingly, there was no overlap of integration patterns between the 3 mice, and the majority of these integration patterns, representing the unique clonal signature of a tranduced HSC were observed once (36 of 51, or 71%). The presence of multiple uniquely marked colonies in each mouse indicates a high degree of polyclonal reconstitution consistent both with high level polyclonal HSC expansion *ex vivo* and an absence of significant clonal dominance *in vivo*. Moreover the mean number of proviral integrations per *HOXB4*-transduced HSC was 2.2±1.8 (range: 1-4). The average number of integrations was equivalent for clones detected once versus more than once. This further reinforces the conclusion that there was no dominance of those clones that carried multiple integrations and thus had a potentially increased risk for integration into genes conferring a growth

advantage. Furthermore, this data also demonstrated life long persistence, up to 19 months, of many expanded *HOXB4*-transduced HSCs under nonmyeloablative conditions.

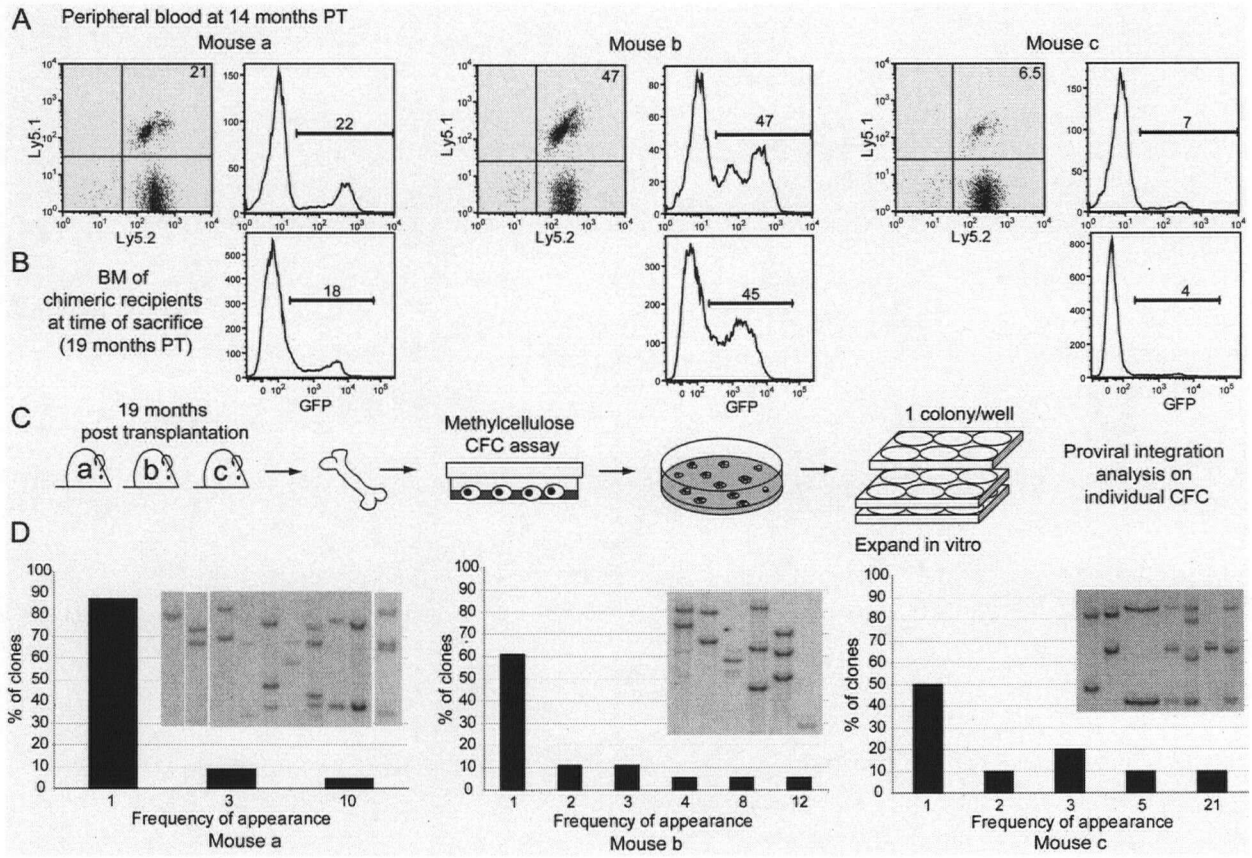


Figure 3.4 Assessment of the degree of polyclonal chimerism from *ex vivo* expanded HSC.

(A) and (B). Flow cytometry profiles documenting chimerism in PB at 14 months post transplantation and for BM at 19 months post transplantation for the three mice used to assess the clonality of transduced HSCs.
Mouse a and b received the progeny of 260,000 original 5-FU BM cells; mouse c received the progeny of 26,000 original 5-FU BM cells.
(C) Schematic diagram of the method used to carry out proviral integration analysis on clonally expanded CFC isolated from BM of mice at the time of sacrifice (19 months post transplantation).
(D) Graphs showing the summary of the data for each of the three mice analyzed. The abscissa shows how many times a specific pattern was found. The ordinate displays what percentage of the clones appeared with the frequency specified on the abscissa. Representative patterns obtained by Southern blot are shown for each of the three mice.

3.2.4 Expanded HSCs contribute to high level chimerism in the BM in addition to the PB

The above studies document that high level chimerism can be achieved in the mature peripheral blood cell compartment and in unseparated BM cells of sublethally conditioned mice transplanted with *ex vivo* expanded *HOXB4*-transduced cells. To assess whether the measured level of chimerism extended to more primitive hematopoietic cells and, importantly, to the HSC compartment, BM was harvested from two primary recipients at 12 or 15 months post transplant. The recipient sacrificed at 12 months had been initially transplanted with 1000 HSCs post expansion (corresponding to an estimated 15 starting HSCs). The second recipient sacrificed at 15 months had been transplanted with 100 HSCs post expansion (corresponding to an estimated 1 to 2 starting HSCs) (Figure 3.5). For both mice, BM cells were analysed by flow cytometry to measure chimerism and by limit dilution transplantation assay in secondary lethally irradiated recipients to assess the level of *HOXB4*-transduced HSCs recovered. The flow cytometry profiles revealed 60% *HOXB4*-transduced cells in the BM of the mouse that received the highest transplant dose and 10% *HOXB4*-transduced cells in the BM of the mouse that received the lower transplant dose. Subsequent quantitation of HSC numbers in these recipients demonstrated that the mouse initially transplanted with 1,000 HOXB4 expanded HSCs, had regenerated transduced HSC numbers to 22,000 (estimated range of 11,500 to 24,200) at the time of sacrifice, or essentially to normal numbers of HSCs seen in unmanipulated normal mice. In the mouse initially transplanted with 100 HOXB4 expanded HSCs the number of HSCs had recovered to an estimated 1,200 at the time of sacrifice or approximately 10% of normal HSC

numbers. The levels of HSC regeneration are thus highly concordant with the levels of chimerism achieved in the BM.

Starting cells transplanted	Estimated CRU content in transplant on day0	Estimated GFP+ CRU in transplant on day11	Estimated GFP+ CRU in BM of primary recipients 12-15 months post transplantation	BM chimerism in primary recipients 12-15 months post transplantation
80000	13	1052	22000	60%
8000	1-2	105	1200	12%

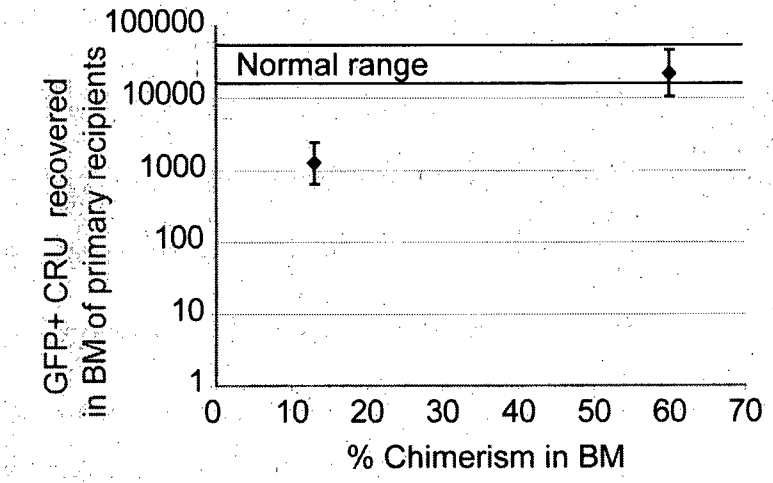


Figure 3.5 HSC contribution to chimerism in the bone marrow. Two primary recipients from the experiments described in Figure 3.4 were sacrificed at 12 months and 15 months post transplantation. Flow cytometry analysis was performed on the BM to assess the proportion of GFP+ cells. Furthermore BM cells were transplanted in limit dilution assay in secondary recipients with the transplantation doses ranging from 1 x 10^3 to 1 x 10^6 primary mouse BM cells per secondary recipient. All secondary recipients were analyzed at 6 months post transplantation.

3.3 Discussion

Many HSC-based therapies would benefit from having protocols to expand HSCs. Up to now expanding HSCs was a difficult task but with newly discovered factors, it is becoming a reality within reach. With the data reported here, we provide

evidence of the value and the applicability of HSC expansion strategies for an important clinical application such as nonmyeloablative BMT (Baron and Storb, 2006). HOXB4 has emerged as a powerful HSC expanding factor, therefore we tested *HOXB4*-transduced and expanded HSCs in nonmyeloablated recipients. The protocol we used for this study to attain HSC expansion requires treatment of BM cells with 5-FU, transduction with a retroviral vector and extended *ex vivo* culture of genetically modified cells. These *ex vivo* manipulations hold the possible risk of modifying some important features of HSCs and affecting the pathways that are normally activated in fresh HSCs, making them less competitive than unmanipulated HSCs. Goebel *et al.* studied the chimerism achieved after transplantation of retrovirally transduced BM cells (Goebel et al., 2002). They found that BM cells stimulated into cycle by 5-FU and transduced with a retroviral vector had decreased engraftment capacity compared to fresh BM cells. In contrast, our data reveals that HOXB4 *ex vivo* expanded HSCs retain the features of fresh unmanipulated cells as manifested by sustained BM and PB chimerism of nonmyeloablated mice. We tested different proportions of the expanded culture at the end of 7 days in mice that received nonmyeloablative conditioning of 200-2.5 Gy. In different experiments, starting from very limited numbers of HSCs we were able to achieve significant engraftment, up to 50% starting with 40-80 HSCs and up to 25-30% starting with 10-20 HSCs. Importantly the chimerism was measured at different time points and it was stable up to 19 months post transplantation. These data show that chimerism levels achieved with the transplantation of expanded HSCs is consistent with the chimerism that would be achieved with the transplantation of the estimated numbers of HSCs recovered after expansion, but unmanipulated. This concordant chimerism confirms that expanded HSCs behave like fresh unmanipulated HSCs and that the competitiveness of these manipulated cells is preserved. Moreover, when

HOXB4-transduced cells were transplanted at the end of the transduction, they behaved exactly like the control-transduced cells, demonstrating that HOXB4 does not alter the *in vivo* growth properties of these cells. On the other hand, when *HOXB4*-transduced HSCs were transplanted after 7 days of culture, the engraftment achieved in the control arm was very poor while HOXB4 expanded HSCs performed dramatically better, giving rise to robust and sustained lymphomyeloid chimerism. This dramatic difference in engraftment between expanded and non expanded HSCs confirms that the final chimerism achieved in nonmyeloablated mice strictly depends on the actual amount of HSCs that is transplanted and not on hypothetical properties imparted by HOXB4. Previously published reports suggested that the final percentage of donor chimerism in nonmyeloablated mice is determined by the ratio of host to donor stem cells (Colvin et al., 2004; Rao et al., 1997) and our data confirms that the final percent donor chimerism is contributed by the amount of HSCs transplanted. Knowing that this is the relationship ruling the outcome of the PB chimerism then the winning strategy for achieving therapeutic range of chimerism after nonmyeloablative BMT is to out-compete at the stem cell level.

In previous studies of HOXB4 it was shown that when transduced HSCs are transplanted in lethally irradiated mice, these are capable to expand *in vivo* until HSC numbers are restored to the normal range, even when relatively small *HOXB4*-transduced cells are transplanted. At this point, these HSCs sense feedback signals from the environment and stop expanding (Sauvageau et al., 1995; Thorsteinsdottir et al., 1999). In light of this finding, we analyzed the behaviour of *HOXB4*-transduced cells *in vivo* in nonmyeloablated mice and monitored if in this setting *in vitro* expanded HSCs are also still responsive to regulation *in vivo* that control the HSC compartment size. The data presented in this chapter demonstrate that transduced HSCs are still subject

to regulation from the environment. These expanded HSCs show an extremely high proliferative potential and are capable of expanding almost 2 logs *in vitro* and an additional 1 log *in vivo*. Nonetheless, in the mouse that received the highest dose of expanded cells, HSC numbers were found to be within the normal range, indicating that in the nonmyeloablative setting as well, *HOXB4*-transduced cells sense the signals from the environment. Although we have not quantified the proportion of HSCs that are spared after a 2 Gy radiation it has been reported that a radiation of 1 Gy is toxic at the stem cell level (Stewart et al., 2001), therefore it is not likely that the total of HSCs in this recipient is above the normal range. On the other hand, in the mouse that received the lower dose of expanded cells, transduced HSCs were only contributing to 10% of the normal range. This could be attributed to the fact that these cells, after expanding 2 logs *in vitro* and 1 log *in vivo*, might have reached the limit of their proliferation potential or alternatively might lack the proliferative stimulus from the cytokine storm that is normally triggered in lethally conditioned mice and is responsible for the early HSC regenerative phase. To elucidate if they stopped proliferating because of environmental signaling or because of proliferation exhaustion, these cells should be further tested in lethally conditioned recipients.

The protocol used to achieve HSC expansion is based on genetic manipulation of BM cells with retroviral vectors. Concerns are raised due to the possibility of creating malignant HSC clones by insertion of the *HOXB4* transgene near host genes with oncogenic potential (Baum et al., 2003; Suzuki et al., 2002; Wu et al., 2003). This would lead to repopulation of the recipients by a mono/oligoclonal population of HSCs bound to trigger malignant transformation. In order to exclude this possibility, we analysed the composition of the HSC pool contributing to the high PB chimerism of these nonmyeloablated mice at very late time points post transplantation (19 months).

We showed through genomic proviral integration analysis of the DNA of progenitor cells derived from HSCs that the contribution to the HSC pool is from a highly polyclonal population of HSCs. This is in accordance with previous data in lethally conditioned mice where it was shown that the majority of the HSCs in the bulk BM culture are targeted by the retroviral vector carrying *HOXB4* and are subsequently expanded with no preference (Antonchuk et al., 2002; Thorsteinsdottir et al., 1999).

Although polyclonal hematopoiesis was documented, the risk of occurrence of malignant clones associated with this transduction procedure remains. In order for this strategy to be safely used in clinical settings, it would be recommended to transiently express the gene or to have the protein product delivered to the cells as a soluble factor over a defined period of time. To circumvent the retroviral integration approach, Krosl *et al.* tested recombinant human TAT-HOXB4 protein carrying the protein transduction domain of the HIV transactivating protein (TAT) as a potential growth factor for stem cells (Krosl et al., 2003a). Experiments are in progress to test different systems to achieve transient expression of HOXB4.

From the experiments presented in this chapter we can conclude that the behavior of HSCs expanded *in vitro* by HOXB4 is equal to fresh unmanipulated cells and that the yield of HSCs is highly beneficial for pre-clinical applications such as nonmyeloablative HSCT.

CHAPTER 4 ENHANCED *EX VIVO* EXPANSON OF HSCs AND IMPROVED CHIMERISM IN NONMYELOABLATED MICE USING *NUP98-HOX* FUSION GENES.

4.1 Introduction

In the previous chapter HOXB4 was shown to provide an effective strategy for the *ex vivo* expansion of HSCs to achieve enhanced levels of chimerism in nonmyeloablated mice. As reported in the literature and confirmed in the studies described in Chapter 3, HOXB4 overexpression can yield *ex vivo* expansion of transduced HSCs to the order of 15 to 80-fold after 7 days *ex vivo* culture. Recent findings as reviewed in Chapter 1, section 1.4.4 now indicate that even greater levels of *ex vivo* HSC expansion can be obtained using the combined overexpression of HOXB4 and knockdown of the Hox cofactor PBX1 (Krosl et al., 2003b) or the engineered expression of novel fusion proteins of Nucleoporin 98 and HOXB4 or HOXA10 (Ohta et al., 2007; Pineault et al., 2004). Indeed with both of these strategies, expansions of greater than 1,000-fold in 6 days are possible. In the studies reported in this chapter, the promising *NUP98-HOX* fusion genes were tested for their improved potency and utility in the context of *ex vivo* HSC expansion and transplantation in nonmyeloablated recipients.

4.2 Results

4.2.1 *Ex vivo* expansion of HSCs using *NUP98-HOXB4* or *NUP98-HOXA10* for transplantation in nonmyeloablated recipients

In order to determine the outcome of HSCT in nonmyeloablative conditions using more powerful HSC expanding factors, fusion genes of *NUP98* and *HOXB4* or *HOXA10* (termed NUP98-HOXB4 or NUP98-HOXA10, respectively; see Figure 1.5) were introduced into primary murine BM using an MSCV-based retrovirus carrying the fusion gene cDNA upstream of an IRES-linked GFP selectable marker. Both fusions encode the N-terminal domain of NUP98 and the 60 amino acid homeodomain and limited flanking sequence as contained in the second exon (Lam and Aplan, 2001; Moore, 2005; Pineault et al., 2004). Two separate experiments were performed using the same conditions. After the 4 day transduction procedure, the cells were kept in culture for an additional 6 days at which point the HSC content was assessed by limit dilution assay for CRU. The results of the measurements of HSC (CRU) frequency before and after the elapsed 10 days in culture and the estimated levels of HSC expansion achieved are shown in table 4.1.

Table 4.1

	CRU FREQUENCY (+/- S.E.)	
	EXP1 (24 weeks PT)	EXP2 (16 weeks PT)
Day of harvest	1/ 6114 (1/9739 - 1/3838)	1/ 5040 (1/8848 - 1/2871)
Day 10 GFP	1/46969 (1/103897-1/21233)	< 1/319395 < (1/860000 -1/118620)
Day 10 NUPB4	1/8 (1/16 - 1/4)	1/47 (1/96 - 1/23)
FOLD EXPANSION	769X	106X
Day 10 NUPA10	>1/3 >(1/8 - 1/1)	1/5 (1/10 - 1/2)
FOLD EXPANSION	>2000X	1000X

The CRU assay confirmed that the HSC expansion achieved *in vitro* was higher than achieved with HOXB4, reaching up to 700 fold or 2000 fold in cultures of *NUP98-HOXB4-* or *NUP98-HOXA10*-transduced cells, respectively. On day 11, cultured cells were also transplanted into nonmyeloablated mice. Mice were treated with 2 Gy TBI and transplanted with the progeny of 8,000 or 80,000 starting cells. The PB chimerism was monitored for 4 months in one experiment and for 7 months in a second experiment. The pooled data are shown in Figure 4.1.

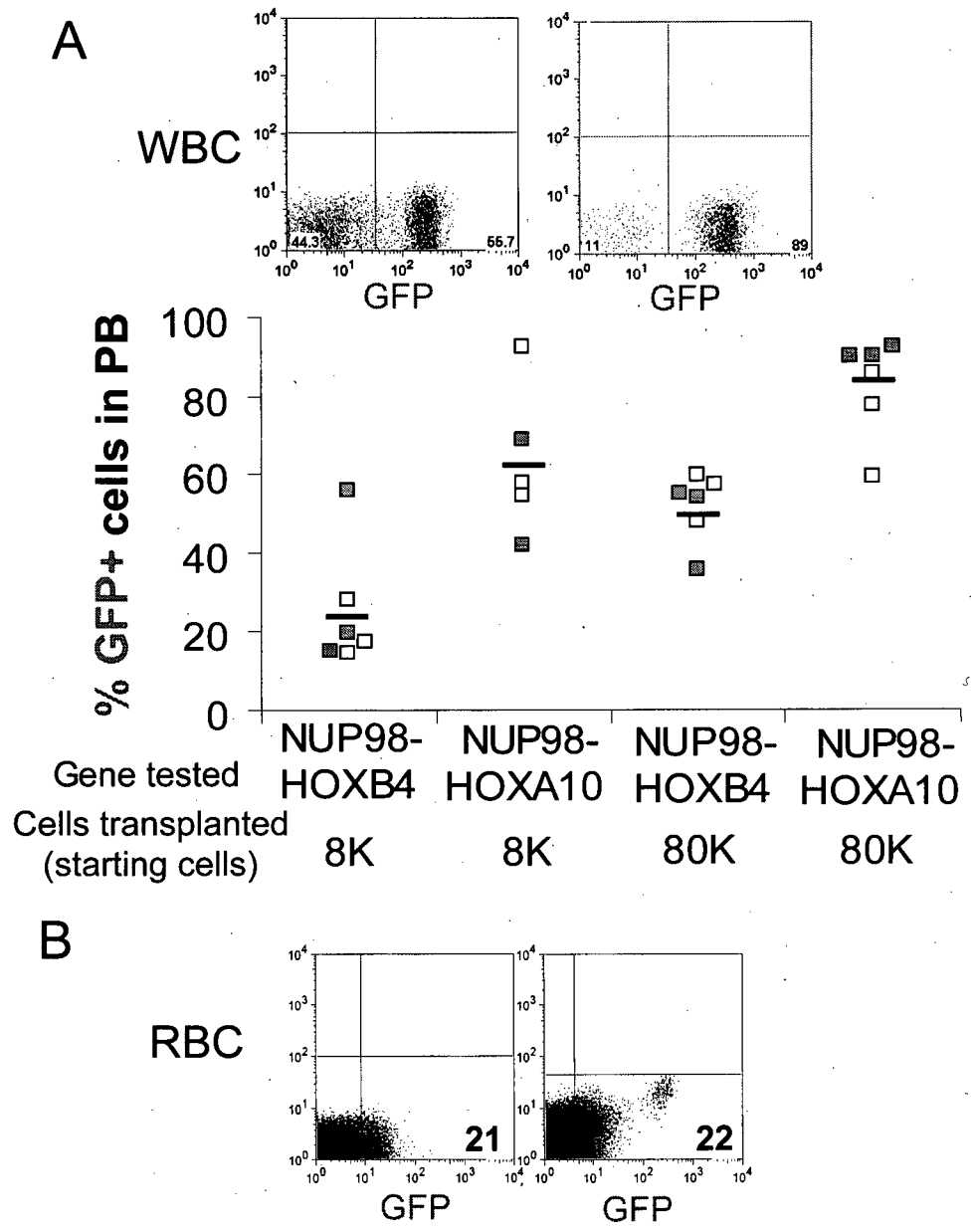


Figure 4.1 Enhanced PB chimerism achieved using different fusion genes.
(A) Representative flow cytometry profile of GFP chimerism in the WBC compartment. Data from one experiment was collected at 4 months post transplantation (open squares) while data from the second experiment was collected at 7 months post transplantation (red squares). In these two experiments 5-FU BM cells were prestimulated in cytokines for 2 days and transduced with oncoretroviral vectors for 2 days. At the end of the infection cells were kept in culture for an additional 7 days and then transplanted in mice that were pre conditioned with 2 Gy. The vectors used for transduction were NUP98-HOXB4 (MSCV NUP98-HOXB4 ires GFP) and NUP98-HOXA10 (MSCV NUP98-HOXA10 ires GFP). In both experiments 2 doses of cells were transplanted: the progeny of 8,000 or 80,000 original 5-FU cells. Each square corresponds to a mouse. Each arm has a total of 5 to 6 mice. The solid horizontal lines indicate the average chimerism in the different arms of the experiment. **(B)** Two representative flow cytometry profiles of RBC showing GFP chimerism from mice transplanted with the higher dose of *NUP98-HOXB4*- and *NUP98-HOXA10*-transduced and expanded cells are shown at the bottom of the figure.

Mice transplanted with the lower dose of *NUP98-HOXB4*- and *NUP98-HOXA10*-transduced and expanded cells, attained chimerism levels of 25% (±16%) and 63% (±19%) respectively. Mice transplanted with the higher dose of *NUP98-HOXB4*- and *NUP98-HOXA10*-transduced and expanded cells, reached even higher levels of chimerism (52% ± 9%; and 82% ± 13% respectively). The higher level of chimerism achieved with *NUP98-HOXA10*-transduced cells at both transplant doses is consistent with the higher magnitude of *ex vivo* HSC expansion documented for NUP98-HOXA10 compared to NUP98-HOXB4. Moreover, both fusion genes enabled higher levels of chimerism compared to previous results with HOXB4, again consistent with the superior ability of either fusion to stimulate *ex vivo* HSC expansion (Figure 4.2).

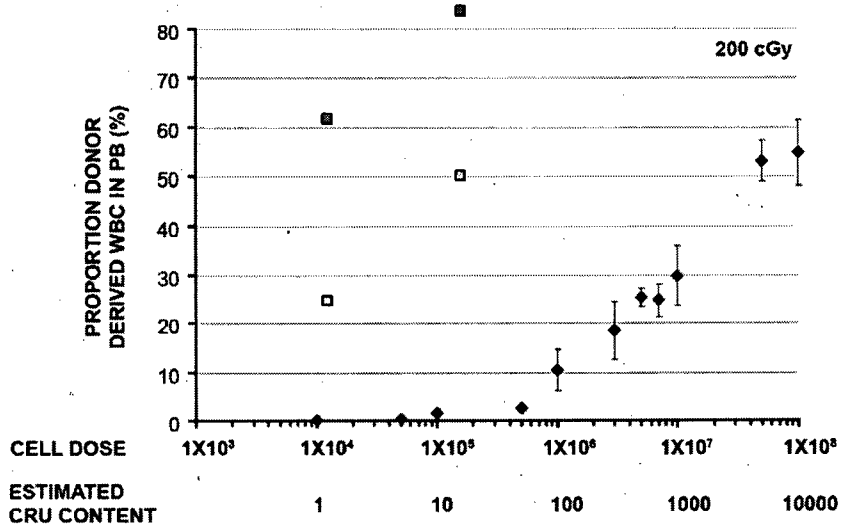


Figure 4.2 Comparison between the chimerism achieved with grafts containing HSCs expanded by fusion genes and unmanipulated cells
Red squares identify the engraftment achieved in mice transplanted with NUP98-HOXA10 expanded cells; yellow squares identify the engraftment achieved in mice transplanted with NUP98-HOXB4 expanded cells. Diamonds represent the cell dose response of average BM engraftment in minimally ablated hosts as described in Figure 3.1.

4.2.2 Assessment of the HSC expanding potential of a fusion of *NUP98* and only the homeodomain of *HOXA10*

A fusion protein of NUP98 with only the homeodomain (hd) of HOXA10 was tested in limit dilution assays and it was demonstrated that the hd by itself fused to NUP98 is sufficient to trigger high levels of HSC expansion, as high as 1000-fold (Ohta et al., 2007). This fusion protein seems to be non-leukemogenic and has no apparent impact on *in vivo* lineage differentiation. I therefore tested this fusion protein in nonmyeloablated mice to prove that in addition to a dramatic increase in the quantity of HSCs in culture, it was also capable of preserving their quality for use in nonmyeloablative settings.

In accordance with previous experiments, BM cells from mice previously treated with 5-FU were transduced with oncoretroviral vectors carrying the *NUP98-HOXA10* or *NUP98-HOXA10hd* fusion genes or a *GFP* control gene. After transduction cells were kept in culture for an additional 6 days at the end of which small portions of the culture were transplanted in nonmyeloablated mice that were treated with 2 Gy TBI. As in the experiments described in the previous paragraphs, the progeny of 8,000 or 80,000 starting cells was transplanted. Mice were analysed at 3 months PT. The PB chimerism achieved for both NUP98-HOXA10 and NUP-HOXA10hd was extremely high while mice transplanted with *GFP*-transduced cells did not show any chimerism.

A major difference observed in this experiment, was the better contribution of transduced cells to the RBC chimerism in mice transplanted with NUP98-HOXA10hd expanded cells compared to mice transplanted with NUP98-HOXA10 expanded cells (Figure 4.3A). All the lineages were represented in the peripheral blood although there was some skewing of transduced cells towards myeloid differentiation (Figure 4.3B).

To confirm that the chimerism achieved with this fusion gene was superior to that achieved when *HOXB4*-transduced and expanded cells were used, the experiment was performed using the same experimental design but testing at the same time *GFP*-, *HOXB4*- or *NUP98-HOXA10hd*-transduced cells.

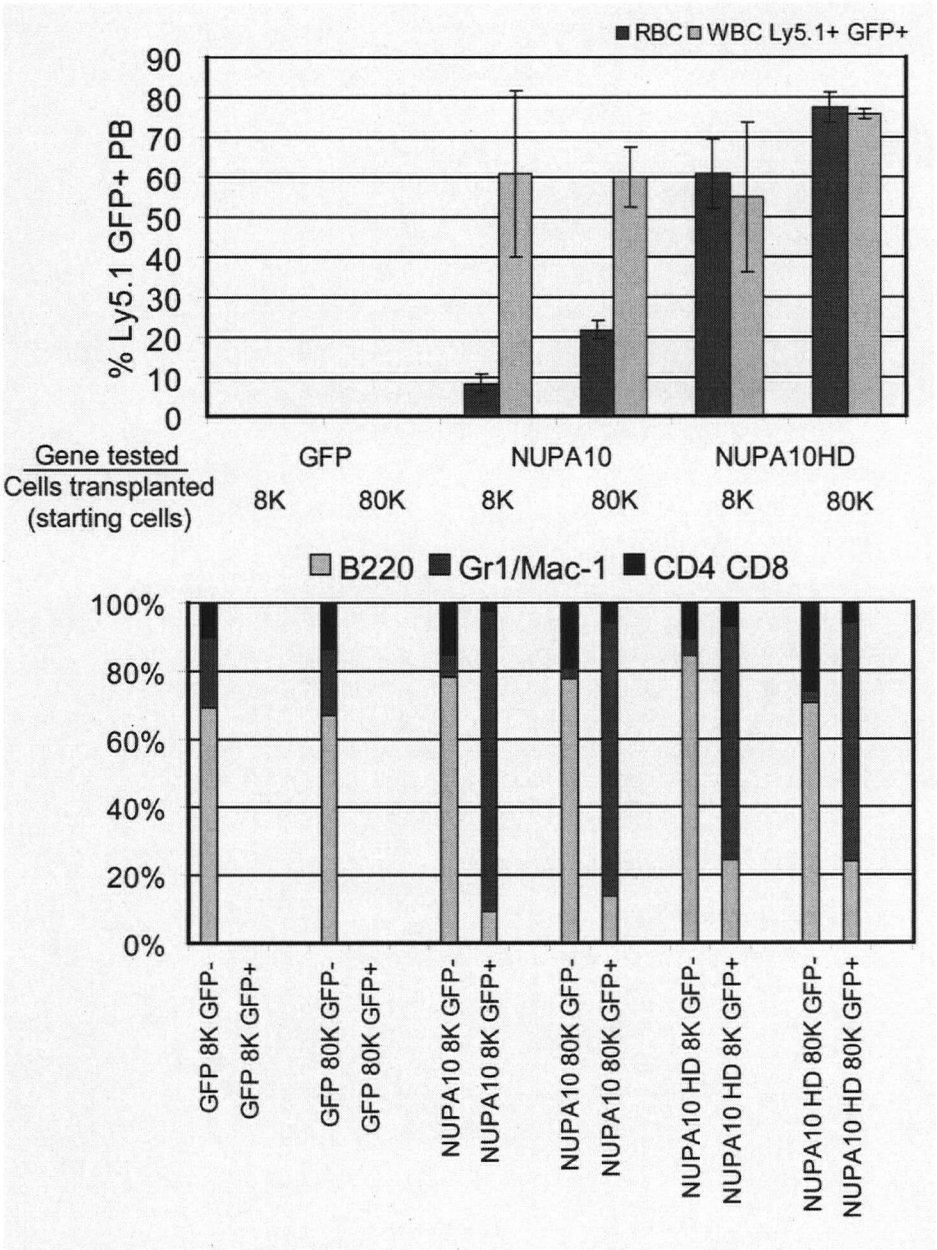


Figure 4.3 (A) PB chimerism of mice transplanted with *NUP98-HOXA10-* or *NUP98-HOXA10hd*-transduced cells after *ex vivo* expansion.
The chimerism is determined by the donor derived (Ly5.1+) GFP proportion of cells in the WBC compartment. Data was collected at 3 months post transplantation. 5-FU BM cells were prestimulated in cytokines for 2 days and transduced with oncoretroviral vectors for 2 days. At the end of the infection cells were kept in culture for an additional 7 days and then transplanted in mice that were pre conditioned with 2 Gy. In both experiments 2 doses of cells were transplanted. Five mice per arm received the progeny of 8,000 and three mice per arm received 80,000 original 5-FU cells. Red bars represent the GFP+ cells in the RBC compartment. Yellow bars represent the GFP+ cells in the WBC compartment. Mean engraftment and SD are shown.
(B) Lineage distribution in the WBC compartment. The lineage distribution was assessed at 3 months post transplantation by flow cytometry analysis. WBC were stained with antibodies that detect B lymphoid cells (anti-B220PE) T lymphoid cells (anti-CD4CD8PE) and myeloid cells (anti-Gr1PE and anti-Mac1PE). The Y axis measures the proportion of lymphoid and myeloid cells in the whole or in the GFP+ proportion of the PB.

In Figure 4.4 the outcome of this comparison is shown. The engraftment achieved with the transplantation of the progeny of 80,000 starting cells was 17% ± 0.4% and 52% ± 3% for HOXB4 and NUP98-HOXA10hd transplanted mice respectively and the engraftment achieved with the transplantation of the progeny of 260,000 starting cells was 24% ± 2% and 73% ± 2%. To confirm that the transduced cells were expressing the HOXB4 and NUP98-HOXA10hd proteins, western blot analysis was performed on BM cells and spleen cells of randomly selected mice from this experiment as shown in Figure 4.5 and 4.11.

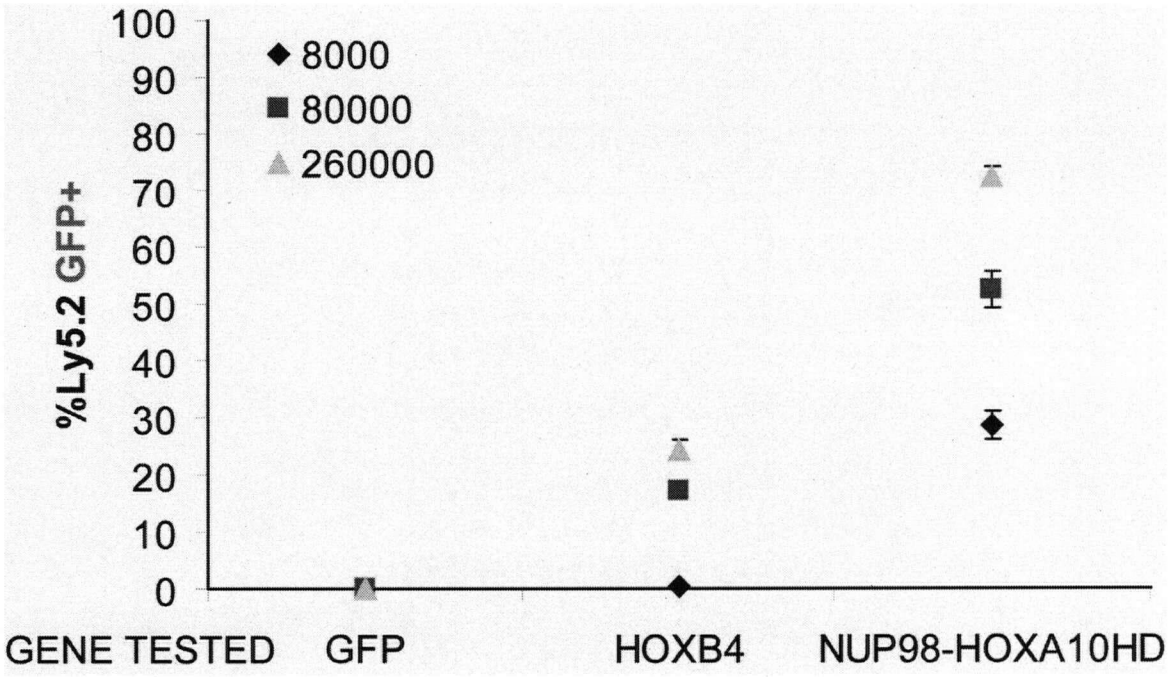


Figure 4.4 Comparison of PB chimerism of mice transplanted with *GFP*-, *HOXB4*- or *NUPA10hd*-transduced cells. The chimerism is determined by the donor derived (Ly5.2+) GFP proportion of cells in the WBC compartment. Data was collected at 6 months PT. 5-FU BM cells transduced with oncoretroviral vectors for 2 days and kept in culture for an additional 7 days. Cells were then transplanted in mice treated with 2 Gy. Three doses of cells were transplanted. Three mice per arm received the progeny of 8,000, 80,000 and 260,000 original 5-FU cells. Mean engraftment and SD are shown.

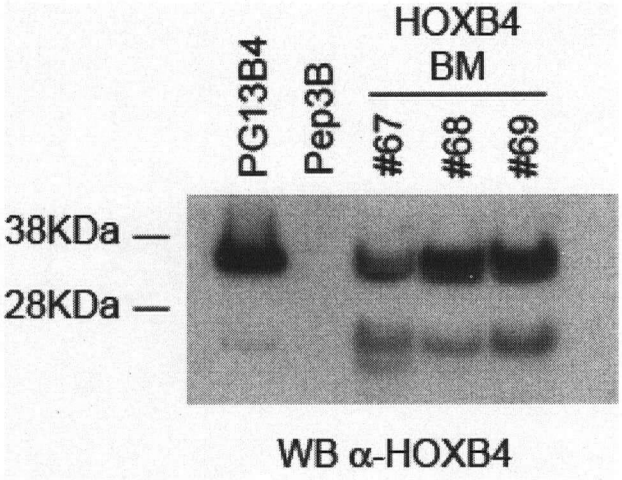


Figure 4.5 HOXB4 expression by BM cells of nonmyeloablated chimeric mice. Representative western blot analysis documenting HOXB4 expression in BM cells of nonmyeloablated mice transplanted with the progeny of 260,000 starting cells transduced with *HOXB4* and expanded in culture. Controls are HOXB4 virus producer cells (PG13B4).

In general, the engraftment achieved in mice transplanted with NUP98-HOXA10hd expanded cells is significantly higher than the engraftment achieved in mice transplanted with HOXB4 expanded cells and this difference correlates with the different HSC expansion potential of these two genes. These findings were confirmed in two separate experiments. In one of these experiments a comparison between the engraftment achieved with non expanded and expanded cells transduced with *HOXB4* or *NUP98-HOXA10hd* demonstrated that the higher engraftment achieved at the end of the *in vitro* culture is due to the enrichment in HSCs since the engraftment before expansion was minimal for both HOXB4 and NUP98-HOXA10hd (Figure 4.6)

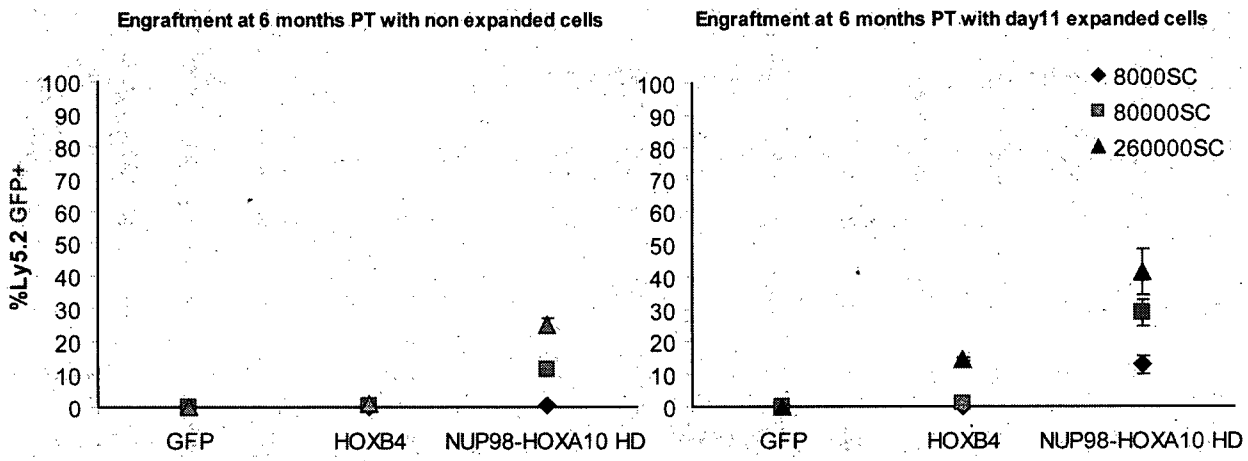


Figure 4.6 Comparison of engraftment achieved with non expanded and expanded cells.

4.2.3 The progeny of limiting numbers of *NUP98-HOXA10hd*-transduced HSCs subjected to *ex vivo* expansion can reconstitute a nonmyeloablated mouse

NUP98-HOXA10hd can stimulate over a three log expansion of HSC in culture and these cells can contribute to high level chimerism in nonmyeloablated recipients. As a further test of the potency of NUP98-HOXA10hd to stimulate HSC expansion and of the quality of expanded HSCs, additional experiments were conducted in which BM cultures were initiated at limit dilution for HSC content and subjected to transduction. After the expansion, the progeny of these transduced and expanded HSCs were tested in both myeloablated and nonmyeloablated mice. In Figure 4.7 the experimental strategy is shown. In brief, 5,000 BM cells from mice previously treated with 5-FU were plated in a 96 well plate (estimated HSC content, 1-2 HSC/well), transduced with *GFP* or *NUP98-HOXA10hd* and kept in culture for an additional 10 days. At day 14, half-well content of two randomly selected wells was transplanted into nonmyeloablated mice while the rest of the cells were transplanted into myeloablated mice in limit dilution assay to measure the HSC content of the well at the time of transplant.

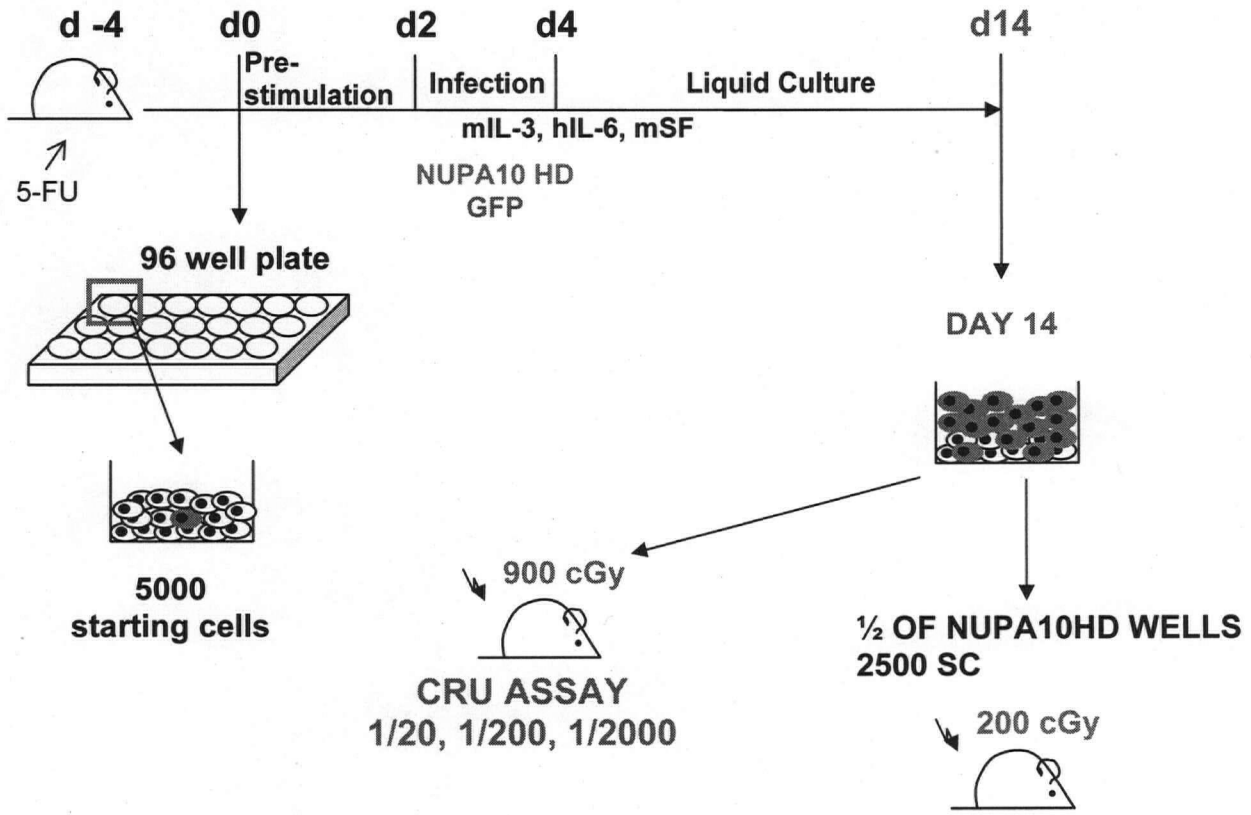


Figure 4.7 Experimental strategy used to achieve the transduction of one or two HSCs in wells containing limited numbers of 5-FU treated cells.
5,000 starting cells were inoculated into a single well of a 96 well plate, pre-stimulated for 2 days with cytokines and transduced with *NUP98-HOXA10hd* or *GFP*. At the end of 10 days culture, 2 wells were randomly chosen and half of each well transplanted in 2 Gy treated mice. The rest of the cells were transplanted in different proportions into lethally conditioned mice to assess the level of HSC expansion achieved during 10 days of culture.

In Figure 4.8 the PB GFP chimerism achieved in nonmyeloablated recipients is shown. As indicated, the progeny of limiting numbers of HSC transduced with *NUP98-HOXA10hd* were capable of giving high-level lympho-myeloid chimerism in the PB of nonmyeloablated mice (40% and 35% at 6 months post transplantation respectively) (Yellow dots in Figure 4.8). These levels of chimerism are consistent with the high HSC content of wells documented by limit dilution assay (over 2,000 HSCs per well) (Red dots in Figure 4.8). Importantly, control *GFP*-transduced cultures started with 100,000

cells and mice transplanted with proportions of these cultures, did not achieve significant chimerism (1-3%).

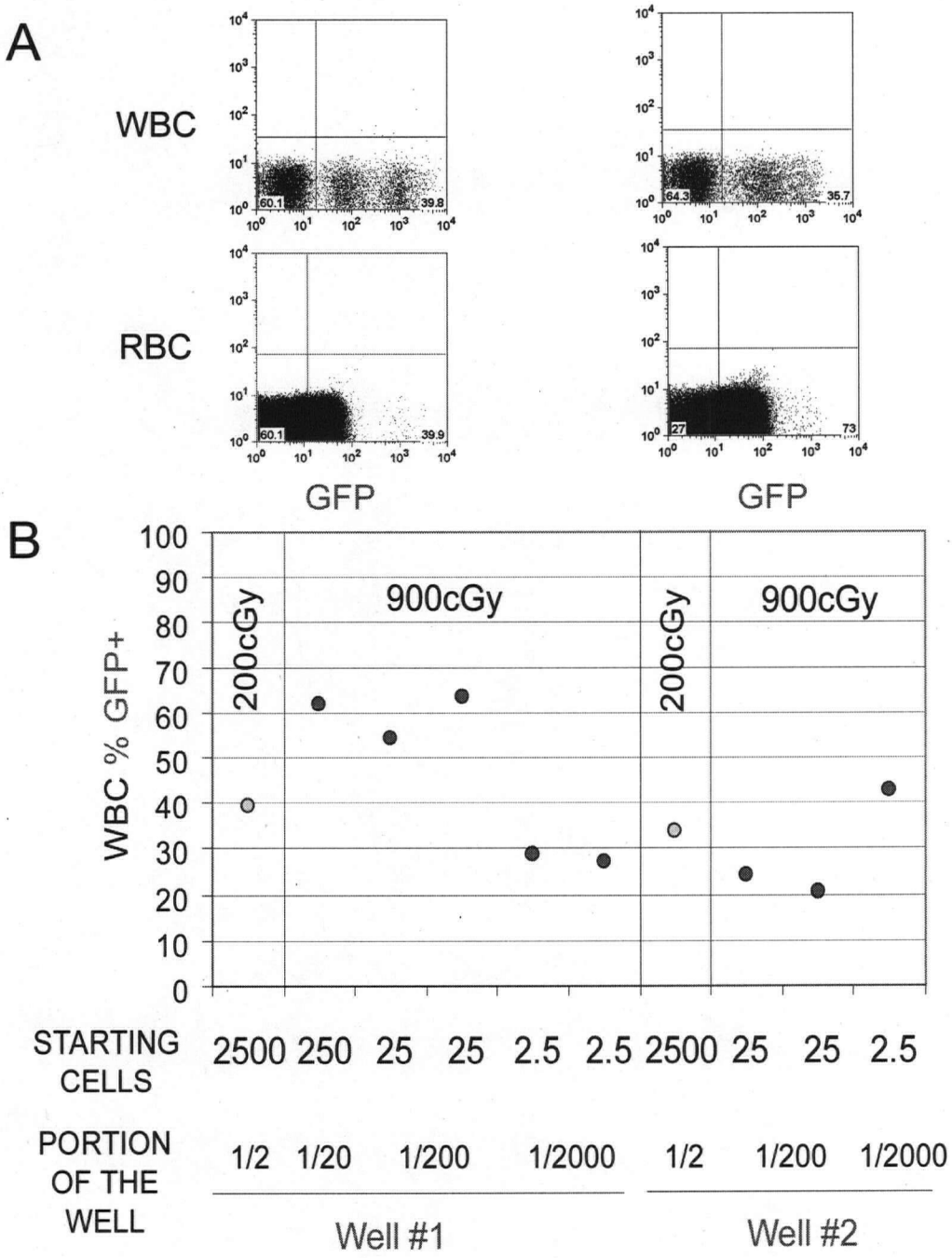


Figure 4.8 PB chimerism of mice transplanted with the progeny of one or two *NUPA10hd*-transduced cell. (A) Flow cytometry profiles of the WBC and RBC compartment of mice treated with 2 Gy and transplanted with 2,500 starting cells transduced with *NUPA10hd* and expanded in vitro for 10 days. **(B)** Summary of the PB WBC chimerism in nonmyeloablated and myeloablated mice. The graph represents the GFP chimerism in the WBC compartment. Data was collected at 6 months post transplantation.

The reconstitution of the nonmyeloablated mice represented in Figure 4.8 was lymphomyeloid and representative flow cytometry profiles of the lineage distribution in the PB is shown in Figure 4.9.

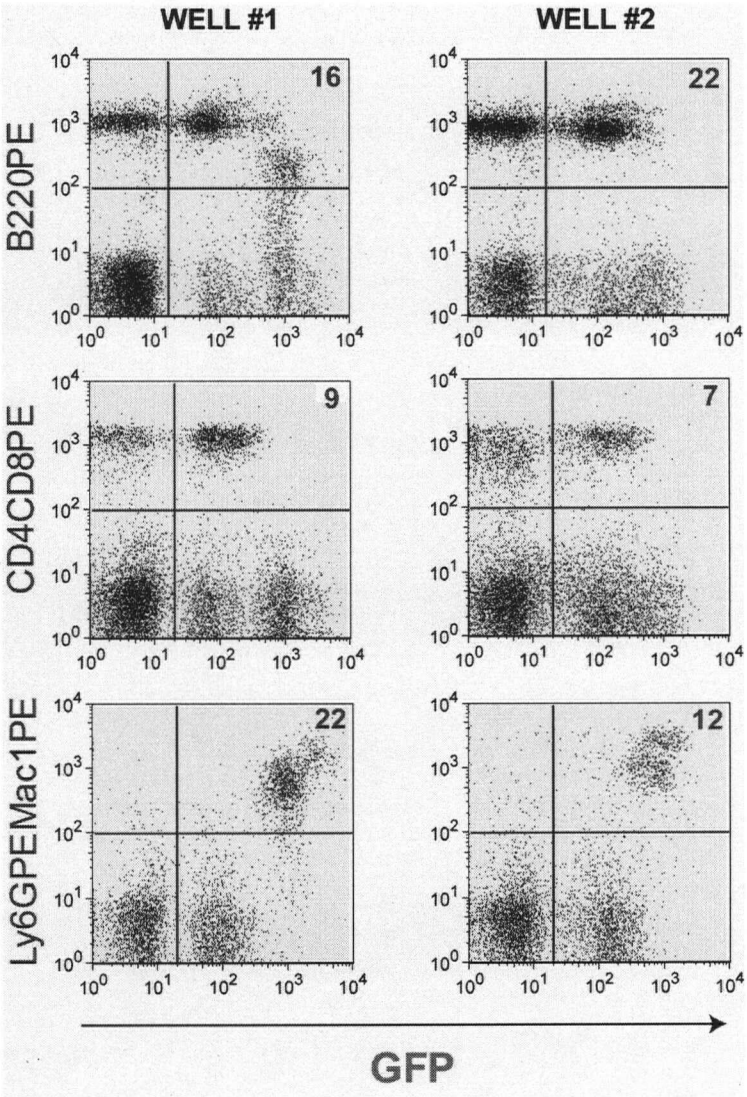


Figure 4.9 Lineage distribution in the PB of mice transplanted with the progeny of few HSCs transduced with *NUPA10hd*.
WBC were stained with antibodies that detect B lymphoid cells (anti-B220PE) T lymphoid cells (anti-CD4CD8PE) and myeloid cells (anti-Gr1PE and/or anti-Mac1PE). The abscissa indicates the GFP fluorescence intensity and the ordinate indicates levels of detected expression for a particular cell surface lineage marker, B220 and CD4CD8 for lymphoid cells, Gr1 (Ly6G) and Mac1 for myeloid cells. Data was collected at 6 months post transplantation.

BM was aspirated from one mouse at 7 months PT and plated in CFC methylcellulose assay. Twelve days later, colonies were visualized, plucked and put in culture for expansion. DNA was extracted from expanded clones and Southern blot analysis carried out to visualize the pattern of integration of the virus. Altogether DNA from 23 colonies was collected and analysed and five unique patterns identified (Figure 4.10). However, from careful observation of the size of the bands detected it seems that there are only two major patterns that acquired subsequent viral integrations arguing that the engraftment was contributed by at most two original HSCs. The results of western blot analysis performed on spleen cells from primary recipients additionally confirmed the expression of the integrated *NUP98-HOXA10hd* (Figure 4.11).

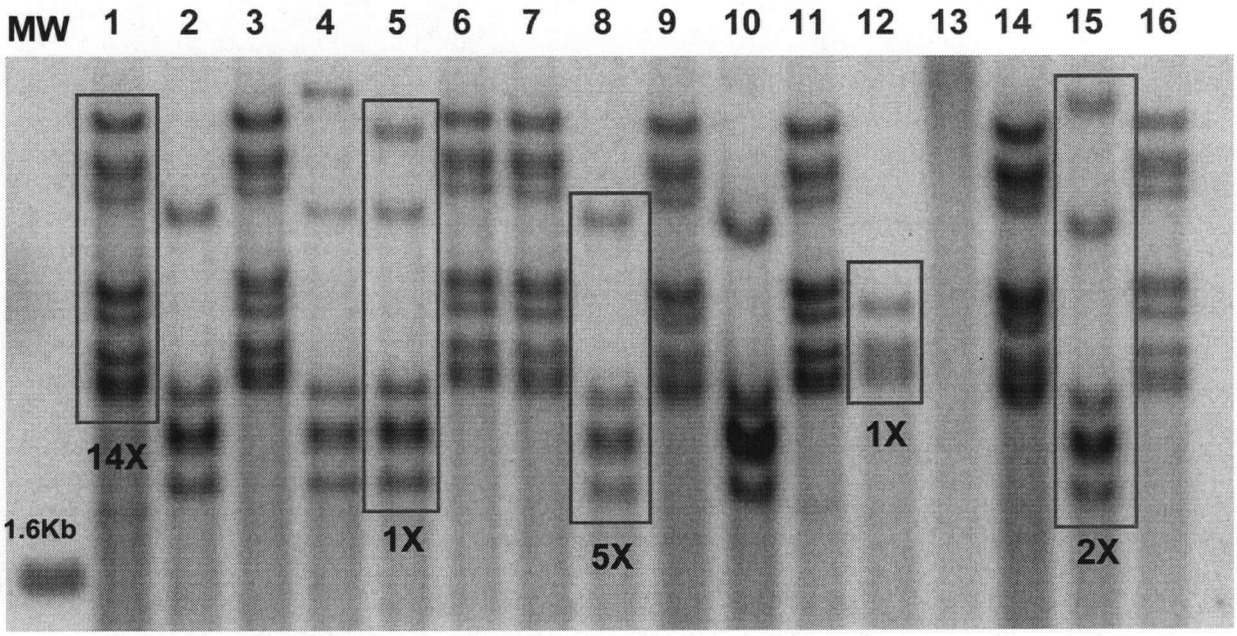


Figure 4.10 Composition of the HSC pool of a mouse transplanted with the progeny of few starting HSCs. Southern blot analysis of genomic DNA from CFC colonies derived from the BM of a mouse treated with 2 Gy and transplanted with the progeny of 2,500 starting cells. BM was aspirated from the primary recipient (Well #2, 2,500 starting cells, Figure 4.8), 7 months post transplantation. The bands visualized in the figure represent specific integrations of the retroviral vector. The numbers at the bottom of the red squares represent the frequency of appearance of a particular pattern of integration of the virus amongst the total of colonies/patterns analyzed.

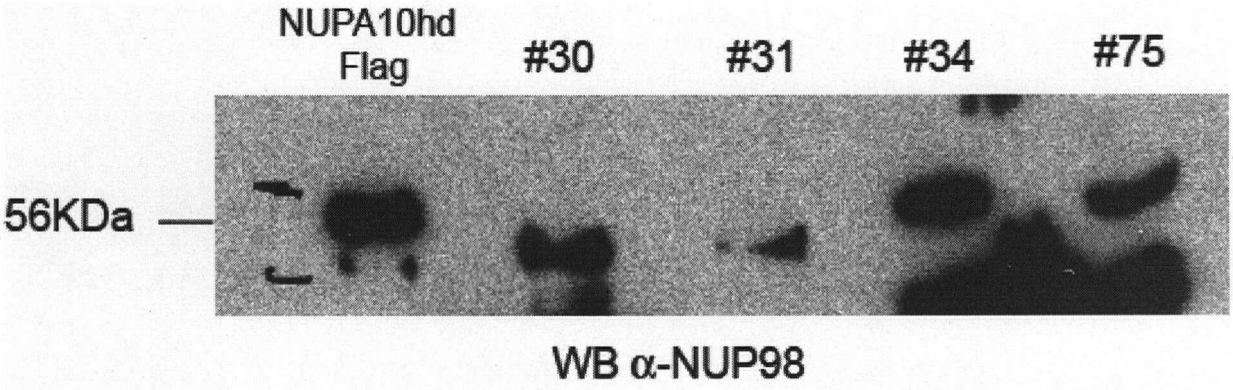


Figure 4.11 NUPA10hd fusion protein expressed by the progeny of transduced cells. Representative WB to assess the protein expression from spleen cells of mice transplanted with *NUPA10hd*-transduced and expanded cells. Recipient mice 30, 31 were myeloablated with 9 Gy and transplanted with the progeny of 25 starting cells (well #1, Figure 4.8). Mouse 34 and 75 were treated with 2 Gy and received the progeny of 2,500 starting cells (well #2, Figure 4.8) and 260,000 starting cells (Figure 4.4) respectively.

Southern blot analysis from whole BM of lethally irradiated mice collected at 7 months PT confirmed that mainly two clones contributed the reconstitution of these mice transplanted at limit dilution (Figure 4.12).

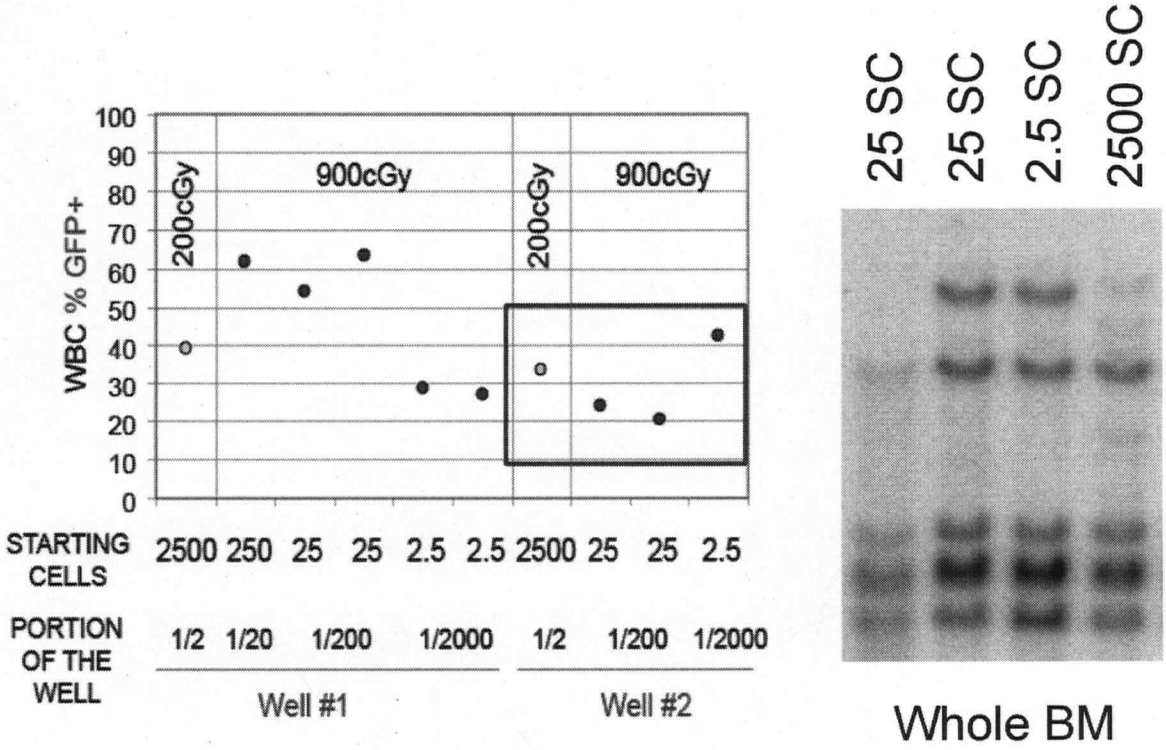


Figure 4.12 Analysis of the composition of the HSC pool in the BM of lethally conditioned mice. Southern blot analysis visualizing the viral integrations in BM cells of recipients treated with 9 Gy and transplanted with small proportions of well #2 (the progeny of 25 and 2.5 starting cells) is shown. BM was collected 7 months PT. Whole BM from a nonmyeloablated mouse recipient of the progeny of 2,500 starting cells from the same well is also shown. BM from this mouse was collected 11 months PT. BM from this mouse collected at 7 months PT was used in clonogenic progenitor assay to produce the CFC colonies shown in Figure 4.10.

4.3 Discussion

Results presented in the previous chapter based on engineered overexpression of HOXB4 provide proof of principle evidence that methods to expand HSCs *in vitro* are a potentially powerful strategy for increasing chimerism in nonmyeloablated transplant recipients. Findings reported in this chapter confirm and extend these conclusions through the demonstration of the remarkable potency of various NUP98-HOX fusions to stimulate even higher levels of *ex vivo* expansion compared to HOXB4 with attendant improvements in chimerism. *HOXB4*- and *NUP98-HOX*-transduced cells performed equivalently (and to *GFP* control transduced cells) when transplanted immediately after

infection (i.e. without extended culture) thus arguing that their beneficial effect in the nonmyeloablative setting is dependent on extended culture and attendant *ex vivo* HSC expansion. This is further argued by the strong correlation between the demonstrated magnitudes of HSC expansion *in vitro* and resultant chimerism levels following transplantation of cultured cells. Such findings add to the evidence that chimerism levels in nonmyeloablative recipients is largely the result of the quantitative competition between transplanted HSCs and the endogenous surviving HSCs and further encourages the optimization and application of *ex vivo* HSC expansion strategies.

In this light, the additional findings obtained using a NUP98-HOX fusion restricted to the 60 amino acid homeodomain of HOXA10 are of interest. NUP98-HOXA10hd retains the potency of NUP98-HOXA10 which has additional sequences flanking the homeodomain. The potency of NUP98-HOXA10hd is dramatically illustrated by the demonstration of high level chimerism achievable with the progeny of cultures set up at or near limit dilution for HSCs. Moreover, for reasons not yet understood, in both the studies reported here and in previous studies in the ablated setting (Ohta et al., 2007), *NUP98-HOXA10hd*-transduced HSC have improved functional characteristics as evidenced by heightened contribution to red blood cell production. In our studies, again consistent with previous analyses, *NUP98-HOXA10hd*-transduced cells, either without or following *ex vivo* expansion did not manifest any leukemogenic potential.

Together these findings encourage further optimization and application of HSC expansion methods building on the potent properties of NUP98-HOXA10hd. In this regard the recent development of protein delivery methods to achieve HSC expansion with HOXB4 (e.g. by production of TAT-HOXB4 fusions) suggest that tests of TAT-NUP98-HOXA10hd fusion proteins will be of interest as a way to avoid genetic

manipulation of target cells. The current results also should stimulate efforts to test such molecules on human hematopoietic stem cells.

CHAPTER 5 *EX VIVO* EXPANDED HSCs IN THE TREATMENT OF BETA THALASSEMIA IN NONMYELOABLATIVE CONDITIONS

5.1 Introduction

Thalassemia and Sickle Cell Disease (SCD) are widespread genetic blood disorders that are life threatening if not treated. The most used life-saving treatment for such pathologies is RBC transfusions that allow for the replacement of malfunctioning RBC with healthy ones. This is a life-long treatment that has to be performed repeatedly because the HSCs that carry the genetic mutation keep replenishing the PB with defective RBC. Blood transfusions carry the undesired side effect of iron overload that require further treatment of the patient with iron chelating therapies. Since HSCs are responsible for perpetuating the disease, a treatment that allows for the replacement of such pluripotent cells with healthy ones, would allow for the ultimate cure of affected patients. The only curative treatment currently available is allogeneic HSCT, but the successful outcome of this procedure highly depends on the age and general medical condition of the patient and an adequate histocompatibility match between the donor and the recipient. Unfortunately, only ~30% of patients have a suitable family donor of HSCs and for unrelated donors the disease free survival has been reported to be 69% (Schrier and Angelucci, 2005). Furthermore, many people affected by β-thalassemia or SCD are not suitable candidates for myeloablative transplantation because of the advanced status of the disease and because of the related morbidity and mortality associated with myeloablation. Reports on the outcome of conventional myeloablative transplantation on thalassemic and SCD patients showed that some patients developed mixed chimerism (simultaneous presence in the BM of cells of donor and host origin) with some patients having only 20% donor cells.

Although mixed chimerism after HSCT is a risk factor for rejection, it was however sufficient to resolve the thalassemic and SCD manifestations (Andreani et al., 2000; Walters et al., 2001). Consequently this observation led to the realization that even a less toxic conditioning regimen might be sufficient to achieve minimal engraftment decreasing the transplant related morbidity and mortality associated with myeloablative transplantation (Gaziev and Lucarelli, 2005). The challenge in a nonmyeloablative transplantation setting is that HSCs from the donor have to outcompete the endogenous surviving population of HSCs of the recipient and to be able to achieve higher levels of mixed chimerism, the ratio of donor to host HSCs should be increased. Therefore, methods to achieve expansion of donor HSCs would be extremely beneficial in this setting.

Over the past decade, many of the mutations causing blood disorders have been characterized at the molecular level. This has allowed for the development of gene therapy strategies by which a functional gene is transferred with viral vectors into the genome of HSCs from affected patients. These corrected HSCs are subsequently transplanted back to the patients, are capable of giving rise to the differentiated cells in the PB and are afterward potentially functional (Klein and Baum, 2004). Therefore another attractive treatment possibility for thalassemia and SCD would be autologous transplantation of genetically corrected HSCs, which would avoid the complications associated with histocompatibility mismatches between donors and recipients. This genetic approach has been considered since the early 1990s to treat blood genetic disorders such as severe combined immunodeficiencies (SCID) and metabolic diseases such as chronic granulomatous disease (Bordignon et al., 1995; Cavazzana-Calvo et al., 2000; Ott et al., 2006) and is now increasingly considered for the treatment of hemoglobinopathies. The success of this genetic approach has been hampered by

technical difficulties. Amongst these is the difficulty to achieve sufficient gene transfer levels in HSCs, to achieve the appropriate regulation of the expression of the transferred gene, especially in the field of globin gene transfer (Sadelain, 2006) and the undesired integration of the transferred gene in or near oncogenic sequences leading to the development of malignant transformation (Hacein-Bey-Abina et al., 2003). Another major hurdle of gene therapy applications is the massive loss of HSCs caused by extended *ex vivo* culture required for the genetic modification. Nevertheless, there has been in recent years considerable progress in this field. Many studies in mouse models of β-thalassemia and SCD have now demonstrated, after many years of struggles with the optimization of viral vectors, the feasibility of permanently transferring a functional β-globin gene into the genome of HSCs of affected mice and stably expressing hemoglobin at a therapeutic level in RBC of reconstituted mice (Imren et al., 2002; May et al., 2000; Pawliuk et al., 2001). The number of cells transplanted in these mice was quite low but the amount of corrected HSCs retrieved at the end of the genetic manipulation was sufficient to achieve a therapeutic level of Hemoglobin A production in RBC and correction of the thalassemic or SCD phenotype. This was possible because a myeloablative treatment before HSCT was used. Unfortunately, if real patients affected by the same disorders were selected for gene therapy pre-clinical trials, very few would be eligible for conventional myeloablative HSCT. This is mainly because many of the patients affected by hemoglobinopathies are older patients and suffer from many of the devastating effects of the disease such as treatment-related organ damage due to prolonged exposure to iron overload (Gaziev and Lucarelli, 2005). In order for these patients to benefit from gene therapy applied to autologous HSCT, milder conditioning regimens have to be used but in such a setting, the major problem is that the modified population of HSCs have to out-compete the large endogenous

HSC population that is spared by the nonmyeloablative treatment. Therefore, methods to expand corrected HSCs after the *in vitro* manipulation would have a major impact and benefit in this field. As shown in the previous chapters, engineered overexpression of HOXB4 or NUPHOX fusion proteins offer a powerful strategy for expanding HSCs *ex vivo* and would offer major advantages in the recovery of HSCs if coupled to gene therapy protocols. In this chapter I have tested whether this approach would enable HSCs to be obtained in sufficient numbers and with retention of sufficient repopulating ability to obtain cures in nonmyeloablated mice with severe β-thalassemia caused by the homozygous deletion of the β-major globin gene (Goldberg et al., 1986; Skow et al., 1983).

5.2 Results

5.2.1 HOXB4 expanded HSCs contribute to high level chimerism and improvement of hematological parameters in the PB of nonmyeloablated β-MDD mice

Autologous transplantation of genetically corrected HSCs using nonmyeloablative conditioning holds great promise for safely treating patients with hemoglobinopathies. This approach, however, is challenged by the large doses of HSCs required to achieve therapeutic levels of chimerism. Engineered overexpression of HOXB4 offers a powerful strategy for expanding HSCs *ex vivo*. In these experiments, to model genetically corrected cells, day 4 5-FU BM cells were harvested from congenic healthy donors and transduced with an MSCV-HOXB4-IRES-GFP virus. Cells were cultured for 10 days following transduction and the progeny of 200,000 starting

cells were transplanted into 3 β-thalassemic (β-MDD) and 4 normal recipients previously given 2 Gy. Figure 5.1 shows a schematic of the experimental design.

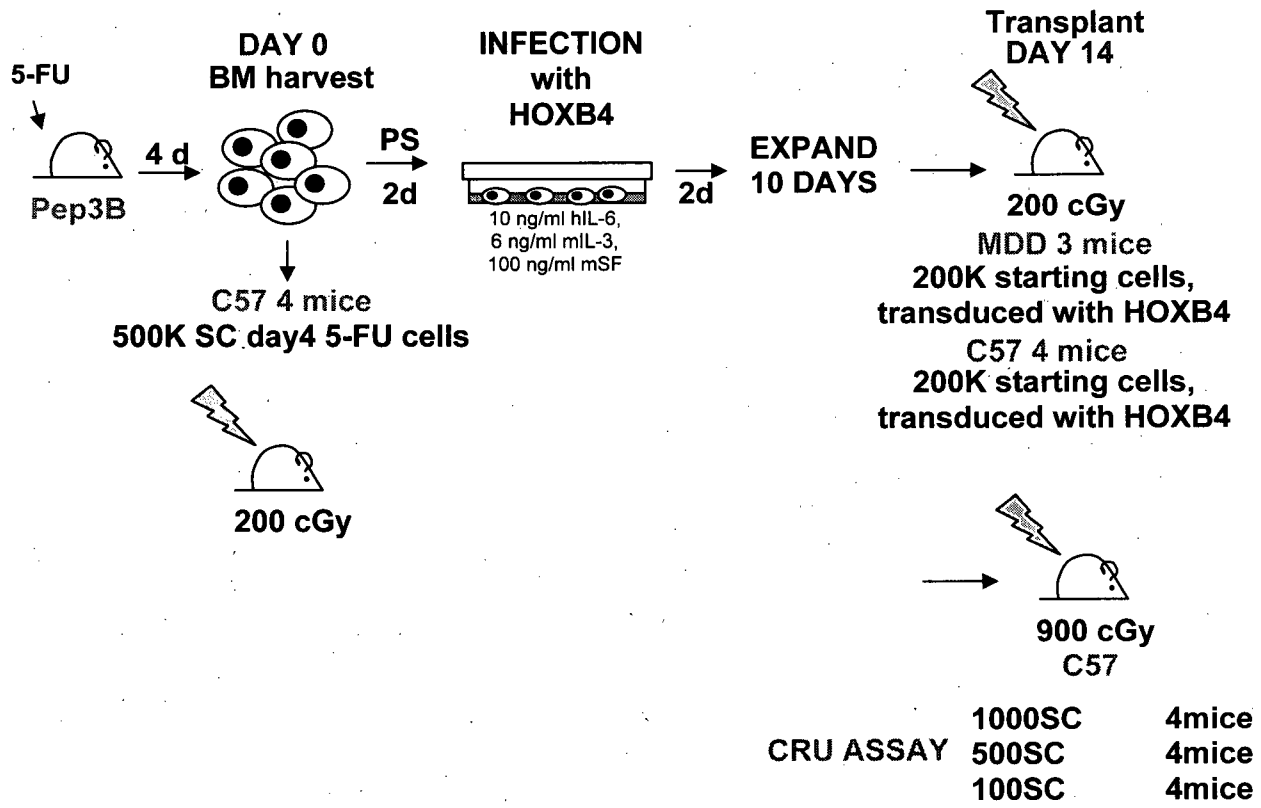


Figure 5.1 Experimental strategy used to transplant β-MDD mice with HOXB4 expanded HSC.

As a control, 500,000 freshly harvested day 4 5-FU BM cells were transplanted into 4 similarly conditioned normal mice. Significant chimerism was not achieved in these mice (1-3% Ly5.1+ in WBC at 5 months). In contrast, all 4 normal recipients of *ex vivo* expanded *HOXB4*-transduced cells exhibited stable, high level chimerism (21 ±6% GFP+ WBC at 5 months). Significant chimerism was also achieved in all 3 β-MDD recipients (18-77% GFP+ RBCs at 10 weeks) (Figure 5.2) and, in 1 of these, the chimerism was sustained at a high level at 5 months PT (34% GFP+ WBCs and 52%

GFP+ RBCs). The sustained donor derived PB chimerism at 5 months post transplant was also associated with substantial improvement in the hematocrit level (36% versus 23% in untreated β-MDD) and hemoglobin production (10.5 g/dl versus 5 g/dl) (Figure 5.3).

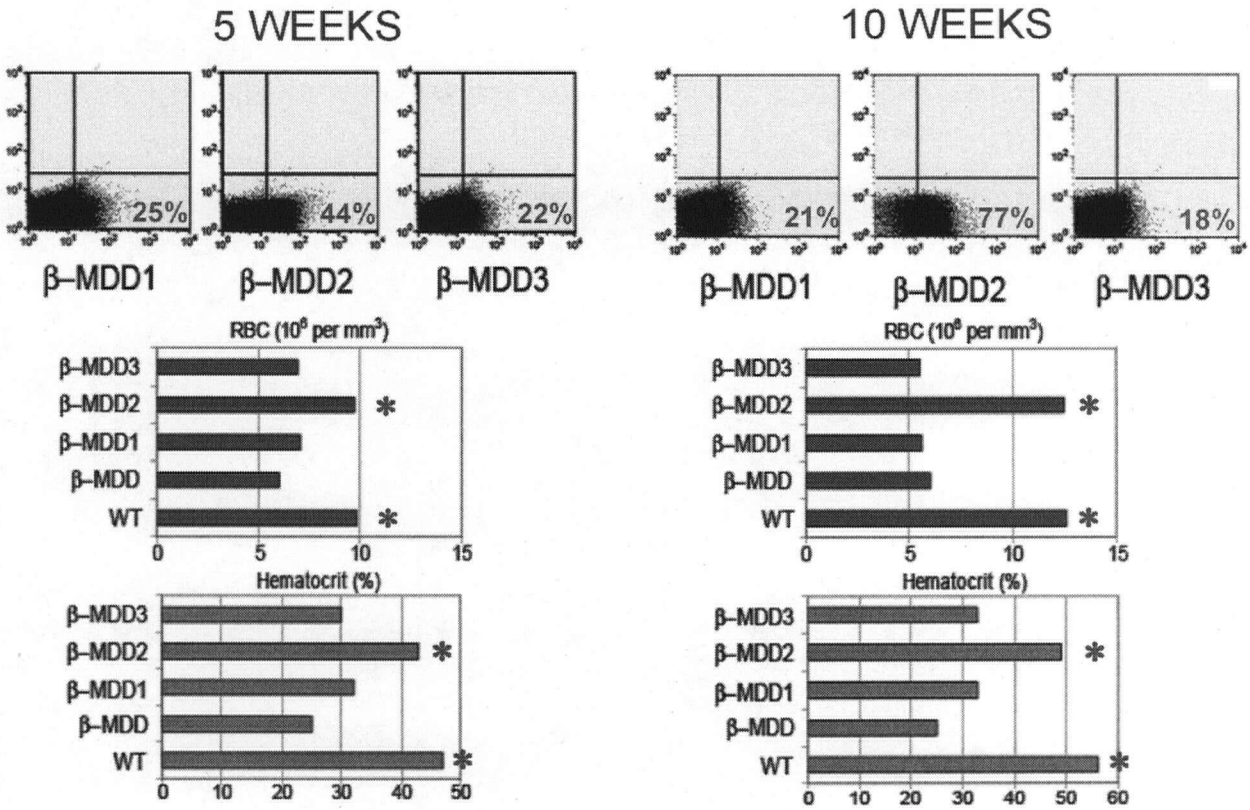


Figure 5.2 RBC GFP chimerism in β-MDD mice receiving *HOXB4*-transduced and expanded HSCs.
Analysis of the PB was performed at 5 (left side of the figure) and 10 weeks (right side of the figure) PT. Flow cytometry profiles in the top section of this figure show the proportion of GFP+ transduced cells in the PB.
In the bottom side of this figure RBC numbers and hematocrit level is represented.
Values shown are of a WT mouse, an unmanipulated β-MDD mouse and of the three mice transplanted with *HOXB4*-transduced and expanded HSCs. The asterisks indicate that the hematocrit values and RBC numbers are within normal range.

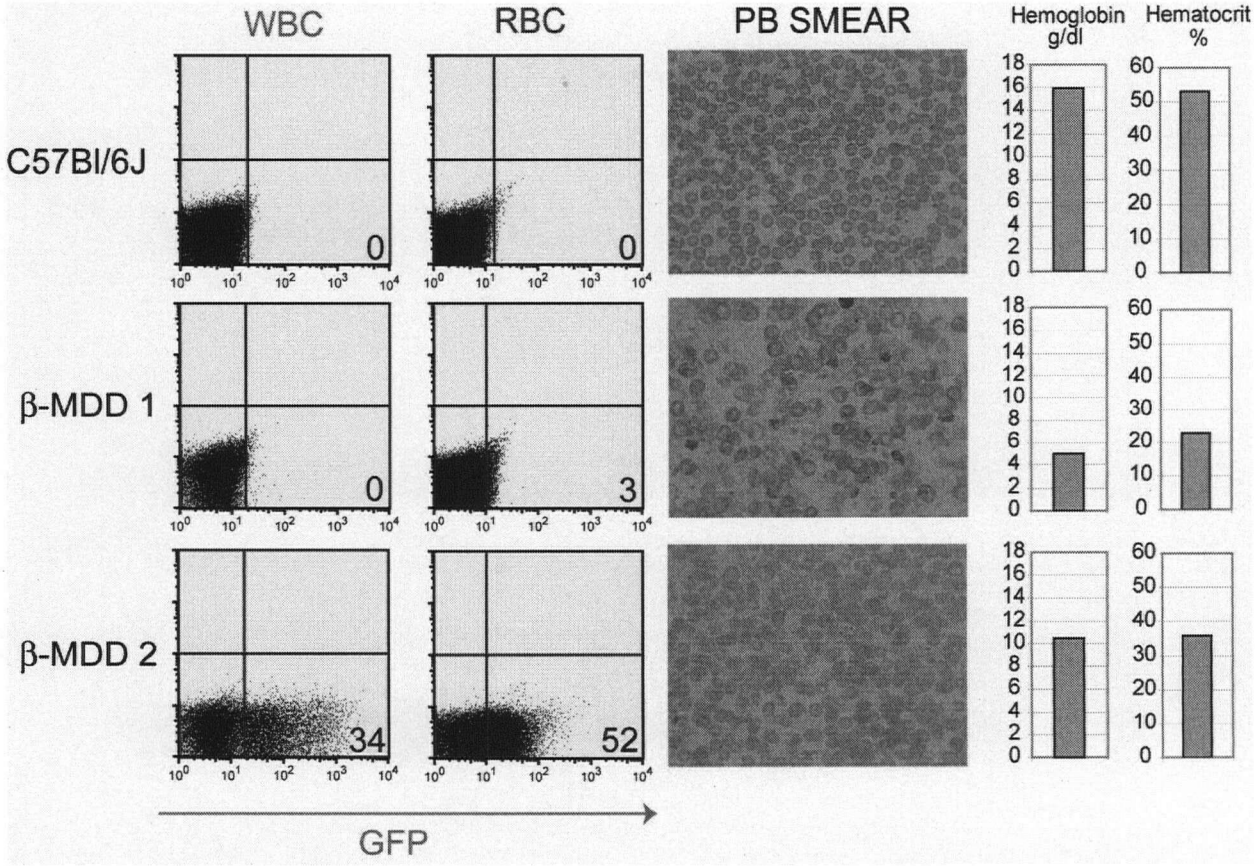


Figure 5.3 β-MDDs PB chimerism, phenotype and hematological parameters contributed by *HOXB4*-transduced and expanded HSCs.
β-MDD mice were transplanted with 200K starting cells *HOXB4*-transduced and expanded. This graph shows the chimerism and phenotype of the PB at 5 months post transplantation. The values for a WT mouse are also shown for comparison.

Furthermore, correction of hematological parameters was evidenced by a significant elevation in RBC numbers as well as a dramatic reduction in reticulocyte numbers (down to 4 % from 25%). Morphologic examination of blood smears from this recipient showed a marked improvement in RBC anisocytosis (irregular size), poikilocytosis (irregular shape), and polychromasia (variation of the hemoglobin content of erythrocytes) and showed that more than 80% of the RBCs were normochromic (normal color) and normocytic (normal size and shape) (Figure 5.3).

5.2.2 PB chimerism in β-MDD mice is contributed by a polyclonal population of HSCs

When dealing with oncoretroviral transduction, insertion of the transgene in sequences with oncogenic potential could lead to the emergence of HSC clones with hyperproliferative capacity and to risk of malignant transformation. In the protocol that was used for the experiments described in this and the previous chapters, clones that have potentially acquired hyperproliferative capacity, could take over during the *ex vivo* culture of transduced cells or *in vivo*, after transplantation. This would result in hematopoiesis derived from only one or few clones expanded *in vitro*. In order to determine the composition of the HSC pool expanded *in vitro* and demonstrate the safety of this approach, we performed proviral integration analysis on individual methylcellulose progenitor colonies derived from the BM of the β-MDD recipient that had the highest chimerism throughout the experiment (Figure 5.4).

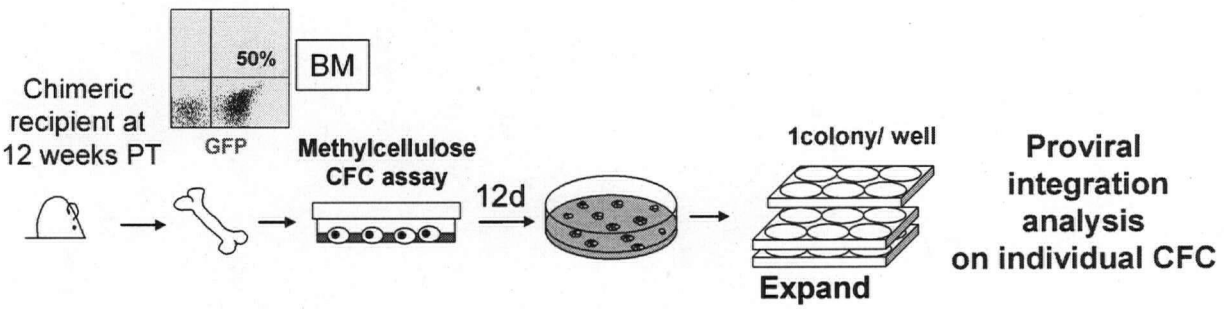


Figure 5.4 Experimental strategy used to assess proviral integrations in CFC colonies.
BM of the chimeric β-MDD recipient was aspirated at 12 weeks post transplantation and plated in methylcellulose CFC assay. Genomic DNA derived from CFC colonies was processed as described in material and methods and analysed for proviral integrations through Southern blot analysis.

At the time of aspiration (12 weeks post transplantation) the chimerism in the BM of this mouse was 50%. DNA was extracted from expanded clones and Southern blot analysis allowed us to establish the presence of a polyclonal population of HSC through the

visualization of different patterns of integration of the virus in different clonogenic progenitors derived from unique HSC clones. Figure 5.5 shows a representative Southern blot showing different specific patterns of integration corresponding to different unique clones.

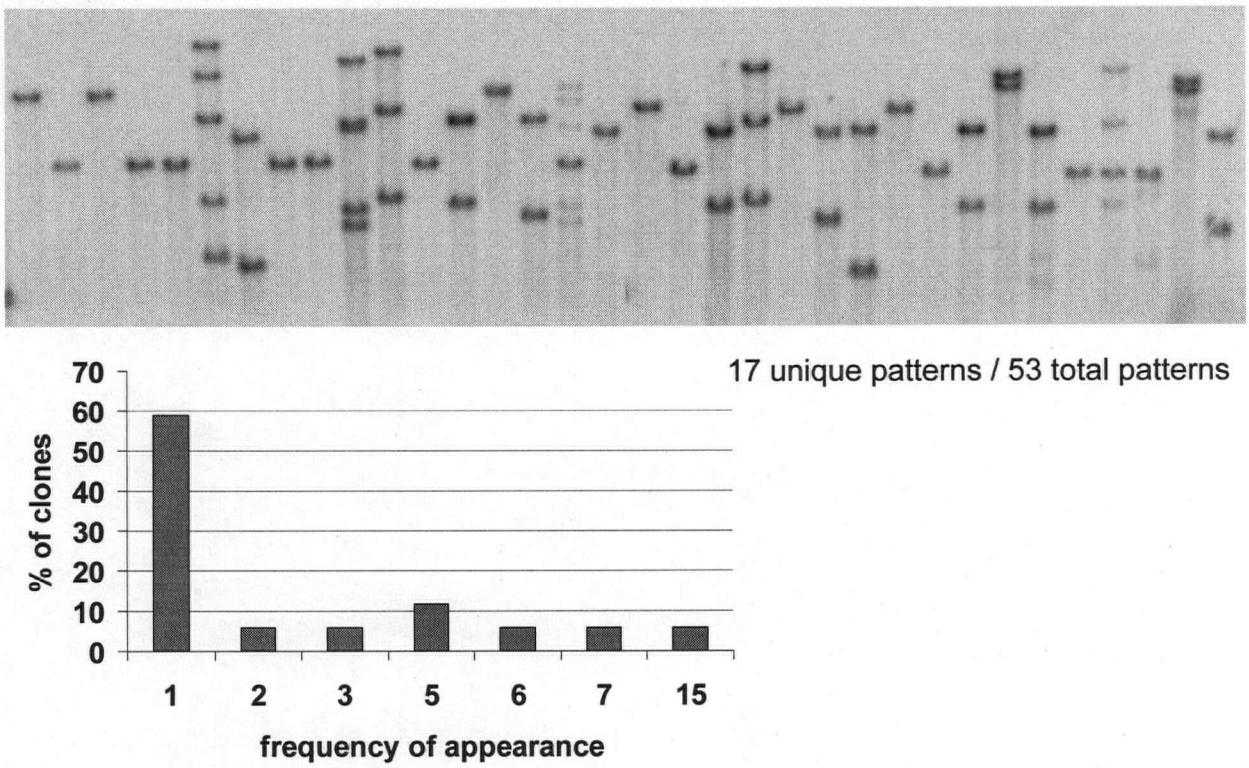


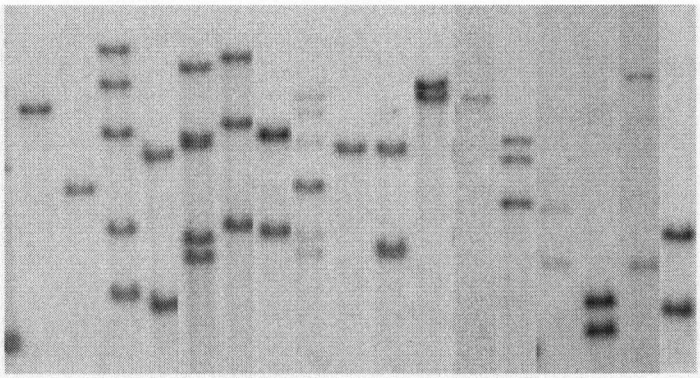
Figure 5.5 Chimerism in β-MDD mice receiving *HOXB4*-transduced and expanded cells is contributed by a polyclonal population of HSCs.
In the top panel of this picture is a representative southern blot showing unique patterns of proviral integration. The bottom graph shows a summary of the frequency of appearance of different clones.

Altogether we identified 17 unique patterns/clones out of 53 colonies analyzed. The graph in the bottom part of Figure 5.5 shows a summary of the frequency of appearance of different clones. Only 7 clones were found more than once, while the

majority (10 clones) was found only once. This indicates that the population of HSCs contributing to the hematopoiesis was polyclonal and that multiple transduced HSCs were expanded in the culture with similar probabilities with few clones expanded with higher rate.

5.2.3 Retroviral integration site analysis confirm the uniqueness of HSCs clones

To further confirm that different bands visualized in the Southern blot corresponded to specific integrations in different sites of the genome and to identify the sequences in the genome where they integrated, linker mediated amplification of retroviral integration sites was performed by Patricia Rosten, as described in chapter 2. This technique allows identification of specific sites of integration of the retroviral vector in the genome. This analysis allowed us to identify 21 unique integations from 14 clones (Figure 5.6).



A B C D E F G H I L M N O P Q R S

Identity of MSCV HOXB4 IRES GFP integration sites

Accession n.	Chromosomal band	Ref Seq gene name	Proposed function	Site of integration (bp)	Proviral integration*	
-	11qE2	intergenic	-	119,742,337	-	A
NM_013672	15qF3	**Promoter of Sp1	TF	102,232,889	R	B
NM_029640.1	15qD3	NIK IKK (beta) bp isoform1	adaptor protein	72,799,478	R	C
NM_008845.2	2qA3	Pip5k2a	Kinase	18,880,992	R	C
-	13qA4	intergenic	-	43,202,242	-	C
-	3qC	mRNA AK042136	unknown	51,468,061	F	D
-	15qD1	intergenic	-	63,448,410	-	D
NM_026353.2	15qF1	4930570C03Rik	unknown	97,615,464	R	E
NM_027900.3	10qD3	R3hdm2	Nucleic acid binding	126,885,153	F	E
NM_028356.1	12qC3	Zbtb25		77,285,581	F	E
NM_008494.2	5qG2	**Promoter of Lnfg	Transferase	140,850,726	-	F
NM_011295	10qA3	**Promoter of Rps12	Rb pt	23,478,146	F	L
-	2qH1	intergenic	-	157,852,628	-	L
NM_013632	14qC1	**Promoter of Pnp	Transferase	49,865,995	F	H
NM_021419	17qA3.3	Rnf8	ligase	29,123,276	F	M
NM_008112.4	13qA1	**Promoter of Gdi2	inhibitor	3,536,981	F	O
-	2qE1	intergenic	-	90,413,818	-	P
NM_001033279	17qA3.3	D17Wsu92e	unknown	27,512,018	R	Q
-	2qA3	intergenic	-	24,986,913	-	R
NM_026350	8qC3	**Promoter of Ccdc130	unknown	87,164,202	F	R
-	6qG1	intergenic	-	136,864,925	-	S

*With respect to transcriptional direction of the gene (F) Forward (R) Reverse

**Integration happened up to 10Kb upstream of a gene

Figure 5.6 Identity of the sites of integration of the MSCV HOXB4 IRES GFP vector

The top panel shows the Southern blot and the clones that have been selected for proviral integration site analysis in red boxes. Each clone is also identified with a letter. The bottom table shows the exact location of the site of integration in the genome.

Roughly, 30% of the integrations were intergenic, 30% were in introns of genes and 30% were in promoter regions (these included integrations up to 10Kb upstream of a gene). Of the genes in which the integration occurred, only 5 had known functions, while the others are still uncharacterized. The integration of the vector in different sites of the genome confirms that the chimerism is contributed by a polyclonal population of cells.

5.2.4 NUP98-HOX expanded HSCs contribute to high level chimerism and cure of nonmyeloablated MDD mice

As a further test of the utility of *ex vivo* expanded HSCs, another experiment was carried out using the more potent *NUP98-HOXA10hd* gene to stimulate high level expansion prior to transplantation. Day 4 5-FU BM cells were harvested from congenic healthy donors and transduced with an MSCV-NUPA10hd-IRES-GFP virus. Cells were cultured for 7 days following transduction and the progeny of 300,000 starting cells were transplanted into 2 β-MDD and 4 normal recipients previously given 2 Gy. As a control, 500,000 freshly harvested day 4 5-FU BM cells were transplanted into 4 similarly conditioned normal mice and these mice as in the previous experiment, did not achieve significant chimerism (2%±1% Ly5.1+ in WBC at 5 weeks post transplantation). In contrast, all 4 normal recipients of *ex vivo* expanded *NUP98-HOXA10hd* -transduced cells exhibited stable, high level chimerism (50±4% GFP+ WBC at 5 weeks). Significant chimerism was also achieved in 2 β-MDD recipients (84-82% GFP+ RBCs at 5 weeks) and, in both of these, the high chimerism resulted in cure of the thalassemic phenotype and in the correction of hematologic parameters to normal range (Figure 5.7). Limit dilution assay quantification of HSCs at 14 weeks post transplantation, confirmed that

there was a 700-fold increase in HSCs in the culture after 10 days of *ex vivo* expansion and the HSC frequency on day 10 was 1 in 7 with a range of 1/14-1/4.

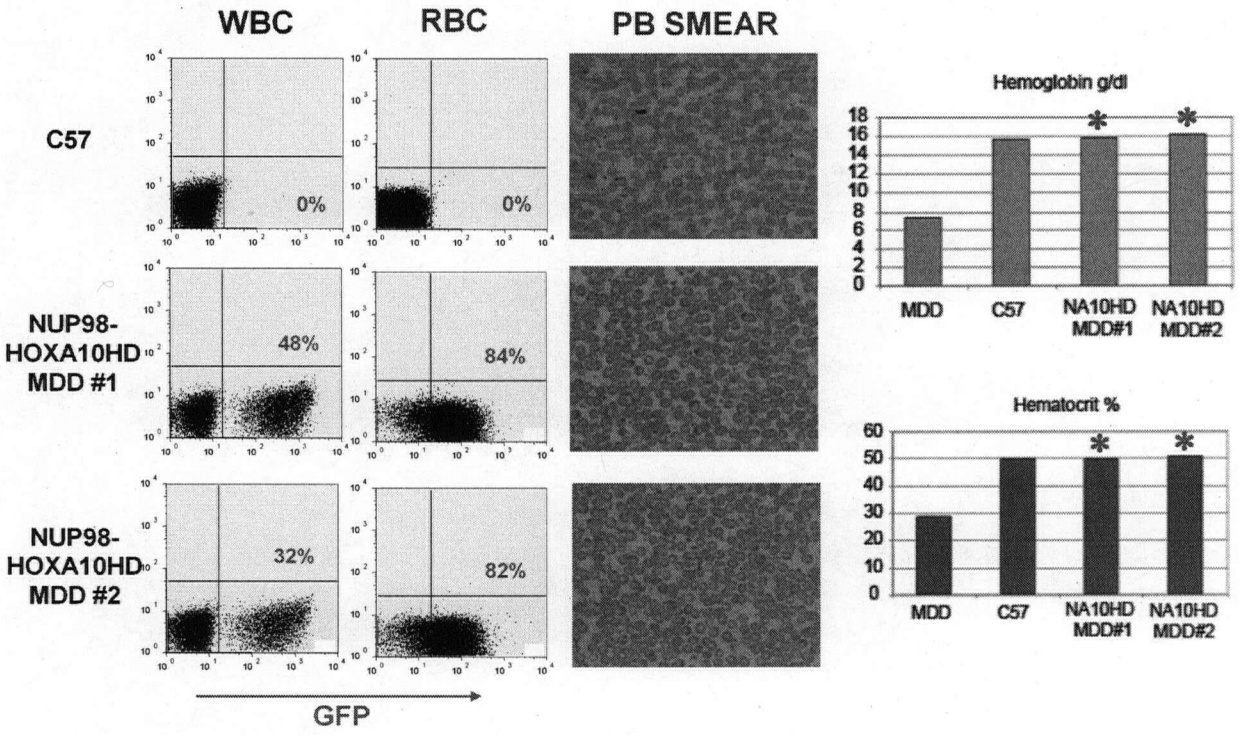


Figure 5.7 β-MDDs PB chimerism, phenotype and hematological parameters contributed by *NUPA10hd*-transduced and expanded HSCs.
β-MDD mice were transplanted with 300K starting cells *NUPA10hd* transduced and expanded. This graph shows the chimerism and phenotype of the PB at 5 weeks post transplantation. The values for a WT and a β-MDD mouse are also shown for comparison. The asterisks indicate that the hematological parameters of the two treated mice are within normal range.

5.3 Discussion

The data presented in this chapter provides proof of principle evidence that expansion of HSCs *in vitro* is a powerful strategy that can be coupled to gene therapy protocols for the treatment of inherited blood disorders.

To model the situation of a minimal number of genetically corrected cells retrieved at the end of genetic manipulations, congenic cells from healthy donors were used and transduced with *HOXB4* or *NUP98-HOXA10hd*, expanded in culture for 10-11

days and then the progeny of a small number of starting cells transplanted into nonmyeloablated thalassemic recipients. This initial amount of HSCs used in these experiments is comparable to that retrieved in the end of gene therapy related manipulations therefore this study potentially shows the benefit of inducing HSC expansion *in vitro* to achieve sustained stable mixed chimerism *in vivo* in nonmyeloablated recipients. We expanded 200,000 and 300,000 starting cells, which roughly contains 40 and 60 HSCs before the expansion. From the curve shown in Figure 3.1 it is evident that this amount of HSCs would produce very minimal chimerism in mice that received 2 Gy TBI pre-transplantation. Instead, our experiments showed that WBC chimerism as high as 34% and RBC chimerism as high as 52% could be achieved in mice transplanted with the progeny of 200,000 starting cells overexpressing HOXB4 and as high as 48% and 80% WBC chimerism and RBC chimerism respectively with the transplantation of the progeny of 300,000 starting cells overexpressing NUP98-HOXA10hd. In both experiments this high PB chimerism produced dramatic improvement of the hematologic parameters and in the case of NUP98-HOXA10hd cure of the thalassemic phenotype. This is likely a reflection of the higher HSC expansion obtained with NUP98-HOXA10hd as opposed to HOXB4.

In the study presented in this chapter and in gene therapy manipulations as well, retroviral vectors are generally used for the delivery of genes. Oncoretroviral vectors carry the risk to integrate near or within important cellular regulatory genes that might give them a selective growth advantage (Hematti et al., 2004; Kustikova et al., 2005). During the *ex vivo* culture these clones might outcompete the other HSC clones leading to a monoclonal reconstitution *in vivo*. In order to demonstrate that in the thalassemic mouse treated with HOXB4 overexpressing cells there is not such an occurrence and the HSC pool is polyclonal we analysed the pattern of integration of the virus in

clonogenic progenitor cells derived from different HSCs. We were able to identify 17 unique patterns out of 53 colonies analysed meaning that the chimerism at 12 weeks post transplantation, was contributed by at least 17 unique HSC clones.

Recently it has also been shown in patients of X-linked chronic granulomatous disease treated by gene therapy and non myeloablative HSCT that the chimerism achieved was contributed by clones that preferentially proliferated due to activating integrations in genes (Ott et al., 2006). In order to confirm that the cure of the thalassemic mouse transplanted with HOXB4 overexpressing cells was not influenced by fortuitous integrations in genes that affect cell proliferation, we performed linker-mediated amplification of retroviral integration sites (see material and methods for details) which allowed us to determine the identity of the site of integration of the vector. Among the 21 unique sites identified, one third were intergenic, one third were in promoter regions and one third were intragenic sequences. None of the genes in which the vector integrated have been implicated in malignant transformation according to published reports, although it is interesting to notice that one clone that was found with high frequency carried an integration in the upstream region of the gene coding for the TF Sp1 (Du et al., 2005; Suzuki et al., 2002).

Altogether, these data demonstrate the curative potential of *ex vivo* expanded HSCs in a preclinical model of β-thalassemia treated with nonmyeloablative conditioning. They also underscore the potential of HOXB4 and NUPHOXA10hd as potent tools to achieve the HSC expansion required.

CHAPTER 6 DISCUSSION

BMT is a procedure that allows for the treatment and cure of a variety of blood disorders ranging from malignant to inherited blood disorders. It was pioneered in the fifties by E. Donnal Thomas (Thomas et al., 1959) and relies on the replacement of abnormal blood forming cells of the affected patient with ones from healthy donors. Its clinical use has become increasingly more widespread in the last few decades due to better understanding and management of the complications associated with it, to increased availability of screening techniques for matching donors and recipients and to better understanding of the population of cells responsible for the reconstitution of the whole hematopoietic system, the HSCs. Currently, the major complications associated with BMT are the immune histocompatibility differences between the donor and the recipient of the graft that can lead to fatal GVHD reactions or engraftment failure, and the regimen related morbidity and mortality. Conventional BMT is associated with elevated toxicity because of the intense myeloablative chemotherapy and/or radiotherapy conditioning given to patients before the procedure. These regimens lead to the depletion of the hematopoietic system and create space for the transplanted cells to engraft but can produce several undesired side effects, such as infertility, neurodevelopmental effects, impaired growth, endocrine dysfunction and secondary malignancy. To decrease the toxicity and broaden the applicability of this procedure to older patients and those whose health is compromised by comorbid conditions, mildly ablative or nonmyeloablative regimens have been tested for their potential and applicability (Baron and Storb, 2006). From studies in mice, large animals and humans it emerged that because of the competition provided by the cells spared in the recipient, engraftment from donor cells would be possible only if the ratio of donor

to recipient cells was increased. In particular, it was observed that by increasing the number of HSCs transplanted, the chimerism in the PB of patients would consequently improve, the recovery time would be faster and the chances of a successful outcome would be higher. Obtaining substantial numbers of HSCs from donors is not always an easy task; consequently ways to achieve HSC expansion would be highly beneficial. In this thesis, I present data on the applicability of HSCs expansion strategies in a mouse model of nonmyeloablative BMT. I evaluate the potential of HSCs expanded *in vitro* by the overexpression of HOXB4 or NUP98-HOX fusion proteins to maintain the capacity to long-term engraft nonmyeloablated mice and to properly differentiate into lineages with lymphoid and myeloid features. Additionally I present findings on the treatment and cure of an important model of a genetic blood disorder in nonmyeloablative conditions with such expanded HSCs.

6.1 HOXB4 HSCs induced *in vitro* expansion for nonmyeloablative HSCT

As of today, many strategies have been attempted to achieve HSC expansion *in vitro* for clinical applications. These rely on two main approaches. One is the manipulation of the environment by addition of different cytokines and the second is to genetically manipulate cells by insertion of genes. Both strategies aim at triggering maintenance of stemness and self-renewal after cell division. While the former strategy has been shown to lead to only modest expansion, strategies that make use of retroviral vectors for the genetic manipulation of HSC have been more successful. One of the genes tested is the human *HOXB4* (Sauvageau et al., 1995). HOXB4 is a TF that plays an important role during development but is also involved in HSC regeneration in situations of stress. It has been shown to trigger *in vitro* up to 40 fold expansion when overexpressed in HSC without impairing normal proliferation and differentiation

(Antonchuk et al., 2002). These features make it a good candidate to test in nonmyeloablative HSCT where the prediction is that transplantation of grafts containing increased numbers of HSCs will achieve better chimerism in the nonmyeloablated recipients (Rao et al., 1997). The questions that I addressed in this thesis are about the quality and the quantity achieved after expansion. Due to the extensive *in vitro* manipulations, it was conceivable that expanded HSCs would not behave like fresh HSC and this would correlate with poor competition with the endogenous HSCs spared in the nonmyeloablated recipient. Additionally, in conventional myeloablative BMT, different inflammatory cytokines are released as a response to the irradiation stress and tissue damage and these might help the transplanted HSCs to proliferate and find their way to the BM while in nonmyeloablative HSCT this cytokine storm would not take place. In the experiments presented in this thesis, I show that the transplantation of the expanded progeny of limited numbers of initial HSCs leads to significantly greater level of chimerism than would be achieved with the original non expanded cells. From previous experiments conducted by Ben Cavilla, it emerges that in order to detect any chimerism in mice that received a nonmyeloablative radiation of 2 Gy, 500,000 fresh BM cells (that contain roughly 50 HSCs) have to be transplanted. By contrast, in one of my experiments, I was able to achieve up to 50% chimerism with the *in vitro* expanded progeny of 50 starting HSCs. The reconstitution was lymphomyeloid and sustained for up to 19 months. This finding was confirmed in multiple experiments and it established that the quality of expanded HSC was preserved and the quantity achieved after the expansion was highly beneficial.

When dealing with high-level *in vitro* HSC expansion, a possibility exists that few original clones are aberrantly transformed and are taking over the whole HSC culture because of acquired mutations and consequent aberrant proliferation. This could be

partly due to the insertion of the oncoretroviral vector in oncogenic sequences that lead to deregulated proliferation. To exclude this possibility, I assessed the constitution of the HSC pool in chimeric animals that received a nonmyeloablative irradiation dose of 2 Gy by analyzing the patterns of integration of the oncoretroviral vector in progenitor cells clonally derived from HSCs. Different HSCs will carry different integrations of the virus in specific sites of the genome and since this integration pattern is unique for each HSC clone, the variety of patterns of integrations indicates how many HSC contributed to the chimerism. I performed this analysis in three different recipients at 19 months post-transplantation and found that in all three cases the population of HSC contributing to the PB chimerism was highly polyclonal excluding the possibility of a pre-leukemic transformation in few selectively expanded HSC clones.

HOXB4 overexpression has also been reported to have an effect on HSC *in vivo* in myeloablated mice, by restructuring the HSC pool to normal numbers after transplantation while normal HSC are capable to reconstitute only 10% of the normal HSC pool of a mouse (Thorsteinsdottir et al., 1999). There are no previous reports on the *in vivo* effect of HOXB4 overexpression in nonmyeloablated mice and I therefore assessed if in this setting a further HSC expansion was possible. Two of the primary recipients were assessed for their donor HSC content 12 to 15 times post transplantation and by limit dilution assay it was possible to show that in addition to the almost 2 logs expansion *in vitro*, these cells expanded also 1 log *in vivo*. Of great interest was the observation that the contribution from donor HSC to the HSC pool correlated with the donor chimerism in the PB. Additionally, while one of the two analyzed recipients had the donor HSC contributing to half of the HSC pool of the recipient mouse, the second mouse that received the lower dose of HSC had the donor HSC contributing to 10% of the HSC pool. The fact that in this recipient HSCs did not

proliferate to reach the normal HSC range could be because of three different situations. One is that the HSCs have finally exhausted their expansion potential and can not proliferate any further. The second is that there are some feedback signals from the BM environment of nonmyeloablated mice that block the further proliferation of these HSCs and a third related reason is that the HSC niches of the recipient are occupied by recipient HSCs therefore there is no need for further proliferation from donor HSCs. Although none of these issues were investigated, the first hypothesis could be addressed by transplantation of these expanded HSCs into myeloablated secondary recipients. Since it is known that HOXB4 overexpressing cells are capable to restore the HSC compartment of myeloablated mice to normal numbers, if donor HSCs still have proliferative potential then in this setting they would be able to manifest it. The third hypothesis could be addressed by quantifying the recipient HSCs in secondary recipients with a limit dilution assay.

Altogether this data confirmed that HOXB4 expanded cells maintains features of fresh HSCs and are a useful tool for achieving high levels of PB chimerism in nonmyeloablative BMT/HSCT.

6.2 Expanded HSCs for the treatment of β-Thalassemia in nonmyeloablative conditions

Many patients affected by genetic blood disorders such as hemoglobinopathies suffer many health complications associated with long-term transfusion treatments and organ damage. These patients affected by multiple comorbid conditions have a high risk of mortality if they are put through conventional myeloablative BMT. Nonmyeloablative HSCT is a safer alternative for these patients but requires the transplantation of higher HSC numbers, therefore HSC expansion strategies would be

highly beneficial for the treatment of these diseases in non myeloablative conditions. Additionally, HSCT is the only curative option for many inherited blood disorders but despite the world registry network, which includes more than 3 million volunteers characterized for their histocompatibility markers, about 40% of patients still fail to find a suitably matched donor. This makes allogeneic HSCT a risky procedure with high chances of immune complications and potential rejection of the graft. Autologous HSCT coupled to gene therapy protocols is a safer option since it relies on the transplantation of patient's own HSCs that have been genetically modified to correct the original genetic defect. However, the recovery of corrected HSCs expressing sufficient levels of the therapeutic protein in the end of the transduction protocol is minimal and definitely not sufficient for transplantation in nonmyeloablated recipients. Hence, the transplantation of HSCs expressing a therapeutic gene and expanded by the overexpression of HOXB4 would be greatly beneficial. In the experiment presented in this thesis, to model genetically corrected cells, healthy donor cells were transduced with *HOXB4*, expanded *in vitro* and transplanted in nonmyeloablated mice with severe β-thalassemia caused by the homozygous deletion of the β-major globin gene. With this system, we were able to demonstrate that HSC expansion strategies could lead to the improvement of the phenotype of sick mice by increasing the contribution by healthy cells to the PB chimerism.

To exclude the possibility that the contribution was from a clone that was transformed and hyperproliferative I analyzed the BM of a mouse that exhibited high PB chimerism and through the visualization of unique patterns of integrations in clonogenic progenitors demonstrated that the high PB chimerism was contributed by a polyclonal pool of expanded HSCs.

Recently, in two patients who received gene therapy for the treatment of X-linked chronic granulomatous disease coupled to nonmyeloablative bone marrow conditioning, it was found that the therapeutic effect was enforced by the integration of the retroviral vector in genes that gave a proliferative advantage to the corrected cells (Ott et al., 2006). Although this did not lead to overt malignant transformation, concerns were raised about the safety of this procedure. Therefore, to investigate if the same situation applied to our nonmyeloablated mice, we analyzed the different sites in the genome where the retroviral vector integrated. We were able to identify 21 unique integrations from 14 different clones. Of these, roughly one third were in promoter regions, one third were intergenic and one third were in introns of genes. Many of these genes are not characterized yet and the ones that are, have not been reported to be implicated in malignant transformations (Kustikova et al., 2006). Independently of the results achieved in these experiments it would be safer to avoid the genetic manipulations, therefore studies are ongoing to optimize transient delivery of HSC expanding factors.

6.3 *NUP-HOX* fusion genes to achieve higher levels of HSC expansion *in vitro*

Despite the striking HSC expansion obtained with HOXB4, many studies are ongoing to understand the mechanism of HSC self-renewal to further identify the genes that are responsible for triggering this process. Some of the genes so far implicated have been identified in part from for their involvement in leukemic transformation. Recently Pineault *et al.* characterized the biologic effect of some fusion proteins composed by the N-terminal portion of the Nucleoporin 98 and the C terminal portion of HOX proteins (HOXA10 or HOXB4) in hematopoietic cells and he observed that they were capable to expand short term repopulating cells and block differentiation (Pineault

et al., 2004). Subsequently, Ohta *et al.* tested these same fusion proteins for their HSC expansion potential and observed up to 1000-fold HSC amplification when these fusion proteins were overexpressed in HSCs. Since from different studies performed in nonmyeloablated mice in the last few decades it has emerged that the higher the amount of HSC transplanted, the higher the chimerism achieved, we tested HSCs expanded by these genes for their repopulation capacity in nonmyeloablated recipients. With these genes we were able to explore a wider range of HSC expansion and transplantation doses for nonmyeloablative HSCT. In the experiments presented in this thesis I was able to show that further expansion of HSCs gave rise to a significantly higher PB chimerism. The reconstitution in all mice tested was lymphomyeloid and sustained for up to 15 months PT. Of great interest was the observation that HSCs expanded clonally from few starting HSCs by a fusion gene carrying *NUP98* and the homeodomain only of the *HOXA10* gene were capable of significantly contributing to the PB chimerism of nonmyeloablated mice. This would lead to major clinical benefit when using genetically modified HSCs since it would allow for pre-screening of clones and identification of those that have safe genetic integrations.

With the availability of such powerful HSC expanding factors, I further assessed if the improved chimerism in nonmyeloablated mice would help the prompt recovery and cure of mice affected by a genetic blood disorder such as β-thalassemia.

As mentioned above, in gene therapy applications coupled to autologous HSCT there is the urgency to expand corrected HSCs prior to transplantation. The remarkable HSC expansion achieved with *NUP98-HOX* fusion genes allowed us to obtain prompt therapy and cure of two nonmyeloablated thalassemic mice which had all the hematologic parameters within normal range.

6.4 Unanswered questions and future directions

The findings presented in this thesis add evidence in support of the hypothesis that the final chimerism in a nonmyeloablated recipient highly depends on the ratio of donor to recipient HSCs. The experiments described in this thesis were conducted in a murine system and no reports are available at this point on the effect of HSC expansion strategies in non-human large animal models in nonmyeloablative conditions. Therefore studies are needed to confirm the findings in mice to higher models closer to the human system.

To confirm and extend the findings in mice on HOXB4 mediated HSC expansion to the human system for possible future clinical applications, HOXB4 is under intense study in human and in nonhuman primate models. Current evidence indicates that the ability of HOXB4 to promote expansion of HSC extends to human, dog and non human primates. While the levels of expansion so far achieved appear to be somewhat less than observed in the murine model, the findings encourage further testing and optimization of HOXB4 and even more potent molecules such as NUP98-HOXA10hd as described here. In gene therapy applications the use of retroviral vectors for the delivery of genes has emerged as an unsafe procedure with the associated risk of insertional mutagenesis. Furthermore, it is not clear what the long-term effect on HSC could be, especially of genes that trigger HSC proliferation. For this reason, better methods for the transient expression of genes or for the transient delivery of proteins have to be engineered. To this end, a fusion protein of HOXB4 with the protein transduction domain of the HIV transactivating protein (TAT) has been produced and tested as a potential growth factor for stem cells (Krosl et al., 2003a). This strategy has avoided the potential risk of insertional mutagenesis but the effect documented on

murine HSCs was low. Work is in progress to improve the stability of this protein and efficacy of this delivery system. Furthermore the TAT fusion with NUP98-HOXA10hd is also being produced and shortly will be tested in our laboratory for its biologic effect on hematopoietic cells.

It is my hope that the findings described in this thesis help further the understanding of the dynamic of engraftment in nonmyeloablative HSCT. The efforts put into finding HSC strategies for future clinical applications will allow for better use of different HSC sources such as UCB and will hopefully lead to safer HSC therapies for the treatment of inherited blood disorders.

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