NOVEL METHODS FOR HIGH LEVEL *EX VIVO* EXPANSION OF HEMATOPOIETIC STEM CELLS

By

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B.Sc. Molecular Biology and Physiology, University of Belgrade, 2001

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In

THE FACULTY OF GRADUATE STUDIES

(Medical Genetics)

THE UNIVERSITY OF BRITISH COLUMBIA

September, 2005

ABSTRACT

The development of strategies to extensively expand HSCs *ex vivo* could greatly improve the utility of hematopoietic stem cell (HSC) – based therapies. In addition to potential clinical applications, such an advance would provide an invaluable tool for studying the mechanisms underlying HSC self-renewal.

Engineered overexpression of the homeobox transcription factor HOXB4 has emerged as a powerful stimulator of hematopoietic stem cell (HSC) expansion *ex vivo* (>40-fold net increase in 2 weeks). More recent studies of the properties of natural and engineered *NUP98-HOX* fusion genes, initially of interest to us for their role in human AML, suggested these molecules might have similar effects on HSCs.

To examine whether specific *NUP98* and *HOX* fusion genes stimulate murine HSC expansion in short term liquid cultures, $3x10^6$ marrow cells from mice given 5-fluorouracil 4 days previously were prestimulated with IL-3, IL-6 and SF, retrovirally transduced with MSCV-IRES-GFP retroviral vectors also encoding *NUP98-HOXB4, NUP98-HOXA10,* or *HOXB4* (only) or nothing as controls and then cultured for another 6 days with the same growth factors. Limiting dilution assays were used to determine the frequency and hence number of Competitive long-term (>4months) lympho-myeloid Repopulating Units (CRU) present before and after culture. The results of these experiments showed that the CRU content of the cultures of *NUP98-HOXB4-*, and *NUP98-HOXA10-* transduced cells increased 290-fold and >2000-fold, respectively, i.e. ~4 and

>25x the effect obtained with *HOXB4* and $>10^4$ and $>10^5$x the yield of CRU in the control cultures. Similar results were obtained in cultures of *NUP98-HOXA10*-transduced cells that were initiated with limiting numbers of CRUs (1-2), demonstrating that the cells targeted were not a rare subset of HSCs. Additional studies of the same design showed that the effect of *NUP98-HOXA10* on HSC expansion was preserved when sequences flanking the homeodomain were removed, thus identifying the homeodomain as the key *HOX* gene sequence required in concert with the N-terminal region of NUP98.

These findings demonstrate a greater potency of *NUP98-HOX* fusions as novel agents for HSC expansion *ex vivo*, reveal the essential contribution of the DNA-binding homeodomain to achieve this effect and set the stage for the design of minimal HOX-based fusion proteins for future studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

5-FU	5-fluorouracil
AML	acute myelogenous leukemia
Antp	Antennapedia
ANT-C	antennapedia complex
BM	bone marrow
B-cells	B-lymphocytes
CB	cord blood
CD	cluster of differentiation
CFC	colony forming cell
CFU	colony forming unit
CFU-E	colony forming unit - erythroid
CFU-G	colony forming unit - granulocyte
CFU-GM	colony forming unit - granulocyte macrophage
CFU-GEMM	colony forming unit granulocyte, erythroid, macrophage, megakaryocyte
CFU-M	colony forming unit - macrophage
CFU-S	colony forming unit - spleen
cGy	centiGray
CRU	competitive repopulating unit
CRUs	competitive repopulating units
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid

FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FL	fetal liver
Flt3-L	Flt3-ligand
G-CSF	granulocyte-colony stimulating factor
GFP	green fluorescence protein
GVHD	graft versus host disease
HD	homeodomain
HF	Hank's balanced salt solution with 2% FBS
HOM-C	homeotic complex
HSC	hematopoietic stem cell
HSCs	hematopoietic stem cells
IL	interleukin
IRES	internal ribosomal entry site
LC	liquid culture
Lin	lineage markers
LTC-IC	ling term culture-initiating cell
LTR	long terminal repeat
LTR-cells	long-term repopulating cells
NA10	NUP98-HOXA10
NA10hd	NUP98-HOXA10hd
NB4	NUP98-HOXB4
NK cells	natural killer cells

NOD/SCID	non-obese diabetes / severe combined immunodeficiency
NUP98	nucleoporin-98
PB	peripheral blood
PBX1	pre-B-cell leukemia transcription factor 1
PI	propidium iodide
PS	prestimulation
RBC	red blood cell
RBCs	red blood cells
RU	repopulation unit 1
Sca-1	stem cell antigen
SF	steel factor
SL cells	$Sca1^{+}Lin^{-}$ cells
SP	side population
STR-cells	short-term repopulating cells
T-cells	T-lymphocytes
WBC	white blood cell
WBCs	white blood cells

ACKNOWLEDGEMENTS

I am deeply grateful to my supervisors, Dr. Keith Humphries and Dr. Connie Eaves, for the opportunity to do graduate training at Terry Fox Laboratory and for their fervent support and selfless guidance throughout this project. I also truly appreciate their encouragement to present the results of this research at the International Society for Experimental Hematology 34th annual scientific meeting.

I would like to thank Dr. Hideaki Ohta for providing invaluable initial leads pointing us towards the high potency of engineered *HOX* fusion genes to stimulate murine HSC expansion *in vitro* and Silvia Bakovic for pleasant and productive team work during the project. They were both very helpful in teaching me the assays for hematopoietic cells. I would also like to thank Dr. Suzan Imren for her constant encouragement in both academic and personal endeavors throughout my time in the lab. Dr. Pamela Hoodless served on my graduate committee and I am thankful for her assistance in project discussions.

Finally, I send my thanks to members of the Humphries and the Eaves lab (Bob, Eric, Carolina, Nick, Lars, Rhonna, Fredrick, Patty, Adrian, Christy, Brad and Andrea) for making TFL such a fun place to work.

Lastly, but far from the least, I would like to thank my parents, my sister and my husband for their endless love and encouragement.

CHAPTER 1 INTRODUCTION

1.1 Hierarchical Model of Hematopoiesis and the Critical Role of Hematopoietic Stem Cells (HSCs)

Hematopoiesis is the life-long process of blood cell development. Because the lifespan of most mature blood cells is relatively short (days), their replacement and the mechanisms that control this process are essential for survival. The establishment and permanent maintenance of hematopoiesis relies on the presence of a small subset of pluripotent hematopoietic stem cells (HSCs) that can amplify their own numbers and/or differentiate as needed. A pool of these cells is retained in the bone marrow (BM) throughout adult life, although they are estimated to comprise only 0.01% of the total BM compartment. The hematopoietic system of mice and humans is organized as a hierarchy of cell types with differing capacities for self-renewal, proliferation, and differentiation. Hematopoiesis is thought to proceed irreversibly through a series of lineage commitment steps, during which the most primitive pluripotent HSCs give rise to multipotent myeloid- or lymphoid-restricted progenitors that in turn give rise to committed progenitors - the final output being the mature functional circulating blood cells. The myeloid lineage includes those cells responsible for carbon dioxide and oxygen transport (erythrocytes), blood clotting (platelets) and those involved in mounting a phagocytic response to foreign organisms (granylocytes, monocytes, macrophages). The lymphoid lineage includes cells involved in humoral (B-lymphocytes) and cellular immunity (T cells and natural killer cells) (Figure1.1). Despite their common origin, cells belonging to the myeloid

lineages are produced in the BM, whereas many cells of the lymphoid lineages undergo further development in the spleen, thymus and lymph nodes.

HSCs are defined by their functional attributes, the potential to generate and maintain a lifetime output of all of the terminally differentiated lymphoid and myeloid cell types that comprise the blood, BM, spleen, and thymus (Jordan and Lemischka, 1990, Szilvassy and Cory, 1994). These cells include the eight major hematopoietic lineages: B and T lymphocytes; erythrocytes; megakaryocytes/platelets; basophils/mast cells; eosinophils; neutrophils, and monocytes/macrophages. To support this potential, HSCs possess an extremely high proliferative potential. It is estimated that in normal humans there are approximately 50 million HSCs, some of which can generate up to 10^{13} mature blood cells over a normal lifespan (reviewed in Szilvassy, 2003). In mice, it has been shown that a single stem cell can regenerate and maintain a significant proportion of the lymphohematopoietic system following transplantation into an irradiated or immunocompromised host (Dick et al., 1985; Keller et al., 1985, Lemischka et al., 1986). Proliferation and differentiation are not necessarily tightly coupled, and in the most primitive HSCs this renders a variable capacity for self-renewal, the cardinal property of all stem cell types. Self-renewal is critical for HSCs because they are constantly subjected to physiological stresses that stimulate their recruitment into maturational pathways. HSC self-renewal, at least at the population level, thus ensures that sufficient numbers of HSCs are available to meet the demands of hematopoiesis over a normal adult lifespan. Furthermore, their potential for reconstituting the hematopoietic system has allowed

the development of powerful clinical therapies such as for leukemias and genetic blood disorders based on HSC transplantation.

Given the pivotal role of HSCs, much effort has been directed at developing tools for their detection and in understanding and ultimately exploiting the molecular mechanisms that control their self-renewal. A longstanding major goal has been to develop methods that would enable significant levels of HSC self-renewal to occur *in vitro*. Such a development would enhance efforts to understand the mechanisms controlling self-renewal and enable broader and safer application of HSC-based therapies. In this thesis work, I have focused on a recently developed strategy for achieving HSC expansion *ex vivo*, which is based on the forced expression in primitive hematopoietic cells of HOX transcription factors and related molecules. In the following sections, the key concepts, assays and regulatory mechanisms of HSC function that guided and enabled the research undertaken, are briefly reviewed.

HEMATOPOIETIC ASSAYS

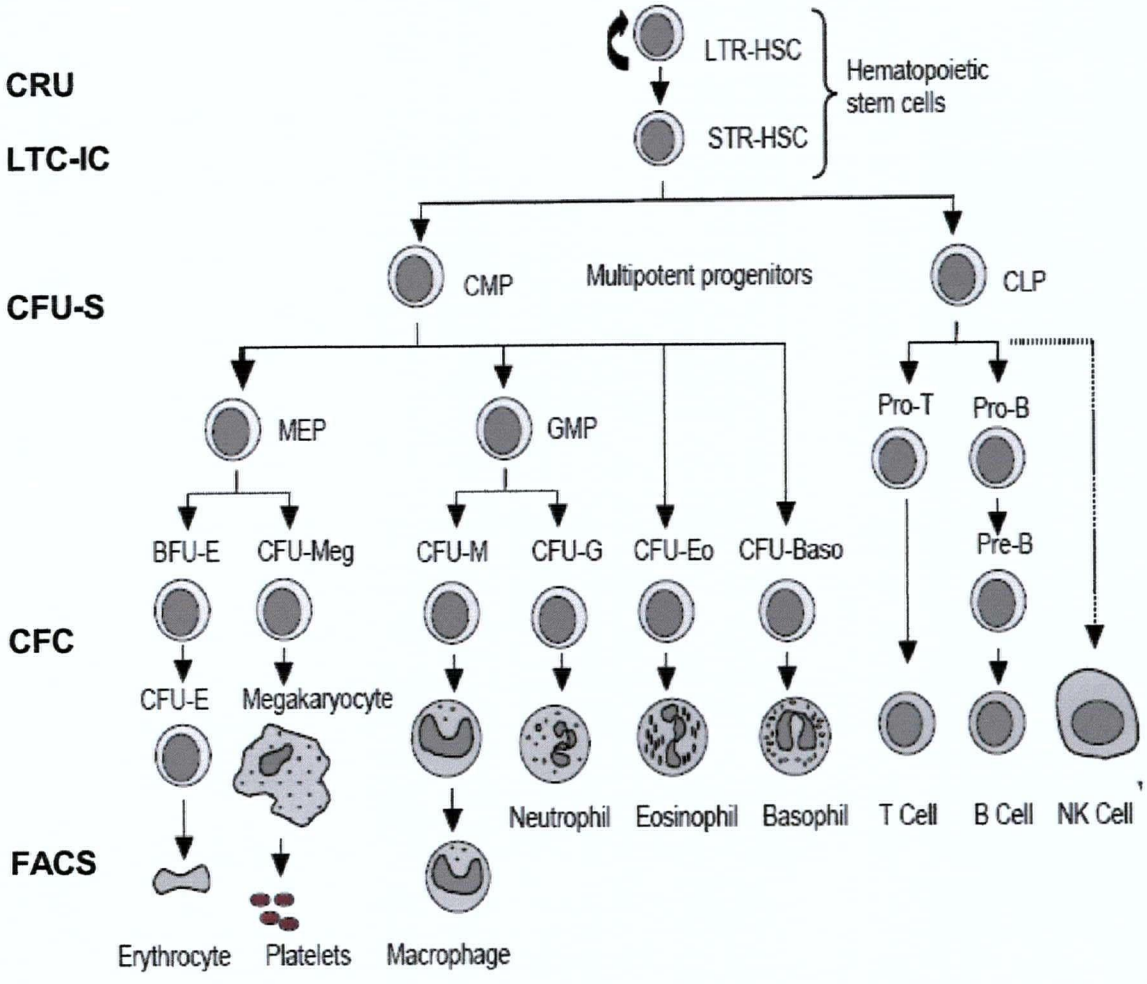


Figure 1.1: The hematopoietic hierarchy. Pluripotent HSCs are shown at the top of the hierarchy (the circular arrow indicates HSC self-renewal). Longterm repopulating (LTR) HSCs give rise to short term repopulating (STR) HSCs, which give rise to multipotent progenitors. Commitment to either myeloid or lymphoid lineages produces common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs), which give rise to more lineage-restricted megakaryocytic-erythroid progenitors (MEP) and granulocyte-macrophage (GMP) progenitors. Further commitment events produce unilineage progenitors, which mature into functional end cells. Some hematopoietic assays are aligned with the developmental level of the cell type they detect. Potentials for proliferation and self-renewal both decrease with differentiation.

1.2 Hematopoietic Stem Cells (HSCs)

1.2.1 Existence of HSCs

Early indications that hematopoiesis is sustained by the continuous differentiation of cells from a common pluripotent stem cell population and that such cells may exist came from studies in which the transplantation of chromosomally marked cells into lethally irradiated mice resulted in the long-term recovery of both lymphoid and myeloid cells that shared the same unique chromosomal markers (Wu et al., 1968). Later, these findings were also confirmed by studies that demonstrated common sites of retroviral integration in lymphoid and myeloid cells regenerated following bone marrow transplantation (Dick et al., 1985; Keller et al., 1985, Capel et al., 1989).

Additional direct evidence for HSCs has most recently derived from methods to enrich for rare hematopoietic subpopulations and the demonstration of the capacity of multilineage repopulation capacity of single transplanted cells (Osawa et al. 1996, Ema et al., 2000; Uchida et al., 2003). Moreover, plating of such single cells into tissue culture wells, revealed repopulating cells within the first division progeny – demonstrating self-renewal capacity. When the two daughter cells were separated, however, retention of long-term repopulating ability could sometimes be found in only one of the two wells, indicating that asymmetric self-renewal divisions occur *in vitro*.

1.2.2 Detection and quantification of HSCs

One of the first methods for detecting and quantifying primitive hematopoietic cells *in vivo* was the colony-forming unit – spleen (CFU-S) assay (Till and McCulloch, 1961). In this assay, hematopoietic cells obtained from the bone marrow or spleen of donor mice are transplanted intravenously into syngeneic recipients, previously exposed to a lethal dose of irradiation. Approximately, 10% of the injected cells become lodged in the spleen (Lord and Hendry, 1973), where they proliferate and generate clonal macroscopic colonies within 1-2 weeks. Counts of the colonies obtained then provide a measure of the CFU-S frequency in the initial cell suspension injected. The number and types of cells comprising each colony can also be determined to assess the proliferation and differentiation potential of the founder cell. Self-renewal potential can be evaluated by re-transplanting cells from individually excised colonies into secondary irradiated hosts to determine whether CFU-S were regenerated during the formation of the primary colony (Siminovitch et al., 1963).

Originally, this assay was thought to detect HSCs with longterm regenerative potential. However, subsequent studies showed that this was not the case, although some HSCs may form spleen colonies (Spangrude and Johnson, 1990). The strongest evidence that many CFU-S are different from HSCs has come from cell separation experiments where it was possible to physically separate CFU-S from HSCs detected by endpoints of long-term repopulation of transplanted recipients (Jones et al., 1990; Ploemacher and Brons, 1989).

The results of the early studies of CFU-S nevertheless led to recognition of the importance of quantitative assays specific for cells with long-term *in vivo* lympho-myeloid reconstitution ability and catalyzed many important concepts and models concerning the properties and regulation of HSCs. Currently, the abundance and properties of HSCs are best determined using transplantation–based assays. HSCs are identified by their unique ability to give long-term lymphomyeloid reconstitution of intravenously transplanted myeloablated recipients pretreated by exposure to lethal or near-lethal doses of ionizing radiation. The injected HSCs home to the BM and re-establish multilineage hematopoiesis. However, heterogeneity exists amongst cells that are capable of some multi-lineage reconstitution and the most primitive subsets can be further defined by the durability of their capacity to sustain donor-derived hematopoiesis.

The repopulation unit (RU) assay developed by Harrison (1980) measures the ability of a cell sample to repopulate irradiated hosts relative to another reference source of repopulating cells (e.g. 10^5 normal BM cells) contained in the transplant innoculum. The test sample is injected, along with a standard dose of freshly isolated and genotypically distinguishable competitor cells, into a congenic irradiated murine recipient. After 3-4 months, the relative repopulation of myeloid and lymphoid cells by the 2 donor sources is calculated, and an RU value calculated by the formula: RU=(P*C)/(100-P), where P is the measured percentage of test cell-derived hematopoiesis, and C is the number of competitor cells used. The magnitude of the RU value reflects both HSC number (quantity) and its cell output capacity. This assay thus compares the stem cell activity of a cell population relative to some

reference population, but does not measure HSC frequency directly. Additional statistical methods can be applied to the variance data obtained from such experiments to calculate the frequencies of the input HSCs if certain assumptions are made about their output potential, which we now know is not an invariant feature of different HSC populations (Rebel et al., 1996).

A method for measuring HSC frequency based on limiting dilution assay of cells with longterm competitive lympho-myeloid repopulation activity is the Competitive Repopulating Unit or CRU assay (Szilvassy et al., 1990, Szilvassy et al., 2002). This procedure uses the principles of limiting dilution analysis to measure the frequency of cells in a given suspension that have transplantable long-term repopulating ability and can individually generate both lymphoid and myeloid progeny (Szilvassy et al., 2002). As recipients, normal mice are pretreated with a lethal dose of radiation (myeloablative treatment), or c-kit mutant mice whose HSCs are defective (Miller et al., 1996) are treated with a sublethal dose of irradiation. This treatment of the hosts maximizes the sensitivity of the assay and reduces the competing endogenous HSC population to a minimum, creating an environment in which the engrafting HSCs will be optimally stimulated. In order for a limiting dilution analysis of the HSC content of the test cell suspension to be performed, the recipients must be able to survive regardless of whether they receive any HSCs in the test cells injected. Survival of normal recipients is assured by co transplanting them with hematopoietic cells of the same genotype that contain sufficient numbers of short-term repopulating cells but minimal numbers of long-term repopulating cells (e.g. 10^5 normal bone marrow cells). Survival of c-kit mutant hosts is similarly

assured by pretreating them with a dose of radiation that allows significant numbers of endogenous cells to survive and avoids the requirement of transplanting additional cells. The differentiated blood cell progeny of the test cells and the recipients must be genetically distinguishable and assessed at a time when the regenerated cells can be assumed to represent the exclusive output of cells with life-long HSC potential. Strains of mice congenic with the C57B1/6 mouse are typically used to allow the blood cell progeny of the test cells to be uniquely identified by CD45 (Ly5) allotype markers (Szilvassy and Cory, 1993).

Quantification of HSCs is achieved by application of Poisson statistical analysis on the proportion of animals that test negative for the test cell-derived repopulation at each cell dose transplanted, where the dose at which 37% of animals are negative is estimated to contain 1 CRU. In practice, a threshold of ≥1% test cell-derived myeloid and lymphoid peripheral blood (PB) cells detected >4 months post-transplant has been shown to rigorously detect a long term lympho-myeloid repopulating cell. Using this assay, the frequency of HSCs in the BM of a mouse has been estimated to be about 1 in 10,000 nucleated cells.

Methods for detecting and assaying human HSCs have also been developed based on the remarkable observation that primitive human hematopoietic cells can engraft and contribute to long-term lympho-myeloid hematopoiesis in certain xenogeneic recipients that are adequately immunocompromised (e.g. fetal sheep and immunodeficient mouse strains (Zanjani et al., 1994; Dick, 1996). Immunocompromised mice bearing non-obese diabetes (NOD) and severe combined immunodeficiency (SCID) genotypes can tolerate hematopoietic grafts

from human sources (Cashman et al., 1977). This has allowed for the development of a quantitative *in vivo* functional assay for human HSCs (Bhatia et al., 1997; Conneally et al., 1997). Limiting doses of the test sample are injected into semi-lethally irradiated NOD-SCID hosts, and transplanted mice are scored as positive (engrafted) if they contain a threshold level of human lymphoid and myeloid cells. As in the murine CRU assay, Poisson statistics are then employed to quantitate the HSC frequency.

1.2.3 Detection and quantification of progenitor cells

The discovery of lineage-restricted progenitor cells in the late 1960s led to a model whereby cells become irreversibly committed to lineages, with progressive restrictions in potentiality, as they progress through hematopoietic differentiation. *In vitro* systems for supporting both murine and human hematopoiesis have also been developed and adapted in various ways to allow specific subpopulations of hematopoietic progenitor cells to be detected and quantified. The colony-forming cell (CFC) assay is a functional assay to test for the presence of progenitor cells that can form colonies of mature progeny in semi-solid medium containing appropriate growth factors (Bradley et al., 1967; Ichikawa et al., 1966). CFCs can produce from one (uni) to many (multi) lineages and the greater the colony size and content of the different lineages, the more primitive the cell from which the colony is thought to be derived. For example, a multi-lineage progenitor, CFU-GEMM (colony forming unit – granulocyte, erythroid, macrophage, megakaryocyte) gives rise to a very large colony composed of granulocytes, macrophanges, erythrocytes and megakarocytes,

and is more primitive than a uni-lineage progenitor, CFU-E (colony forming unit – erythroid) which gives rise to smaller colonies of only erythroid cells (Metcalf, 1984). Although colonies have been shown to be clonal (Metcalf and Moore, 1971), most CFCs display a limited or no capacity for self-renewal as no or very few colonies are normally formed when primary colonies are re-plated into secondary assays (Siminovitch et al., 1963).

Cell purification techniques further revealed that most CFCs could be separated from more primitive cells with repopulating activity. CFCs are also more numerous than CFU-S and could be detected in spleen colonies as the progeny of CFU-S (Metcalf, 1984). These observations formed the basis of assigning these cells to different steps in a hierarchy of hematopoietic cell differentiation (Metcalf, 2001).

An *in vitro* assay that has shown greater specificity for quantifying hematopoietic cells that appear to be more primitive than most CFCs is the long-term culture initiating cell (LTC-IC) assay. This assay detects a cell that can initiate sustained myelopoiesis when co-cultured on stromal feeder layers (Sutherland et al., 1989). This assay was developed from the observation that mature granulocytes and macrophages can be produced for several months when unseparated suspensions of BM cells are cultured at high density in media containing horse serum and corticosteroids. Subsequent studies showed that the primary need for serum was to generate a competent feeder layer of stromal cells that then stimulate the proliferation and differentiation of very primitive hematopoietic cells in the absence of exogenously supplied growth factors. In order to enable the latter to be

quantified independently of the ability of cells in the test suspension to form a competent feeder layer, irradiated pre-established marrow feeders or irradiated monolayers of a number of human or murine fibroblast cell lines can be used (reviewed in Eaves and Eaves, 2004).

1.2.4 Functional properties of HSCs

Assays for HSCs are based on 3 critical, and in large part unique functional HSC properties: capacity for self-renewal, generating at least one identical daughter cell; and capacity for long-term multi-lineage hematopietic reconstitution. Using these criteria and available assays a number of key properties and regulatory mechanisms of HSCs have emerged.

1.2.5 Phenotypic characterization of HSCs

Currently, HSCs cannot yet be directly and consistently identified on the basis of any unique morphological, physical or cell surface characteristics, although a number of different phenotypic markers can now be used in combination to obtain HSCs in nearly pure form from adult mouse BM. The most commonly analyzed markers are cell surface antigens against which specifically reactive monoclonal antibodies have been made. These antibodies can then be labeled either directly or indirectly (via a secondary antibody) with a unique fluorochrome and used to distinguish cells as positive or negative on the basis of their acquired fluorescence. Multi-parameter flow cytometry has a great power to distinguish cells based on these molecularly-determined features, in an objective and quantitative way, with a high

degree of specificity. In addition, this technology can be used not only for cell analysis but also for their separation into viable subsets defined by the analysis. These isolated cells can then be assayed for their functional attributes. In this way, the phenotype of different functionally defined cell populations can be identified.

Many stem cell markers have been described over the past 20 years (reviewed in Visser and Bekkum, 1990, Civin and Gore, 1993, Uchida et al., 1993, Szilvassy and Hofman, 1995) . Murine HSCs are characterized by their high expression of stem cell antigen (Sca)-1 and low levels of Thy-1. They are also characterized by the absence of lineage (Lin) antigens expressed predominantly on terminally differentiated lymphocytes (CD45R/B220, CD3, CD4, CD8), myeloid (CD11b/Mac-1, Ly-6G/Gr-1), and erythroid (TER-119) cells. Notably, however, low levels of CD4 and Mac-1 can be induced on some HSCs, particularly following activation by 5-FU treatment of donor mice (Szilvassy and Cory, 1993, Weissman et al., 1997). $Sca1^{+}Thy\text{-}1^{lo}Lin^{-}$ cells have the capacity for long term reconstitution of lethally irradiated mice (Uchida and Weissman, 1992). The co-expression of c-kit further enriches for HSCs and $c\text{-}kit^{+}$ murine BM progenitors and therefore the $c\text{-}kit^{+}Sca1^{+}Lin^{-}$ cell population is often used as a source of murine stem cells (Orlic et al., 1993). Human HSCs are characterized by high expression of CD34, intermediate expression of c-kit and Thy-1, and low or no expression of CD38.

Most strategies to isolate HSCs rely on utilizing the cell surface antigens described above, however, other properties of HSCs can be used in addition when isolating these primitive cells. The majority of HSCs in adult BM are believed to be in a quiescent, non-cycling state (Ogawa et al., 1993), although more recent studies

indicating that up to 10% of LTR-HSC randomly enter cell cycle per day, with all HSC entering the cell cycle in 1-3 months (Bradford et al., 1997, Cheshier et al., 1999). Based on the quiescence theory, sorting for HSC residing in G0/G1 cell cycle phase with low levels of RNA, can enrich for these cells. Viable dyes, such as the DNA dye Hoechst 33342 and the RNA dye Pyronin Y, are often used for this purpose (Gothot et al., 1997). Another dye commonly used in stem and progenitor cell enrichment is the mitochondria dye Rhodamine-123 (Rho), which does not stain primitive hematopoietic cells but does stain most other cells in the BM (Li et al., 1992, Spangrude and Johnson, 1990).

Recently, another method based on the propensity of HSCs to actively pump out the Hoecht 33342 dye has emerged. A unique side population (SP) of cells is observed when the fluorescent properties of cells stained with this dye are simultaneously observed at 2 wavelengths. SP cells from mouse BM were found to be highly enriched for CFU-S and multilineage repopulating cells (Goodell et al., 1996). Hoechst is actively excluded by the ABC transporter protein ABCG2 in HSCs, and overexpression of ABCG2 leads to an expansion of cells with an SP phenotype (Zhou et al., 2001). The SP phenotype of the HSCs in adult mouse BM has been useful to devise relatively simple strategies for isolating populations that are at least 40% pure CRUs (Uchida et al., 2003). The SP phenotype initially attracted considerable interest as a potential marker for HSCs from many species because a small SP population can be demonstrated in hematopoietic cells from many species including humans (Goodell et al., 1997). Moreover, HSCs in human fetal liver were

found to have an SP phenotype (Uchida et al., 2001) but this is not the case for HSCs in human cord blood (Fischer et al., 2005).

Unfortunately, many of the available markers for discriminating the most primitive hematopoietic cell types cannot be used to enumerate changes in their numbers reliably, particularly under circumstances where their activation or cycling status may have been altered, because expression of these markers is labile under these conditions (Zanjani et al., 2003; Uchida et al., 2004). Thus, in order to measure levels of HSC expansion in culture is still necessary to rely on functional assays rather than phenotype. However, the increasing refinement in methodologies for phenotype discrimination, when validated by functional assays, do offer great promise for obtaining highly purified populations that can then be used for gene expression and proteomic studies.

1.2.6 Self-renewal of HSCs

HSCs appear early in embryogenesis and subsequently amplify their numbers to levels that are maintained for the lifespan of the individual. During ontogeny, there is a great expansion of all hematopietic cells, including HSCs, to meet the growing needs of the body. The murine fetal liver (FL) at 12 days post-conceptus (dpc) contains approximately 40 HSCs, as detected by the CRU assay. By 16 dpc this number has expanded 30-fold to 1500 HSCs (Ema and Nakauchi, 2000) and by adulthood a further 13-fold expansion brings the total HSC cnotent up to 20,000 (Szilvassy et al., 1990). From this expansion we can infer that extensive HSC self-renewal occurs during ontogeny.

HSC self-renewal also occurs in myeloablated hosts undergoing hematologic recovery, as for example, following a BM transplant. This has been formally demonstrated by recovery of cells capable of giving donor-derived long-term repopulation of multiple secondary recipients from mice previously transplanted with only a single HSC (Dick et al., 1985; Keller et al., 1985; Lemischka et al., 1986; Brecher et al., 1993; Osawa et al.,1996). Quantitative analyses have shown that HSCs recovered from transplant recipients can be up to 100-fold higher than the number transplanted (Pawlliuk et al., 1996).

1.2.7 Potential clinical applications of *ex vivo* expanded HSCs

The ability to activate HSCs into division without causing their differentiation would be an immensely useful tool for both experimental and clinical applications. The capacity for sustained self-renewal is fundamental for the increasing application of HSC-based therapies in a wide range of malignant and genetic disorders. From that perspective, the development of strategies to extensively expand HSCs *ex vivo* could greatly enhance the safety and application of HSC – based therapies in treatment of malignancies, gene therapy and other areas. HSC transplantation following a myeloablative conditioning regimen is the only potenially curative treatment for aplastic anemia, hemoglobinophaties and leukemias, as it results in a full or partial replacement of the diseased hematopoietic tissue with normal cells. However, there are significant risks associated with HSC transplantation, including toxicity of conditioning regimens, graft versus host disease (GVHD) and mortality (reviewed in Anasetti et al., 2001). While HSCs obtained directly from the patient

(autologous HSCs) are used for rescuing patients from the effects of high dose of chemotherapy or used as the target for gene therapy vectors, HSCs obtained from another person (allogeneic HSCs) are used to treat hematological malignancies by replacing the malignant hematopoietic system with normal cells.

The number of HSCs injected is believed to be critical to ensure the efficacy of BM transplantation procedures (Mavroudis et al., 1996, Sierra et al., 2000). Therefore, amplification of HSC numbers would be useful in several contexts, both to overcome existing limitations and to develop new transplantation approaches. A successful HSC expansion strategy could also potentially allow the use of much smaller harvests of sources of HSC (e.g., mobilized peripheral blood, bone marrow) thereby reducing the cost and the risk of HSC collection. It might also accelerate the rate of HSC recovery following BM transplantation and allow use of T-cell depleted donor grafts, which reduce the incidence of and severity of GVHD. When such grafts were used in pediatric transplantation, the rate of T cell recovery was dependent on the dose of the $CD34^+$ cells that were administered. High doses of $CD34^+$ cells ($>20x10^6$ cells per kg) led to a marked decrease in the time required for T cell reconstitution (Handgretinger et al., 2001, Lang et al., 2004). However, in many cases, this high number of CD34+ HSCs could not be collected from the donor.

Another promising new source of HSCs for transplantation is cord blood (CB), which gives a reduced incidence of GVHD due to the naïve immunological state of the cells (Rocha et al., 2001). However, there is also a higher rate of mortality because of failed or delayed engraftment. This latter issue relates to the small size of CB grafts and their consequently low stem cell content. Higher HSC doses have

consistently been found to correlate with improved disease-free survival and reduced transplant-related mortality (Mavroudis et al., 1996, Sierra et al., 2000). Until HSC expansion methods are dramatically improved, the use of CB material will remain limited in adults.

HSC-based gene therapy is a growing treatment option for patients with hematological defects (reviewed in Cavazzana-Calvo and Hacein-Bey-Abina, 2001). It involves collecting HSCs from the patient and genetically modifying them with a therapeutic transgene. These procedures require *ex vivo* culture of hematopoietic cells and usually result in significant HSC losses, since in most culture conditions differentiation is favored over expansion. Moreover, genomic integration of retrovirus-based vectors requires target cells to be proliferating (Sadelain et al., 2000). Activation of HSCs into the S/G2/M phase of the cell cycle also results in the transient loss of engraftment potential (Habibian et al., 1998 Glimm et al., 2000). Therefore, an ability to expand HSCs *ex vivo* could greatly improve the clinical outcomes of HSC-based gene therapies.

In addition to these potential clinical applications, an ability to increase the number of HSCs in culture would provide a useful tool and source for studying the molecular mechanisms underlying HSC self-renewal.

1.2.8 Extrinsic regulators of HSC function

While the clinical imperative is high, harnessing and enhancing HSC self-renewal potential remains a formidable challenge (see Sauvageau et al., 2004. for recent review). A variety of *in vitro* conditions have now been described that permit

enormous expansion of CFCs and substantial expansion even of LTC-ICs (Petzer et al., 1997; Zandstra et al., 1997; Hoffman, 1999). However, the *in vitro* expansion of rigorously defined HSCs has not been achieved under the same conditions.

The largest reprodicble *in vitro* expansion of murine HSCs to date, using exogenous growth factors, is a 4-fold net increase of CRUs that is obtained in serum-free medium containing a combination of interleukin-11 (IL-11), flt3-ligand (flt3-L) and Steel factor (SF) (Miller and Eaves 1997). In a similar fashion, human CB HSCs were found to undergo a 2-4-fold net increase after 4-8 days culture in serum-free medium containing a rich cocktail of growth factors: flt3-L, SF, granulocyte colony-stimulating factor (G-CSF), IL-3 and IL-6 (Bahtia et al., 1997, Conneally et al., 1997). Intrinsic differences in cytokine requirements from different sources of HSCs were demonstrated, since human HSCs have different optimal cytokine combinations from murine HSCs.

Clues to extrinsic mediators of HSC self-renewal are also emerging from a broader understanding of the key receptor signaling pathways involved in the development and maintenance of the hematopoietic system. Perhaps, some of the most compelling evidence of early developmental growth factors impacting of HSC self-renewal have emerged from studies where presentation of the Notch1 ligand as an engineered immobilized form (Delta1), together with a cocktail of growth factors (SF, flt3-L, IL-6 and IL-11), resulted in a several log increase in the number of cells capable of short term lymphoid and myeloid repopulation after 28-day culture (Varnum-Finney et. al., 2003). Furthermore, addition of the soluble form of Sonic Hedgehog protein along with a cocktail of hematopoietic growth factors (flt3-L, SF,

G-CSF, IL-3 and IL-6) to a liquid cultures of human BM cells, also enhanced recovery of human HSCs over 7 day culture period (Bhardwai et al., 2001). Purified Wnt3A was shown to synergize with low doses of SF to induce proliferation of HSC-enriched cells placed in single-cell cultures (Willert et al., 2003). Such findings raise optimism that further refinement of culture conditions, growth factors etc. may enable striking increases in HSC numbers.

1.2.9 Intrinsic regulators of HSC function

The decision made by individual HSCs to self-renew (or not) has long been thought to be largely determined by stochastically regulated intrinsic mechanisms. This concept was first developed from studies showing a large variability in the numbers of CFU-S generated in individually assessed spleen colonies (Siminovitch et al., 1963) and the demonstration that this variability is predicted by a probabilistic model (Till et al., 1964) in which the likelihood of each CFU-S producing at least one progeny CFU-S throughout the formation of a spleen colony is only slightly higher than 0.5 (Vogel et al., 1968). It is important to note that the concept of stem cell self-renewal outcomes being described as probabilistic at a population level does not mean that the probability of stem cell self-renewal cannot be influenced. The limitations to this model were that they did not accommodate the possibility of pre-existent CFU-S hetergeneity in self-renewal potential or variations in the micro-environment in which each spleen colony develops (reviewed in Till and McCulloch, 1980). Later, the role of the environmental variability as a determining factor was largely ruled out by replicating these findings *in vitro* (Humphries et al., 1981).

However, even when self-renewal responses appear to be optimally supported by external factors, intrinsic mediators may be limiting resulting in a stochastic picture of response outcome at the population level.

Through the use of forward-genetic approaches, particularly loss-of-function or gain-of-function mouse models, some of the genes and related signaling pathways that are important in these outcomes have been identified. However, the identification of important HSC regulatory genes is likely incomplete. Thus deciphering the way in which these various regulatory pathways interact and identifying key common molecular target(s) remains a major challenge.

While much has been learned with regard to extrinsic factors that can promote the survival and proliferation of HSCs, growth factor-stimulated pathways that might promote self-renewal have proven more difficult to elucidate. A hematopoietic cell's developmental state is reflected by its complement of expressed genes. However, specific regulatory programs that control the gene expression repertoire to maintain the HSC state or trigger their differentiation remain unknown. Nevertheless, analysis of genes involved in leukemia and/or early development have begun to provide some important clues and, in particular, have drawn attention to various regulators of gene expression such as transcription factors, cell cycle regulators and chromatin modifiers.

Interestingly, gene expression experiments have shown that multipotent progenitor cells express many lineage-associated genes at low levels (Hu et al., 1997). Development along a given lineage thus appears to involve both activation of lineage-specific maturation factors and repression of genes associated with alternate

lineages. Transcription factors act on both processes, thereby promoting lineage choices. They act at all hematopoietic branch points, including specification to the hematopoietic fate, branching of the major lymphoid and myeloid lineages and commitment of bipotent progenitors to single lineages (reviewed in Orkin, 2000, Shivdasani and Orkin, 1996).

Genes required for the specification of hematopoietic potential include *SCL* (also called *tal-1*) and *LMO2* (also called *rbtn2*), which encode basic helix-loop-helix and LIM-domain type transcription factors, respectively. Mice lacking either of these genes have a complete absence of primitive hematopoiesis and die at approximately 10 dpc (Porcher et al., 1996, Warren et al., 1994). SCL and LMO2 proteins interact physically (Larson et al., 1996, Wadman et al., 1994) and their similar loss-of-function phenotypes suggest co-operative transcriptional control of hematopoietic-specific genes. Both are further required for erythroid differentiation, where they form a complex together with the transcription factors GATA1, E2A and Ldb1 (Wadman et al., 1997). Ectopic expression of SCL or LMO2 in T-cells leads to the generation of T cell acute lymphoid leukemia (Brown et al., 1990, Larson et al., 1996).

Ikaros is an intrinsic factor, which acts at the lympho-myeloid branching point, promoting specification to the lymphoid lineage. Mice lacking *Ikaros* lack all B-lymphocytes and precursors, as well as fetal T lymphocytes, although a few CD4+ T cells are aberrantly produced (Wang et al., 1996). Ikaros represses transcription of non-lymphoid genes via recruitment of histone deacetylase complexes to specific promoters (Kim et al., 1999), thereby altering chromatin accessibility.

Further specification within the lymphoid lineage comes from factors such as Pax5, which directs cells along the B-lymphiod lineage while repressing differentiation along alternate lineages. *Pax5* knockout mice lack mature B-cells and precursors (Urbanek et al., 1994) and pro-B cells from these mice will reconstitute T but not B lymphoid cells in transplanted mice (Rolnik et al., 1999).

Lineage specification within the myeloid system comes in part from cross-antagonism between GATA-1 and PU.1. GATA-1 promotes erythroid and megakaryocyte differentiation, while PU.1 acts on disparate lymphoid and myeloid lineages. GATA-1 and PU.1 directly interact with and inhibit one another (Nerlov et al., 2000, Rekhtman et al., 1999). Thus the ultimate lineage choice will be decided and then reinforced by the relative levels of these 2 transcription factors.

Recently, HOX family transcription factors have emerged as important regulators of hematopoiesis, acting at various levels of the hematopoietic hierarchy. The following section will discuss the roles of these proteins in hematopoiesis and their potency to expand HSCs *ex vivo*.

1.3 *HOX* Genes in HSC Function

1.3.1 *Hox* gene organization and expression

The *Homeobox* (*HOX*) genes were first discovered in *Drosophila melanogaster* (Lewis, 1978.). The term "homeobox" takes its origin from an old genetic term, the homeotic mutation, which describes *Drosophila* mutations in which the identity of one body segment was transformed into that of another: for example, the development of legs in the position where antennae are normally located. D. *melanogaster* has 8 homeobox genes divided in 2 clusters, *Antennapedia* (*ANT-C*) and *Bithorax* (*BX-C*) complexes. Together the 2 groups of clustered genes make up the *Drosophila homeotic complex* (*HOM-C*).

A large number of genes involved in pattern formation and morphogenesis during fruit fly development are homeobox genes. It was quickly recognized that these genes are present in all animal genomes, including man and mouse and play crucial roles in pattern formation and tissue identity throughout the animal kingdom (Akam 1989).

In mammals there are 2 main groups of *HOX* genes: class I, or the clustered *Hox* genes that have high homology to *Antennipedia* and class II, a diverged group of *homeobox* genes that have low homology to *Antennipedia* (Krumlauf, 1994). There are 39 class I *HOX* genes known in mammals, organized in four clusters, A-D, each containing 9-11 genes on 4 different chromosomes (Boncicelli et al., 1989, Scott, 1992, Zeltser et al., 1996). Based on homology, *HOX* genes in separate clusters can be aligned in groups, resulting in 13 paralogs (i.e. *HOXA4, HOXB4,*

HOXC4 and *HOXD4*). The high homology within paralogs suggests that a quadruplication of a single gene cluster has occurred during evolution (Holland et al., 1994, Kappen et al., 1993, Schughart et al., 1989). Figure 1.2 shows the organization of the clusters and comparison with the corresponding *Drosophila homeobox* genes.

HOX genes are expressed during embryonic development co-linear with chromosomal order. In *Drosophila*, genes at the 3' ends of clusters were found to be expressed earliest in development, with more 5' genes expressed later. Co-linearity also extends to the spatial domains of *HOM-C* gene expression, with 3' genes expressed in more anterior structures and more 5' genes having sequentially more posterior expression domains. This temporal and spatial co-linearity is also true for mammalian *HOX* gene expression during development (Dolle et al., 1989, Graham et al., 1989, Izpisua-Belmonte and Duboule, 1992), suggesting that gene order might have been conserved in order to maintain this tightly linked expression pattern. Regulation of *HOX* gene expression, which is not fully understood, involves most likely complex cross- and auto-regulatory mechanisms since it is known that *HOX* genes can affect the expression of each other (Zappavigna et al., 1991).

HOX proteins are DNA-binding transcription factors. They have domains for DNA binding and for protein-protein interactions. The most prominent structure of all HOX proteins is their homeodomain (reviewed in Gehring et al., 1994). The structure of homeodomain, 60-amino acid DNA binding domain, has been deduced by NMR (Billatar et al., 1990, Qian et al., 1989) and X-ray crystallography (Kissinger et al., 1990, Li et al., 1995). At the N-terminal end is flexible arm, followed by alpha helix I,

which is connected by a loop to alpha helix II. The helix-turn-helix sequence connecting helices II and III forms a highly conserved structure common to many DNA binding proteins. Footprinting, EMSA and trans-activation assays have shown that HOX proteins bind DNA as monomers to 5'-TAAT-3' core motif (Kalionis and O'Farrell, 1993). Helix III acts to recognize this sequence and binds DNA in the major groove. The flexible N-terminal arm binds to bases in the minor groove and the loop between helices II and III binds to the DNA backbone (Otting et al., 1990).

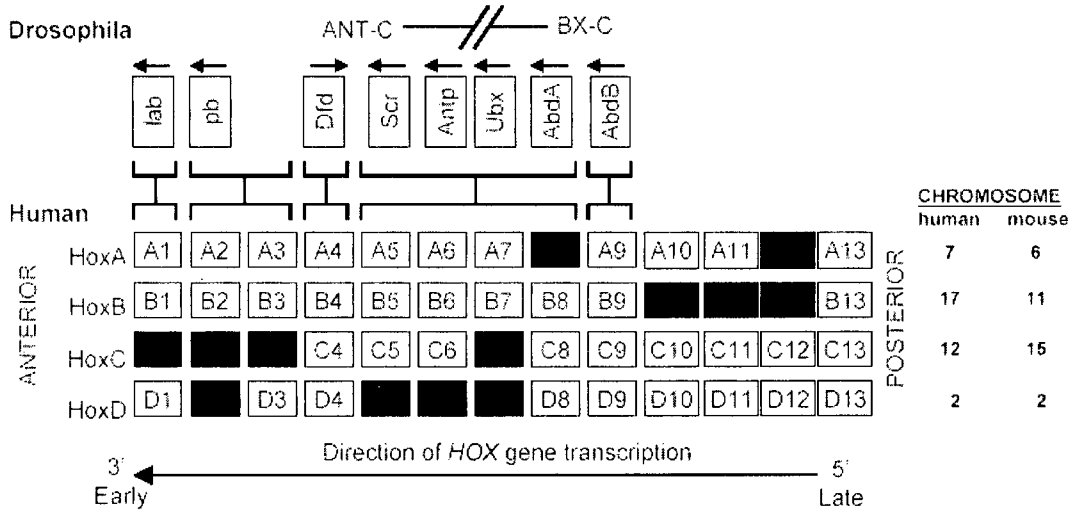


Figure 1.2 *HOX* chromosomal organization. The 4 mammalian *HOX* clusters (A-D) are shown, with alignments to the *Drosophila ANT-C* and *BX-C*. Groups 1-13 are called paralog groups sharing high sequence homology in the homeodomain. Black boxes indicate lack of gene. Genes at the 3' ends of the clusters are expressed earliest and most anterior, with sequentially later and more posterior expression of more 5' genes.

1.3.2 *HOX* gene expression and roles in hematopoiesis

A role for *HOX* genes in hematopoiesis was first demonstrated in human and murine hematopoietic cell lines. The first reports indicated lineage specificity of the Hox clusters, e.g., *HOXB* genes predominantly expressed in erythroid cell lines while *HOXA* genes were active in myeloid cells and *HOXC* in lymphoid cell lines (Lowney et al., 1991, Mathews et al., 1991, Lawrence et al., 1993). This lineage specificity is, however, not true for all *HOX* genes, where some show a much broader expression pattern. Members of the *HOXD* cluster were found not to be expressed in hematopoietic cells (Thompson et al., 2003). Expression analysis of primitive human hematopoietic CD34+ subpopulations revealed that nine of 11 *HOXA* genes; 8 of 9 *HOXB* genes and 4 of 9 *HOXC* genes tested, were expressed in this population (Giampaolo et al., 1994, Moretti et al., 1994, Sauvageau et al., 1994). Expression of the *HOXA* genes was strongest, followed by the *HOXB* genes and thereafter the *HOXC* genes. *HOX* genes located 3′ in the cluster, such as *HOXB3* and *HOXB4*, were mainly expressed in the primitive subset of $CD34^+$ cells and then down-regulated as the primitive cells start to mature. In contrast, 5′ located *HOX* genes, such as *HOXA9* and *HOXA10*, had prolonged expression and were also found in more differentiated populations (Sauvageau et al., 1994). Such findings suggested that *HOX* genes play functional roles in early stages of hematopoietic growth and differentiation.

Further expression analysis and gain- or loss- function studies in mouse models indeed confirmed that *HOX* genes have the ability to specifically regulate different stages of hematopoietic development, including the self-

renewal/proliferation of HSCs (Thorsteinsdottir et al., 2002; Antonchuk et al., 2001; Buske et al., 2002) and the differentiation of myeloid and lymphoid lineages (Owens and Hawley, 2002; Buske and Humphries, 2000). In recent years, HOX genes have been strongly linked to human leukemia (Lawrence et al., 1999; Rozovskaia et al., 2001; Kawagoe et al., 1999Afonja et al., 2000; Imamura et al., 2002) by their observed aberrant expression and by translocations involving their cofactor PBX1 (Kamos et al., 1993) and upstream regulators (Ayton et al., 2001). Moreover, the discovery of chromosomal translocations involving a growing list of Abd-B HOX genes provided support for the direct involvement of HOX genes in the pathobiology of human leukemia (Slape and Aplan 2004). Further elucidation of the cellular and molecular processes that are involved in normal and/or leukemic hematopoiesis and controlled by the complex HOX-based regulatory network, holds promise for developing new tools to expand HSCs and for providing a deeper understanding of mechanisms underlying HSC self-renewal.

1.3.3 *HOXB4* – a potent stimulator of HSC expansion

The effects of *HOXB4* gene on hematopoietic cells were initially discovered by using a retroviral-expression vector (Sauvageau, G. *et al.*, 1995). The most dramatic effect observed in recipients of *HOXB4*-transduced cells was an enhanced regeneration of donor-derived HSCs. Thus when lethally irradiated mice were reconstituted with BM cells that were transduced with a control vector, HSC numbers in the BM regenerated to only 5–10% of normal levels, whereas BM cells transduced the *HOXB4* vector regenerated normal numbers of HSCs. However, further

expansion of the HSC compartment did not occur (Thorsteinsdottir et al., 1999), showing that the effects of *HOXB4* are still subject to normal homeostatic controls.

Studies indicating an ability of HOXB4 to enhance the self-renewal of HSCs *in vivo* provided a basis for investigating its potential to promote an expansion of HSCs *in vitro* (Antonchuk et al., 2002; Krosl et al., 2003; Krosl et al., 2003). These studies demonstrated that *HOXB4* can stimulate the *ex vivo* expansion of HSCs, when supplied either as a transduced cDNA or as externally delivered protein and the HSCs produced retain their normal differentiation and long-term repopulation potential. Adult mouse HSCs engineered to overexpress *HOXB4* expand 40-fold after 2 weeks of culture in media containing IL-3, IL-6 and SF, while the number of untransduced of GFP-transduced HSCs decreased by 30-60-fold (Antonchuk et al., 2002). The rapid *ex vivo* HSC expansion induced by HOXB4 has further been exploited with the development of HOXB4 fusion proteins with the protein transduction domain of HIV TAT protein, that can be delivered directly to tissue culture medium to achieve HSC expansion in short term liquid culture (Krosl et al., 2003). TAT fusion proteins moved freely between the medium and intracellular compartments. However, the majority of TAT-HOXB4 protein is lost after a 4 hour incubation in serum-containing media and the half-life of intracellular HOXB4 is only approximately 1 hour. HSCs exposed to TAT-HOXB4 for 4 days expanded by about 4 to 6-fold and were 8-20 times more numerous than HSCs in control cultures. These findings indicate that HSC expansion induced by TAT-HOXB4 is comparable to that induced by *HOXB4* retrovirus during a similar period of observation and

encourage further development of more potent Hox-based molecules that could be adapted to delivery as proteins rather than by gene transfer.

Stimulatory effects of *HOXB4* have also been demonstrated on human HSCs following retrovirally-engineered overexpression. Studies by Buske et al showed 5-fold expansion of CB cells that repopulate NOD/SCID mice after just 1-2 days in culture (Buske et al., 2002). In addition, studies by Baum's group showed increased levels of *HOXB4*-transduced CD34+ cells in SCID recipients compared to controls (Schiedlmeier et al., 2003).

The mechanism of *HOXB4*-mediated expansion of HSCs is not well understood. It is known that *HOXB4* can co-operatively dimerize with PBX1 (pre-B-cell leukaemia transcription factor 1) (Krosl et al., 1998) which is encoded by a proto-oncogene that is required for the maintenance of definitive (adult) hematopoiesis (Dimartino et al., 2001). Downregulation of PBX1 expression using a vector that co-expressed both a PBX1 antisense sequence and HOXB4 resulted in a further increase in HSC expansion relative to that observed using *HOXB4* alone (Krosl et al., 2003).

1.3.4 Other *HOX* genes that have an ability to promote HSC expansion

Although *HOXB4* has been the most extensively studied *HOX* gene for its ability to increase HSC self-renewal, this potential may not be unique to *HOXB4*, and may extend to other intact or variant *HOX* genes. For example, *HOXA9* was initially studied for its strong involvement in acute myeloid leukemia. However, when compared with controls, recipients of *HOXA9*-transduced cells had about a 15-fold

increase in transplantable lymphomyeloid long-term repopulating cells, during the preleukemic phase of the disease (Thorsteinsdottir et al., 2002). In the same study, it was demonstrated that overexpression of *HOXA9* greatly enhances HSC regeneration in transplantation chimeras, leading to an expansion of myeloid CFCs and accompanied by a partial block in B lymphopoiesis. These data, together with the preferential expression of *HOXA9* seen in primitive hematopoietic cells (Sauvageau et al., 1994) and the reduction in HSC numbers seen in *Hoxa9* homozygous mutant mice (Lawrence et al., 1998), suggest that this gene might qualify as a regulator of primitive hematopoietic cells and be capable also of promoting HSC expansion *ex vivo*.

Another major way in which *HOX* genes have been implicated in leukemia is through their involvement in translocations with nucleoporin 98 (*NUP98*). *Abdominal-B HOX* genes are the most common fusion partners of *NUP98*, identified in patients with myeloid leukemia (Lam and Aplan, 2001). The NUP98 protein is a component of the nuclear pore complex, which regulates nucleocytoplasmic transport of protein and RNA (Radu et al., 1995). All *NUP98-HOX* fusions reported to date 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). They also contain the C-terminus of the *HOX* gene product (A9, A11, A13, C11, C13 or D13), including the intact homeodomain and a variable portion of the flanking amino acids (Lam and Aplan, 2001). As detailed below, findings from studies of properties of natural and engineered fusions have provided intriguing new leads to potent molecules for HSC expansion.

Initially these *NUP98-HOX* fusion genes were analyzed for their effects on transduced murine BM transplants, which demonstrated the leukemogenic activity of fusions containing *Abdominal*-B members (i.e., *NUP98-HOXA10)*, but not *Antennapedia* members (i.e., *NUP98-HOXB4* or *NUP98-HOXB3)*(Pineault et al., 2004). It was also found that these *NUP98-HOX* fusion genes have a potent ability *in vitro* to block hematopoietic differentiation and to promote the self-renewal of primitive progenitors, as indicated by serial replating of CFCs or massive expansion of CFU-S numbers in short term (7 day) cultures. Interestingly, *NUP98-HOXA10* had a much more potent activity in this regard than did *NUP98-HOXB4*.

1.4 Thesis objectives

The experiments in this study were driven by a primary goal to develop new strategies for achieving higher levels of HSC expansion *ex vivo* than can be obtained with HOXB4. Based on the work showing that NUP98-HOX proteins have a more powerful ability to suppress early hematopoietic cell differentiation than HOXB4, my work focused on the potential use of *NUP98-HOX* fusion genes as potential stimulators of HSC expansion in an *ex vivo* system. The major aims of these studies were as follows:

1) To delineate the potency of *NUP98-HOX* fusion genes for HSC expansion *ex vivo;*
2) To determine if the *HOXA10* homeodomain sequence was sufficient as part of the *NUP98-HOXA10* fusion gene to stimulate HSC expansion;
3) To assess the quality of *NUP98-HOX*-transduced and expanded HSC in regard to long-term lympho-myeloid repopulation ability;
4) To demonstrate clonal ex-vivo expansion of *NUP98-HOX* transduced HSCs.

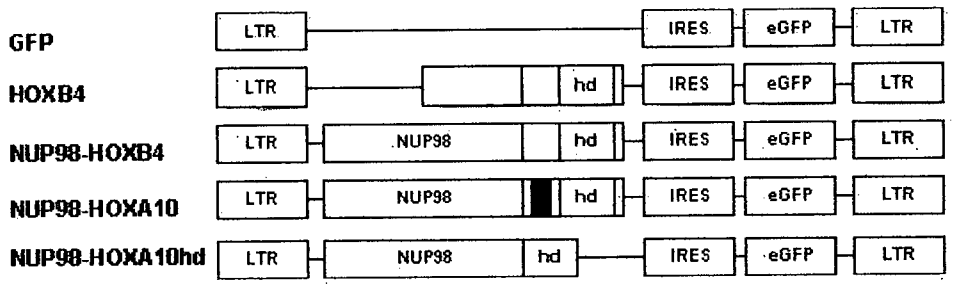
CHAPTER 2 MATERIALS AND METHODS

2.1 Retroviral Vectors

Vectors were based on the murine stem cell virus (MSCV) vector originally described by Hawley which has virtues of expressing well in HSC and later cells and is not prone to expression silencing (Hawley et al., 1994). All vectors used an internal ribosomal entry site (IRES) to enable efficient translation of 2 proteins from a single LTR driven transcript. All vectors also contained a *GFP* cDNA in the second position to provide a convenient marker to identify and select transduced cells and serve as a surrogate marker of the co-expressed *HOX* gene. All vectors (Figure 2.1.) have been described previously (Antonchuk et al., 2001; Pineault et al., 2004). Constructs were validated by sequencing and correct expression and transmission were confirmed by Western blot and Sothern blot analysis. Production of high-titre helper-free retroviruses 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 transducer the ecotropic packaging cell line $GP^{+}E86$ (Markowitz et al., 1988).

Figure 2.1. Structures of retroviruses used in the study

Three new *NUP98-HOX* fusion genes were engineered by fusing the cDNA sequence corresponding to the homeobox-containing exon of *HOXA10* (*NA10*) or *HOXB4* (*NB4*), or just homeodomain (hd) of *HOXA10* (*NA10hd*) to that of *NUP98*. Only *NA10* retained its Pbx-interacting motif, which is indicated as a black rectangle.



2.2 Mice

Mice were bred and maintained at the British Columbia Cancer Research Centre animal facility according to the guidelines of the Canadian Council on Animal Care. All BM donors and recipients were chosen based on their CD45 cell surface marker genotype. Transplant donor/recipient pairs were either C57Bl/6Ly-Pep3b

(Pep3b) mice that express Ly5.1 and C57Bl/6-W^{41}/W^{41} (W^{41}) mice that express Ly5.2, or Pep3b mice that express Ly5.1 and C57BL/6J (B6) mice that express Ly5.2, or B6 mice that express Ly5.2 and Pep3b mice that express Ly5.1.

2.3 Infection of Primary Murine BM Cells

Primary mouse BM cells were transduced as previously described (Antonchuk et al., 2002). Briefly, BM cells were extracted from mice injected intravenously 4 days previously with 150 mg/kg 5-fluorouracil (5-FU) (Faulding, Underdaler, Australia) and the cells were then cultured for 2 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 10 ng/ml human IL-6, 6 ng/ml murine IL-3, and 100 ng/ml murine SF. Media, serum and growth factors were purchased from StemCell Technologies (Vancouver, BC, Canada). After stimulation, the cells were harvested and infected by co-cultivation on irradiated (4,000 cGy X-rays) GP+E-86 viral producer cells with the addition of 5 μg/ml protamine sulfate (Sigma, Oakville, ON, Canada). Loosely adherent and nonadherent cells were recovered from these co-cultures after 2 days and cultured a further 6 days in the same medium without protamine sulfate. For bulk culture experiments, $3x10^6$ cells were seeded in a 10 cm dish at day 0, and the equivalent of $3x10^5$ starting cells (day 0) were replated into the same size dish on days 6 or 7. For cultures initiated with small numbers of bulk BM cells (5000) or sorted Sca-1^+ Lin^- cells (500), cells were cultured in a 96-well at day 0, and replated into a 24-well plate at day 7.

2.4 CRU Assay

HSCs were detected and evaluated using a limiting dilution transplantation-based assay for cells with competitive, long-term, lympho-myeloid repopulating function. The basic procedure (Szilvassy et al., 1990) and a modification employing sublethally irradiated W^{41} recipients (450 cGy ^{137}Cs gamma radiation) with an endogenous source of competitor cells, have been described in detail previously (Miller et. al. 1997, Antonchuk et al., 2002; Antonchuk et. al., 2001;). Briefly, lethally (810 cGy of X-ray) or sublethally (450 cGy of ^{137}Cs –gamma radiation) mice were injected with the cultured cells in varying dilutions, and their PB cells were collected and analyzed by flow cytometry ≥ 16 weeks post-transplant to look for evidence of regenerated lymphoid and myeloid cells derived from the transduced (GFP^+) cells injected. Mice that had >1% donor-derived (GFP^+) cells in all subpopulations of myeloid cells (Gr-1^+ and/or Mac-1^+), B cells ($B220^+$), and T cells ($CD4^+$ and/or $CD8^+$) were considered to be repopulated with transduced cells. CRU frequencies were calculated by applying Poisson statistics to the proportion of negative recipients at different dilutions using Limit Dilution Analysis software (StemCell Technologies).

2.5 Flow Cytometry

For analysis of transplant recipients, 100 μl of blood was extracted from the tail vain, and the erythrocytes were lysed with ammonium chloride (StemCell Technoligies). Leukocyte samples suspended in HF were incubated sequentially on ice with the following monoclonal antibodies: a combination of biotinylated anti-Ly5.1

(anti-Ly5.2) and either phycoerythrin (PE)-labeled B220 or combination of PE-labeled Gr-1 and Mac-1 or combination of PE-labeled CD4 and CD8 and then allophycocyanin (APC)-labeled streptavidin. All antibodies were purchased from Pharmingen (San Diego, California, USA). All samples were washed with HF and 1μg/ml PI prior to analysis.

2.6 Purification of Sca-1^{+}Lin^{-} Cells

BM cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-Sca-1, allophycocyanin (APC)-labeled anti-c-kit, and PE-labeled anti-Gr-1, anti-B220, anti-CD4, anti-CD8 and Ter119 antibodies. Sorting was performed on a FACSAria system (Becton Dickinson, San Jose, CA). Sorted cells were counted using a hemocytometer and plated into a 96-well plate.

2.7 Proviral Integration Analysis

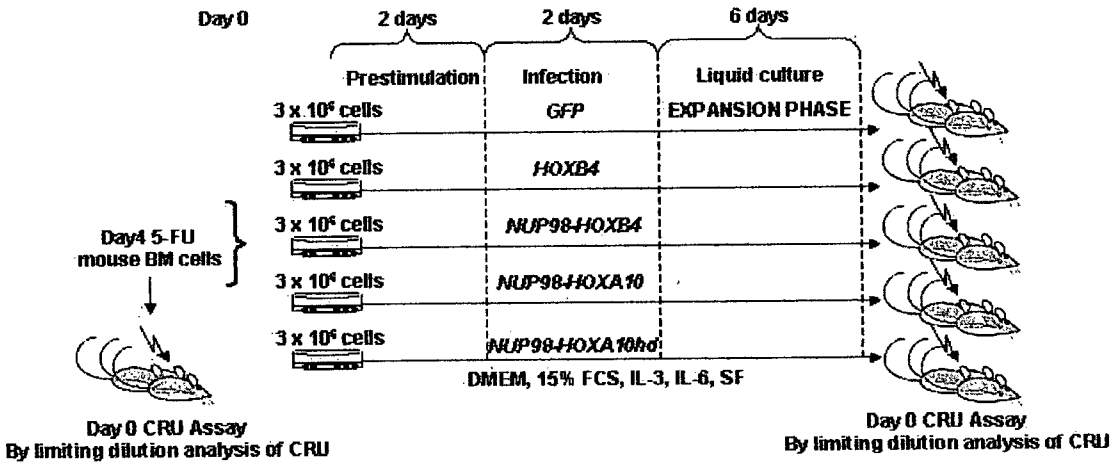
Genomic DNA was isolated with DNAzol reagent (Invitrogen, Carlsbad, CA), as recommended by the manufacturer, and Southern blot analysis was performed as previously described (Sauvageau et. al., 1994). Unique proviral integrations were identified by digestion of DNA with *EcoRI*, which cleaves once within the provirus and at various distances outside in the host genome. Digested DNA was then separated in 0.8% agarose gel by electrophoresis and transferred to zeta-probe membranes (Bio-Rad, Mississauga, ON). Membranes were probed with a [^{32}P] dCTP *GFP* sequence.

CHAPTER 3 RESULTS

3.1 *NUP98-HOX* Fusion Genes Stimulate A Very Large Expansion of HSCs in Culture

A first series of experiments were designed to test the possibility that *NUP98-HOX* fusion genes have a similar or even greater potency to stimulate HSC expansion *in vitro* than *HOXB4*, which was previously documented to expand HSCs more than 40-fold in 2-week cultures (Antonchuk et al., 2002). Therefore, HSC numbers were measured in cultures of *GFP*-, *HOXB4*-, *NUP98-HOXB4*- and *NUP98-HOXA10*-transduced mouse BM cells by performing limiting dilution CRU assays before and after 10 days *in vitro* without selection of *GFP*-expressing cells. As shown schematically in Figure 3.1, BM cells were harvested 4 days after intravenous injection of donor mice with 5-FU and individual cultures were initiated (Day 0) with $3x10^6$ cells per culture. Cells were pre-stimulated for 2 days with IL-6, IL-3 and SF prior to being retrovirally transduced with GFP control or *HOXB4*, *NUP98-HOXB4* or *NUP98-HOXA10* vectors (2 additional days of co-culture with virus producers) and were then cultured for another 6 days in suspension with the same growth factors. On day 10, more than 75% of the cells in each culture were *GFP*+ and various doses of starting cell equivalents were transplanted into irradiated recipients.

Figure 3.1. – General experimental design



The contribution of transduced (*GFP*+) cells to the lymphoid and myeloid reconstitution of the transplanted recipients was determined by flow cytometry at 16 weeks after transplantation. Recipients having at least 1% of transduced cells in both lymphoid and myeloid compartments were considered to be positively reconstituted (Figure 3.2.).

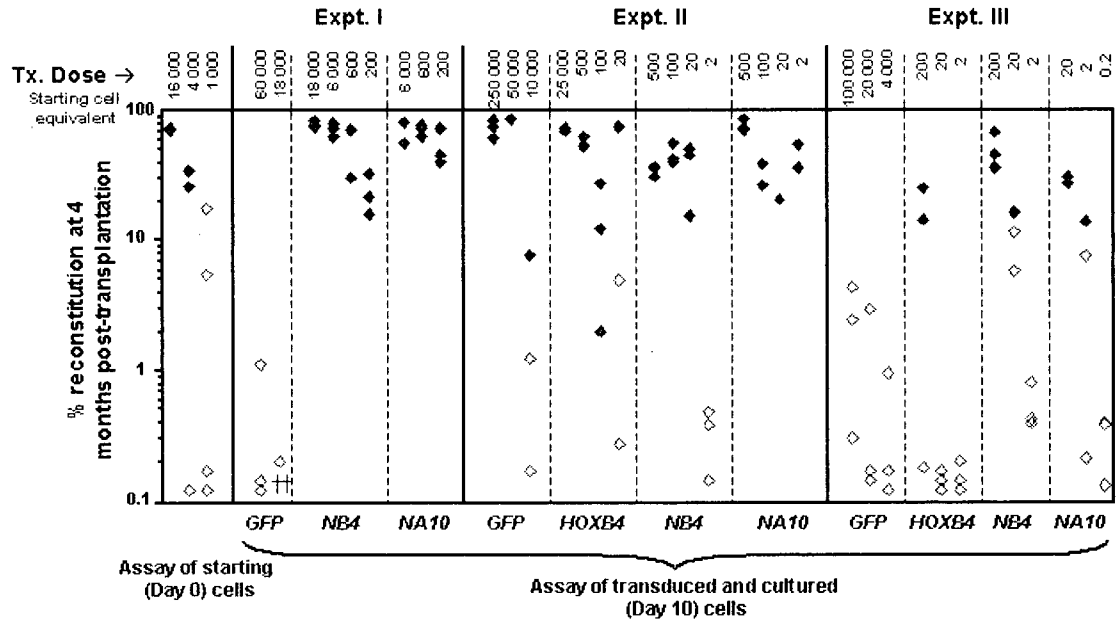


Figure 3.2. - Long-term reconstitution of recipients transplanted with *GFP*-, *HOXB4*-, *NUP98-HOXB4*- or *NUP98-HOXA10*-transduced cells. Shown is donor-derived (Day 0) or donor-derived/*GFP*$^+$ (Day 10) reconstitution of PB at 16 weeks post transplantation. Black diamonds represent reconstituted recipients having > 1% of donor-derived/*GFP*$^+$ cells in myeloid (Gr-1$^+$/Mac-1$^+$) and lymphoid (B220$^+$ and CD4$^+$/CD8$^+$) subpopulations. White diamonds represent non-reconstituted recipients having < 1% of donor-derived/GFP$^+$ cells and/or lacking ability to reconstitute myeloid or lymphoid compartment. Transplantation (Tx) dose is expressed in starting cell equivalents. (Data obtained by Dr. Hideaki Ohta.)

The proportion of "negative" recipients as a function of the number of cells injected was analyzed by Poisson statistics to calculate GFP+ CRU frequencies. At the start of the pre-stimulation culture period, this value was ~1 in 5000 cells. Thus ~600 CRUs were used to initiate each culture. Figure 3.3. shows the CRU frequencies at the end of the 10 days of culture but expressed on the basis of starting cell equivalents for a representative experiment. These show that the yield of CRU dramatically increased in the cultures containing the *HOXB4* and *NUP98-HOX* transduced cells, in contrast to the marked decrease in CRU frequency documented in the control culture (Figure 3.3).

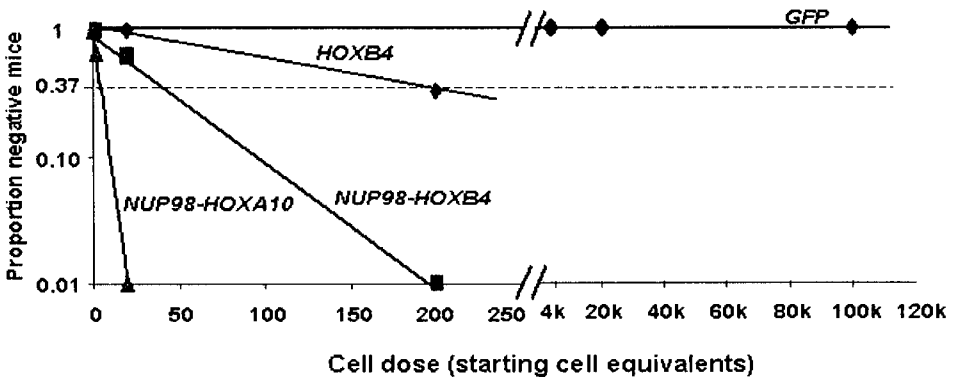


Figure 3.3. - Limiting dilution analysis (LDA) for estimation of CRU frequencies in 10-day cultures of HSCs transduced with various *HOX* fusion genes. If ~37% of recipients transplanted with the given dose are not repopulated or negative, that exact dose should contain 1 CRU. Transplantation dose is expressed in starting cell equivalents. (Data obtained by Dr. Hideaki Ohta.)

These findings were consistent in multiple experiments. Pooled data (at least 3 for each gene tested) are presented in Figure 3.4. The results show an overall net amplification of CRU numbers of 80-fold, 290-fold and >2000-fold in the cultures of *HOXB4*-, *NUP98-HOXB4*- and *NUP98-HOXA10*-transduced cells and an overall net decline in CRU numbers of 50-fold in the control cultures of GFP-transduced cells. The expanded CRU populations were restricted to *HOXB4*-, *NUP98-HOXB4*- or *NUP98-HOXA10*-transduced cells, as there was complete concordance between the presence of regenerated donor-derived cells (identified by CD45 allotype markers) and transduced (GFP+) cells in the reconstituted recipients.

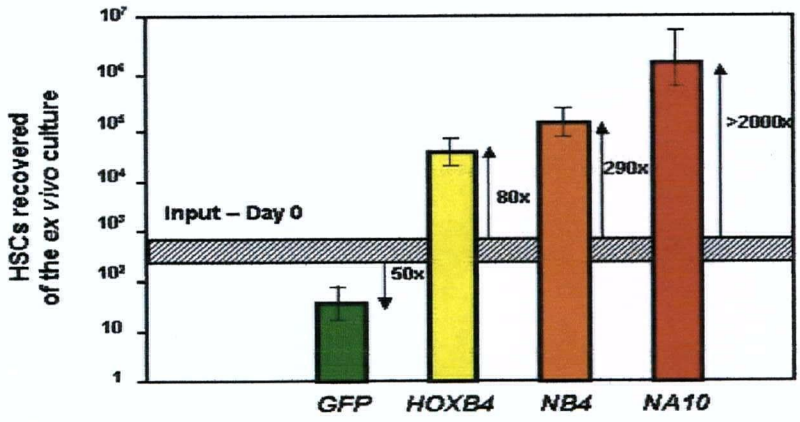


Figure 3.4. - *Ex vivo* expansion of transduced HSCs after 10 dys of culture For each expanding agent tested, results of at least three independent experiments were pooled and expressed as the mean +/- standard error mean (SEM) of the CRU numbers per culture of 3 x 10^6 starting cells. In some experiments, for culture containing *NUP98-HOXA10*-transduced cells, limiting dilution was not reached, thus the level of HSC expansion was estimated to be at least 2000-fold. (Data obtained by Dr. Hideaki Ohta.)

Together, these results establish the ability of the *NUP98-HOXB4* and *NUP98-HOXA10* fusion genes to mimic the ability of *HOXB4* to amplify HSCs *ex vivo* thus extending this activity to fusion genes of *NUP98* and *HOXB4* and the *Abdominal–B* class *HOX* gene, *HOXA10.* Moreover, the potency of *HOXB4* appears enhanced as part of a fusion with *NUP98* with even greater potency obtained with the *NUP98-HOXA10* fusion gene.

3.2 A *NUP98-HOX* Fusion Gene Containing Only the Homeodomain of *HOXA10* Retains the Full HSC *Ex Vivo* Expansion Activity of the Parent Fusion Gene

Following the finding that very marked expansion of HSCs can be achieved *in vitro* by forced expression of an engineered fusion between *NUP98* and the second exon of *HOXA10*, we further analyzed the *HOXA10* sequences required to achieve this effect. The second exon of *NUP98-HOXA10* encodes the homeodomain plus another 16 N terminal amino acids that provide a PBX-binding motif (Chang et al., 1996) and another 15 C terminal amino acids of unknown function. Recent studies of the leukemogenic properties of various *NUP98-HOX* fusion genes, including *NUP98-HOXA10*, have revealed that the homeodomain is essential for blocking hematopoietic differentiation (Pineault et al., 2004) and PBX1 knock down studies have shown that the *in vivo* competitiveness of *HOXB4*-overexpressing cells is enhanced >20-fold (Krosl et al., 2003). I therefore hypothesized that the homeodomain might be necessary and perhaps sufficient to provide the HSC expanding properties of *NUP98-HOX* fusion proteins. To test this hypothesis, a *NUP98-HOX* fusion gene retaining only the *HOX* sequence encoding the 61 amino

acid homeodomain of *HOXA10* was constructed, placed in an MSCV vector (hereafter referred to as the *NUP98-HOXA10hd* vector, Figure 2.1), and then tested for its ability to expand HSCs in culture by comparison to the parental fusion gene using the same protocol as outlined above. On the 10^{th} day of culture, the percentage of GFP^+ cells in each culture was again above 75%. CRU assays performed on the cells before and after the 10-day prestimulation-transduction-expansion culture period showed that the CRU content of the culture containing *NUP98-HOXA10hd*-transduced cells had increased ~1500-fold-essentially identical to the levels obtained previously using the *NUP98-HOXA10* vector (Figures 3.5 and 3.6).

These findings indicate that the DNA-binding homeodomain alone as part of a *NUP98-HOX* fusion gene is sufficient to promote the high levels of HSC expansion in culture achieved with the full length fusion gene and further demonstrate that the PBX-binding motif to be dispensable for this activity.

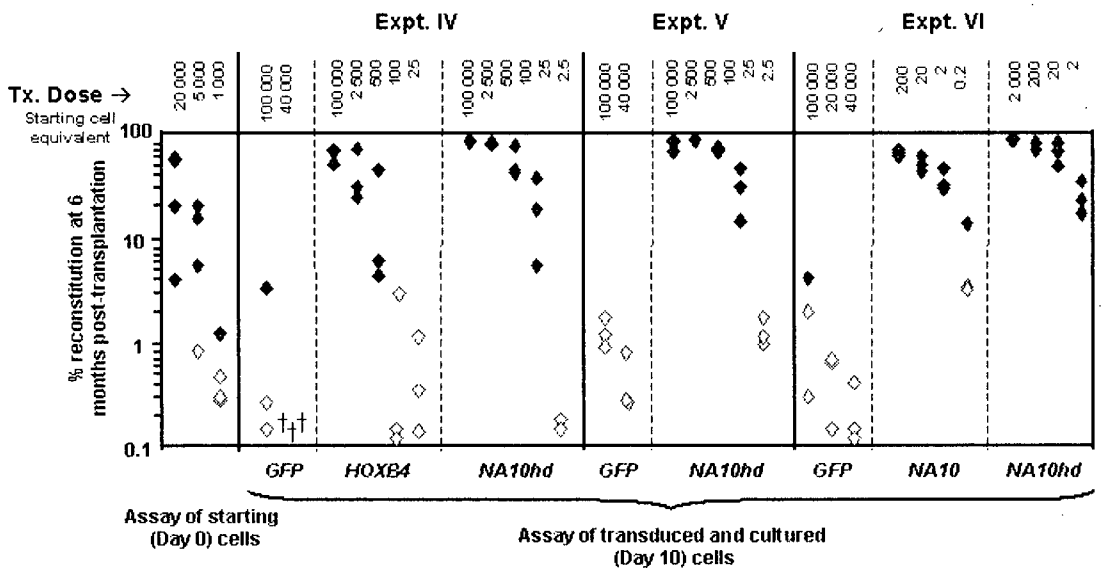


Figure 3.5. - Long-term reconstitution of recipients transplanted with *GFP*-, *HOXB4*- or *NUP98-HOXA10hd*-transduced cells. Shown is donor-derived (Day 0) or donor-derived/*GFP*$^+$ (Day 10) reconstitution of PB at 6 months post-transplantation. Black diamonds represent reconstituted recipients having > 1% of donor-derived/*GFP*$^+$ cells in myeloid (Gr-1$^+$/Mac-1$^+$) and lymphoid (B220$^+$ and CD4$^+$/CD8$^+$) subpopulations. White diamonds represent non-reconstituted recipients having < 1% of donor-derived/*GFP*$^+$ cells and/or lacking ability to reconstitute myeloid or lymphoid compartment. Transplantation (Tx) dose is expressed in starting cell equivalents.

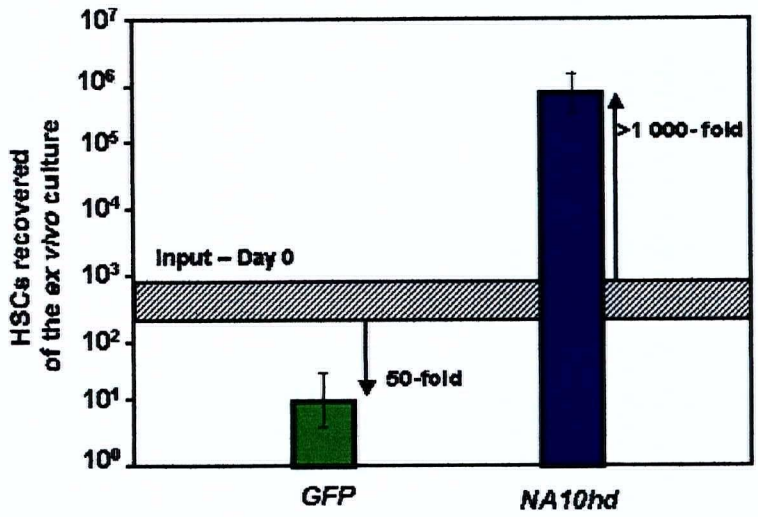


Figure 3.6. - *Ex vivo* expansion of transduced HSCs after 10 days of culture For each expanding agent tested, results of three independent experiments were pooled and expressed as the mean +/- standard error mean (SEM) of the CRU numbers per culture of 3 x 10^6 starting cells. In some experiments, for culture containing *NUP98-HOXA10hd*-transduced cells, limiting dilution was not reached, thus the level of HSC expansion was estimated to be at least 1000-fold.

3.3 *NUP98-HOX*-transduced Cells Retain Multi-lineage Repopulating Ability

To confirm that the expanded HSCs retained full multi-lineage repopulating ability, detailed immunophenotypic flow cytometric analysis was performed on blood samples from mice transplanted ≥ 16 weeks previously. The presence of transduced (*GFP*+) cells in the myeloid, B-lymphoid, T-lymphoid and red blood cell compartments from representative recipients indicated that the expanded HSCs were not compromised in their capacity to differentiate along all myeloid and lymphoid lineages examined (Figure 3.7).

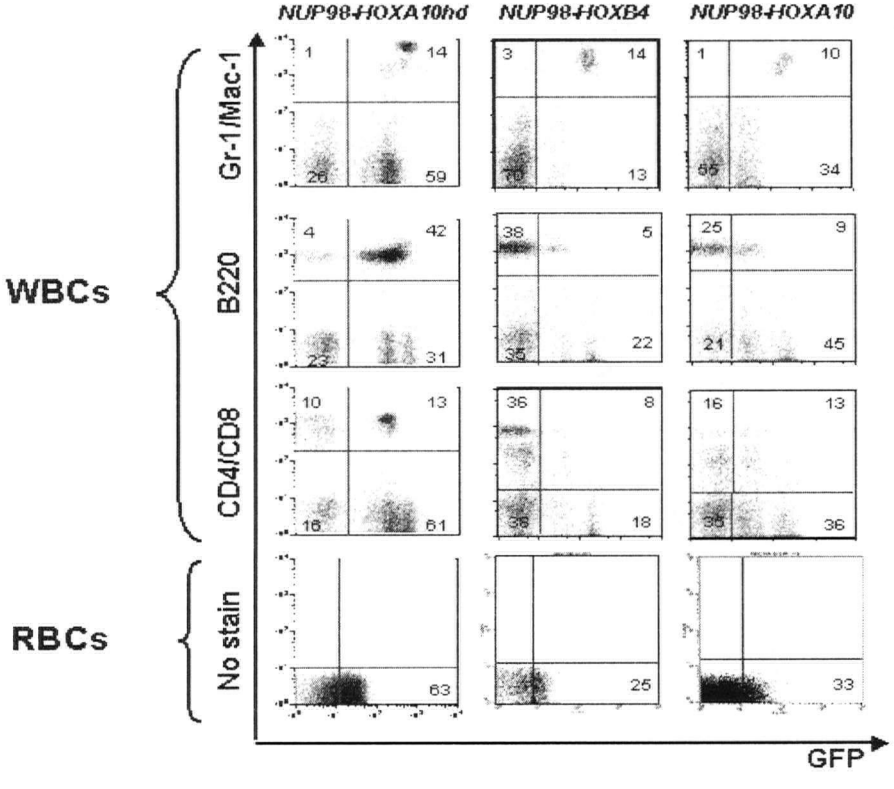


Figure 3.7. - Representative peripheral blood FACS profiles of recipients transplanted with *NUP98-HOXB4-*, *NUP98-HOXA10-* or *NUP98-HOXA10hd-*transduced cells. Representative recipients received equivalent of 200, 200 or 250 starting cells, respectively. *GFP*-expressing cells were present in myeloid (Gr-1$^+$Mac-1$^+$), B-lymphoid (B220$^+$) and T-lymphoid (CD4$^+$CD8$^+$) compartments as well as red blood compartment (RBC) of transplanted recipients.

Recipients transplanted with *HOXB4-* or *NUP98-HOXB4*-transduced cells demonstrated normal lineage distributions, similar to those seen in normal, unmanipulated mice. On the other hand, although recipients of *NUP98-HOXA10*-transduced cells displayed substantial contributions to all lineages, there was a modest increase in proportion of myeloid as compared to lymphoid cells. However, these recipients remained healthy, not showing any signs of an incipient myeloproliferative disorder and/or leukemia during the period of at least one year post transplantation that the mice were followed. Similar detailed analyses carried out on recipients of expanded *NUP98-HOXA10hd*-transduced cells also showed normal lympho-myeloid distributions and high-level contributions to the red blood cell compartment (Table 3.1.).

Table 3.1. – Summary of lineage distribution of *GFP*+ cells in PB of transplanted recipients

Expanding agent	% of GM cells in GFP^+ compartment ± SD	% of B cells in GFP^+ compartment ± SD	% of T cells in GFP^+ compartment ± SD
(-) → CTL	16.27±7.35	52.67±15.28	17.99±9.64
HOXB4	40.68±15.70	46.60±15.62	5.85±2.23
NB4	45.38±16.22	51.44±15.69	8.31±1.55
NA10	70.94±3.65	32.87±7.71	5.52±1.93
NA10hd	27.46±8.30	41.66±8.22	29.52±4.11

To further examine the lympho-myeloid repopulating capacity of HSCs recovered after expansion *ex-vivo*, Southern blot analysis of proviral integrations was performed on DNA extracts from enriched populations of BM myeloid cells, splenic B cells and thymic T cells from representative recipients of *NUP98-HOXA10-* or *NUP98-HOXA10hd*-transduced cells. The identical patterns of proviral integration sites observed confirmed the pluripotent nature of *NUP98-HOXA10-* or *NUP98-HOXA10hd*-transduced HSC clones (Figure 3.8).

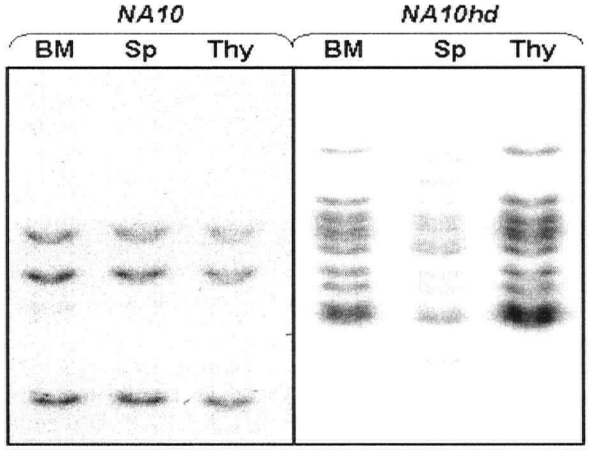


Figure 3.8. - Southern blots, showing common integration patterns of vector DNA in reconstituted myeloid (BM) and lymphoid (Spleen and Thymus) tissues of representative recipients of *NUP98-HOX* expanded HSCs. Representative recipients reconstituted by *NUP98-HOXA10-* or *NUP98-HOXA10hd*-transduced cells, received equivalent of 500 or 200 starting cells, respectively. (Data obtained by Dr. Hideaki Ohta)

3.4 Polyclonal Recovery of *NUP98-HOX*-transduced HSCs

Further evidence of the polyclonal composition of the HSCs obtained in cultures of *NUP98-HOXA10-* or *NUP98-HOXA10hd*-transduced cells was obtained by Southern blot analysis of proviral integrations in BM DNA isolated from recipients of these cells at late time points (>6 months post-transplant). For this purpose, multiple recipients transplanted with various doses of cells harvested from a single culture of *GFP*, *NUP98-HOXA10* or *NUP98-HOXA10hd*-transduced cells were analyzed. Given the marked decline in HSC content in GFP control cultures, recipients of these cells even at the highest transplant dose (250,000 starting cell equivalents) showed a small number of proviral integrations with the same proviral integration pattern in all positive mice (data not shown). In contrast, recipients of cells from the cultures of *NUP98-HOXA10* and *NUP98-HOXA10hd*-transduced cells showed highly complex and distinct proviral integration patterns at much lower transplant doses (e.g. 200 or 20 starting cell equivalents) and the extent of polyclonality was clearly related to the dose of transduced cells transplanted (Figure 3.9). Thus, only in recipients of the lowest dose of *NUP98-HOXA10* and *NUP98-HOXA10hd* cells (~2 starting cell equivalents) was a simple proviral integration pattern apparent consistent with the injection of these mice with a near limiting dilution of transduced HSCs. At these lowest transplant doses, unique patterns were apparent in different recipients, indicating that the >2000-fold HSC expansion *ex-vivo* measured by CRU assays reflected a highly polyclonal population of HSCs in the expansion cultures from which they were obtained. Variations in the autoradiographic intensities of bands that represent these integrations suggest that each recipient of the lowest cell dose

was reconstituted by one or two unique clones with up to 3 proviral integrations per clone. This result would also agree with the CRU frequencies measured (<1 in 2 starting cell equivalents) for the cultures of *NUP98-HOXA10*- or NUP98-HOXA10hd-transduced cells.

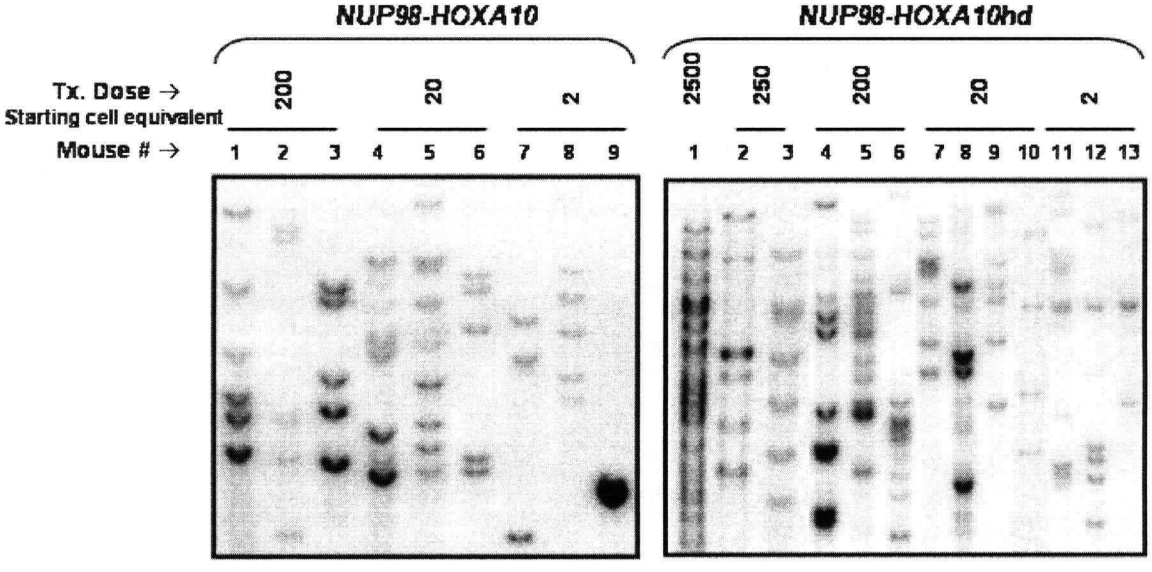


Figure 3.9. - Polyclonal recovery of *NUP98-HOX*-transduced HSCs. Southern blot analysis of proviral integrations in DNA isolated from BM of multiple recipients transplanted with various doses of *NUP98-HOXA10*- and *NUP98-HOXA10hd*-transduced cells, 16 weeks post transplantation. Transplantation (Tx.) dose is expressed in starting cell equivalents. (*NUP98-HOXA10* data obtained by Dr. Hideaki Ohta.)

3.5 *Ex Vivo* Expansion of *NUP98-HOXA10*-transduced BM cells in Cultures Initiated with Small numbers of Input CRUs – Direct Evidence of High Level Clonal HSC Expansion

As a further test of the ability of *NUP98-HOXA10* to stimulate high level clonal expansion of HSCs, additional experiments were carried out in which individual cultures were initiated with small numbers of 5FU-pretreated BM cells per well, estimated to contain 1 or 2 CRUs. Each well was then subjected to transduction, culture and assayed individually for CRU content at the end of the 10 day culture period as for the larger cultures (Figure 3.10).

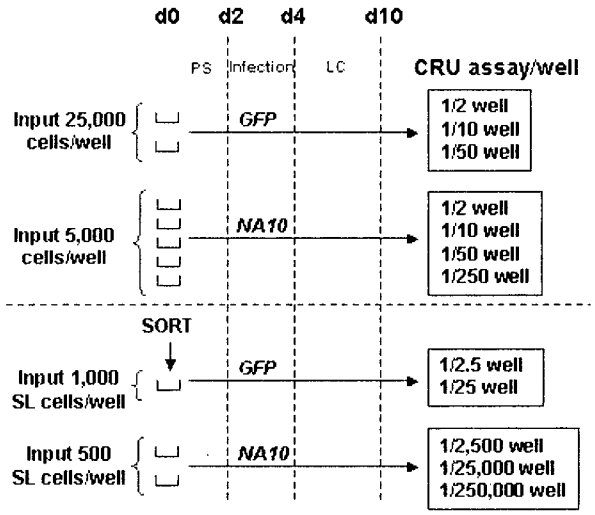


Figure 3.10. – Experimental protocol for examining the ex vivo expansion of *NUP98-HOXA10*-transduced BM cells in cultures initiated with 1-2 CRUs. Cultures were initiated with 25,000 or 5,000 (~1-2 CRUs) 5-FU pre-treated mouse BM cells per well, prestimulated with IL-3, IL-6 and SF and retrovirally transduced with *GFP* control or *NA10* vectors, respectively. 6 days after infection, individual wells were harvested and various fractions of each/single well were transplanted into irradiated recipients. By day 10, percentage of GFP^+ cells in wells containing *GFP*-transduced or *NA10*-transduced cells was above 65%. Also at day 0 5-FU pre-treated mouse BM cells were sorted for $Sca1^+Lin^-$ stem cell enriched population and cultures were initiated with 1,000 or 500 (~2 CRUs) per well. Cells were again prestimulated with IL-3, IL-6 and SF and retrovirally transduced with *GFP* control or *NA10* vectors, respectively. 6 days after infection, individual wells were harvested and various fractions of each/single well were transplanted into irradiated recipients. By day 10, percentage of GFP^+ cells in wells containing *GFP*-transduced or *NA10*-transduced cells was above 75%.

In these experiments none of the recipients transplanted with *GFP*-transduced cells showed GFP^+ cells in their reconstituted blood cells, indicating the expected loss of HSCs in these cultures whereas up to 6 recipients transplanted with various fractions of the single well containing *NUP98-HOXA10*-transduced cells demonstrated long-term lympho-myeloid reconstitution. These results confirm the extensive CRU expansion obtainable with these vectors even when starting with limiting numbers of CRUs. The fact that recipients of as little as $1/250^{th}$ of one of these cultures were highly reconstituted points to expansions of 125-fold as suggested by the bulk cultures (Figure 3.11). Moreover, in experiment carried out with phenotypically defined, CRU-enriched $Sca1^+Lin^-$ cells that allowed even smaller numbers of starting cells to be used (i.e., as low as 500 cells, estimated to contain 2 CRUs), measurement of CRU frequencies before and after the culture period again showed a high degree of reconstitution of recipients transplanted with extremely low ($1/2500^{th}$ or even $1/25000^{th}$) fractions of single cultures of *NUP98-HOXA10*-transduced cells whereas no reconstitution was obtained with the *GFP*-transduced cells (Figure 3.11). These findings further document the very high levels of clonal expansion of CRUs obtainable with *NUP98-HOXA10* and suggest that the responsive cells have a $Sca1^+Lin^-$ phenotype.

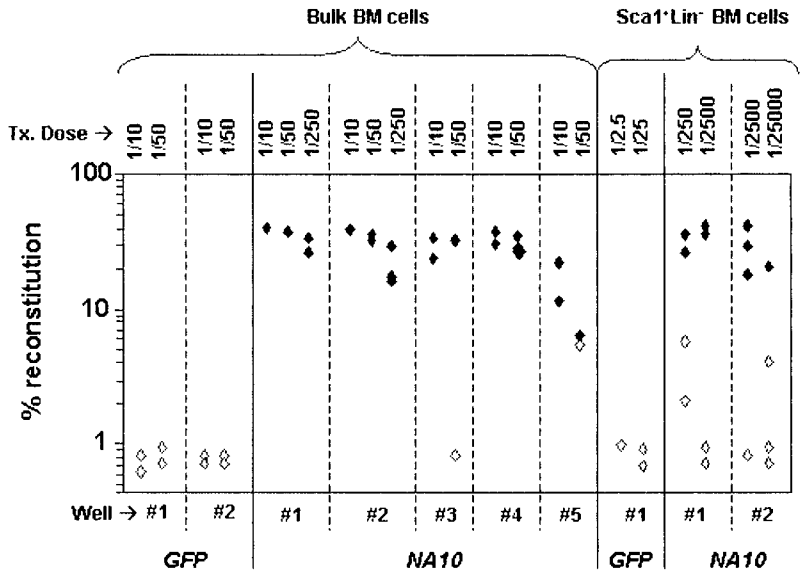


Figure 3.11. - Long-term reconstitution of recipients by *GFP*- or *NUP98-HOXA10*-transduced cells expanded *ex vivo* in cultures initiated with 1-2 CRUs. Shown is donor-derived/*GFP*$^+$ reconstitution of PB at ≥ 16 weeks post transplantation. While the control cultures experienced significant HSC decrease, which was confirmed by inability of *GFP*-transduced cells to reconstitute any of transplanted recipients, the *NUP98-HOXA10* cultures achieved at least 125-fold (bulk BM cells) or 1250-fold (Sca1$^+$Lin$^-$ BM cells) net HSC increase. Black diamonds represent reconstituted recipients having > 1% of donor-derived/*GFP*$^+$ cells in myeloid (Gr-1$^+$/Mac-1$^+$) and lymphoid (B220$^+$ and CD4$^+$/CD8$^+$) subpopulations. White diamonds represent non-reconstituted recipients having < 1% of donor-derived/*GFP*$^+$ cells and/or lacking ability to repopulate myeloid or lymphoid compartment. Transplantation (Tx.) dose indicates proportion of each/single well.

Proviral integration analysis of BM DNA from representative recipients that received various cell doses from single wells also displayed either a single proviral integration pattern (well #1 – bulk BM cells) or not more than two patterns (well #1 – $Sca^{+}Lin^{-}$ BM cells, #2 – bulk BM cells and #3 – bulk BM cells) (Figure 3.12.), confirming that the likelihood that the expansions obtained in these cultures were clonal. Moreover, toward the end of the culture period and just before the transplantation, a portion of a well#1, initially containing $Sca^{+}Lin^{-}$ BM cells, was resorted for this cell population and transplanted into several recipients (Figure 3.12.). Proviral integration analysis of BM DNA from these mice showed the same integration pattern, providing evidence that the expanded CRUs retain these phenotypic features during their generation *in vitro*.

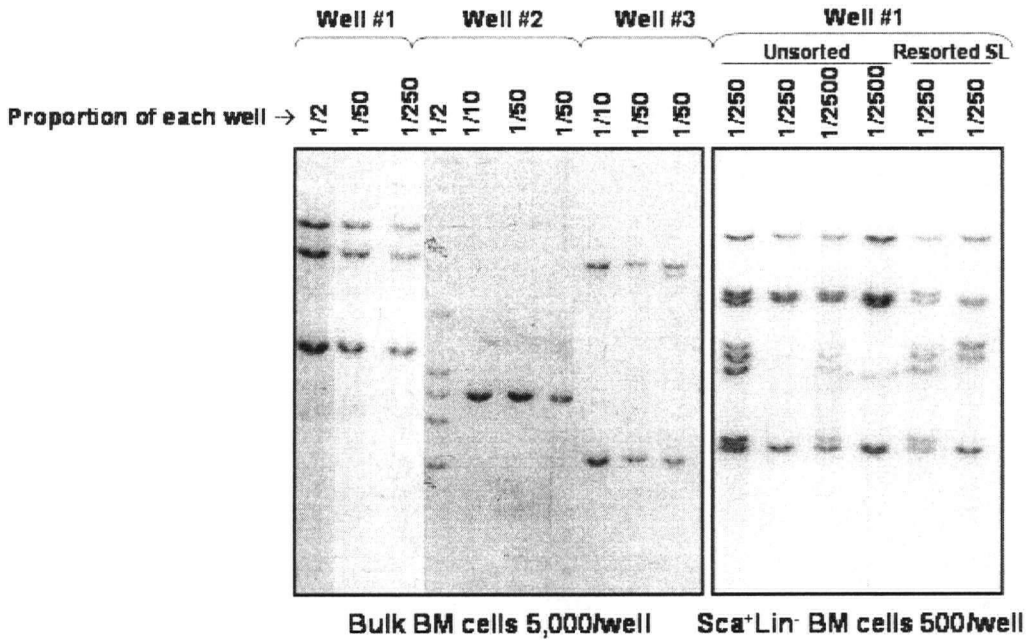


Figure 3.12. - Southern blots of DNA from representative recipients transplanted with *NUP98-HOXA10*-transduced BM cells expanded *ex vivo* from cultures initiated with 1-2 CRUs.

CHAPTER 4 DISCUSSION

Recent studies have provided intriguing evidence that the ability to expand HSCs might not be unique to *HOXB4*, but may extend to other intact *HOX* genes, notably *HOXA9* (Thorsteinsdottir et al., 2002), or engineered *NUP98-HOX* fusion genes, initially studied for their role in leukemogenesis. The recent studies indicating that these fusion genes can promote CFC self-renewal *in vitro* and block their differentiation (Pineault et. al., 2004) suggested that these activities extend to HSCs and allow the enhancement of HSC expansion *in vitro*.

My results reveal a striking potency of multiple fusion genes of *NUP98* and *HOX,* to stimulate multi-log expansion of murine HSCs in short term liquid culture. Interestingly, the *NUP98-HOXB4* fusion gene showed a 7 to 8-fold greater potency than that previously documented for *HOXB4* (average HSC expansion of 300-fold versus 40-fold). Moreover, even higher levels of HSC expansion were achieved with the *NUP98-HOXA10* fusion gene, which stimulated a >2000-fold expansion of HSCs; i.e., a potency 7- and 50-fold greater than that exhibited by *NUP98-HOXB4* or *HOXB4*, respectively. Of further interest, the HSC expansion effect of *NUP98-HOXA10* was preserved when sequences flanking the homeodomain were removed, thus identifying the homeodomain as the key *HOX* gene sequence required in concert with the N-terminal region of NUP98. Analysis of proviral integrations and of cultures initiated with limiting numbers of enriched HSC populations provided strong support for the view that the remarkable output of HSCs achieved after transducing mouse BM cells with *NUP98-HOXA10* is due to clonal expansions of all pre-existing HSCs transduced. However, regardless of the precise nature of the cellular target,

these findings point to *NUP98-HOXA10*-based molecules as promising new tools for stimulating high level *ex vivo* expansion of HSCs.

The underlying molecular mechanism governing this striking increase in potency of *NUP98-HOXA10* and even *NUP98-HOXB4* for HSC expansion compared to *HOXB4* is currently unclear. In part, it may be due to dominant transcriptional activation properties attributable to NUP98 rather than intrinsic properties of intact HOX protein. Alternatively, a potentially increased stability of the HOX component within the fusion protein could be important.

The ability of *NUP98-HOXA10* to stimulate a greater HSC expansion than *NUP98-HOXB4* is consistent with previous observations that *NUP98-HOXA10* is a much stronger inhibitor of hematopoietic differentiation *in vitro* compared to *NUP98-HOXB4* (Pineault et al., 2004). This property of *NUP98-HOXA10* may reflect its ability to affect overlapping but not identical target genes. Given that the fundamental mechanism of HOX function is modulation of gene expression, the identification of NUP98-HOXB4 and NUP98-HOXA10 target genes would be anticipated to help elucidate the mechanisms involved in NUP98-HOX-mediated HSC expansion. In addition, the cultured *NUP98-HOXB4*- and *NUP98-HOXA10*-transduced cells would be expected to contain large numbers of actively self-renewing HSCs and should thus serve as ideal populations for global gene expression approaches to analyze their unique properties and dissect the complex regulatory networks that control HSC fate decisions.

We have clearly shown that homeodomain alone of HOXA10 is sufficient for HSC expansion effect in context of a NUP98 fusion protein. Interestingly, the PBX

binding motif was not required for achieving high levels of HSC expansion. Moreover, with homeodomain alone, no signs of abnormalities in lineage distributions were evident, indicating perhaps that the PBX domain may be responsible for some deleterious effects. Interestingly, *HOXA10* was reported to bind the p21(Cip1/Waf1) promoter and activate p21 transcription in the presence of the cofactors, PBX1 and MEIS1 (Bromleigh, 2000). Combined with the observation that the PBX binding motif is dispensable for *NUP98-HOXA10*-promoted HSC expansion and the HSC expanding potential of *HOXB4* is augmented by antisense PBX1, it might be speculated that *NUP98-HOX* fusions somehow inhibit p21, facilitating HSC entry into the cell cycle. Evaluation of the effect of *NUP98-HOX* fusions on cell cycle or cell division would allow this idea to be further explored.

Finally, it is remarkable that multi-log clonal HSC expansion was achieved within a period of 6 days, indicating at least 10 self-renewal doublings. These must therefore have been symmetrical self-renewal divisions, assuming a cell cycle time of 12-16 hours. Interestingly, upon transplantation, the cells behaved "normally" in terms of their ability to repopulate all lineages with no evident increase in primitive subpopulations. Indeed, preliminary measurements of the number of CRUs regenerated in primary mice suggest that the transduced CRUs reach normal levels but do not exceed these (data not shown). Therefore, for HSC expansion to occur, appropriate extrinsic conditions (e.g., growth factors, absence of inhibitors) are required.

In conclusion, a strategy for consistently achieving very high expansion of HSCs *ex vivo* using a single agent has been developed. These findings raise

optimism that further prolongation of the time in culture or modification of the growth factors used may enable even greater levels of HSC expansion *ex vivo* to be obtained. The approach described here also appears ideally suited to the type of protein delivery system afforded using TAT-fusion protein technology (Krosl, 2003) which might allow HSC expansion without gene manipulation and be of great interest for human applications. Controlled high-level HSC expansion would also provide an invaluable source of cells for studying the molecular mechanisms underlying the HSC self-renewal process.

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