GROWTH PROPERTIES AND GENETIC MANIPULATION OF MURINE HEMOPOIETIC STEM CELLS

by

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ABSTRACT

The development of recombinant retroviral vectors able to transfer exogenous genetic material into hemopoietic target cells has played a pivotal role in our current understanding of hemopoiesis and has played a pioneering role in the field of gene therapy. However, with the efficiency of gene transfer to murine stem cells only 15% the power of recombinant retroviral gene transfer is currently severely compromised by the efficiency of retroviral infection. To optimize the utility of recombinant retroviruses, the human CD24 cell surface antigen was developed as a dominant selectable marker in a retroviral vector to enable the identification and selection of retrovirally transduced murine bone marrow cells, including those with long term in vivo repopulating ability. Following infection of day 4 5-FU treated murine bone marrow cells and selection of retrovirally transduced cells using an anti-CD24 antibody and Fluorescence Activated Cell Sorting (FACS), functional analysis of selected $CD24^+$ cells demonstrated the presence of hemopoietic cells at various stages of development, including in vitro clonogenic progenitors, day 12 CFU-S, and cells with totipotent long-term repopulating potential. Further experiments demonstrated the ability to regenerate the hemopoietic systems of myeloablated recipient mice with cells derived exclusively from provirally marked stem cells and that the transferred CD24 gene was expressed in various phenotypically defined populations of cells in vivo including marrow stem cell candidates defined by the Sca^+Lin^- cell surface phenotype. Thus, CD24 can be utilized not only as a selectable marker but also as a means to track and phenotype transduced cells and their progeny in vitro and in vivo. To provide information on the recovery of hemopoietic stem cells following bone marrow transplant, irradiated recipient mice were injected with various numbers of day 14.5 fetal liver or day 4 5-FU adult bone marrow estimated to contain 10, 100 or 1000 Competitive Repopulating Units (CRU). Analysis of the femoral marrow of primary recipients showed complete recovery of bone marrow

cellularity and clonogenic progenitor content and a near full recovery of day 12 CFU-S numbers irrespective of the number or origin of the cells initially transplanted. While the recovery of donor-cell-derived CRU was incomplete in all cases, fetal liver was markedly superior to those from adult bone marrow. Moreover, proviral integration analysis of mice receiving retrovirally transduced CD24+ selected bone marrow cells provided evidence for a >300-fold clonal amplification of a single transduced stem cell. These studies have provided procedures for the selection, tracking and phenotyping of murine bone marrow cells, including those with competitive long term lympho-myeloid repopulating ability. The availability of such procedures should increase the power of retroviral marking studies, and be advantageous in studies aimed at the genetic manipulation of hemopoietic stem cells and their progeny, as well as in the development of vectors able to optimize the expression of transferred genes in specific target cells of interest for use in human gene therapy trials. Moreover, these findings set the stage for attempts to enhance hemopoietic stem cell regeneration post-transplant by the administration of exogenous agents or the expression of intracellular factors that may enhance the regenerative potential of stem cells.

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LIST OF ABBREVIATIONS

7AAD	7-amino actinomycin D
ADA	adenosine deaminase
AGM	aortic gonadal mesenephros region
AIDS	acquired immune deficiency syndrome
ATTC	American Type Culture Collection
BFU-E	burst forming unit-erythroid
BFU-Mk	burst forming unit-megakaryocyte
BM	bone marrow
bp	base pair
BSA	bovine serum albumin
CAFC	cobble stone area forming cell
cDNA	complementary deoxyribonucleic acid
CD	cluster designation
CFU	colony forming unit
CFU-E	colony forming unit-erythroid
CFU-G	colony forming unit-granulocyte
CFU-GEMM	colony forming unit-granulocyte-erythroid-monocyte-megakaryocyte
CFU-GM	colony forming unit-granulocyte-macrophage
CFU-M	colony forming unit-macrophage
CFU-S	colony forming unit-spleen
cGy	centiGray
CRU	competitive repopulating unit
Cs	cesium
CS	calf serum
dCTP	deoxycytidine triphosphate
DMEM	Dulbecco's Modified Eagles Medium
DNA	deoxyribonucleic acid
EC	embryonic carcinoma cell
EDTA	ethylenediaminetetraacetic acid
Epo	erythropoietin
ES	embryonic stem cell
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein-isothiocyanate
FL	flk2/flt3 ligand
5-FU	5-fluorouracil
G418	geneticin
G-CSF	granulocyte colony stimulating factor
GFP	green fluorescence protein
GM-CSF	granulocyte-macrophage colony stimulating factor
G6PD	glucose 6 phosphate dehydrogenase
Gpi	glucose phosphate isomerase
HBS	hepes buffered solution
HF	Hank's balanced salt solution/2%fetal calf serum
HPP-CFC	high proliferative potential colony forming cell
HSA	heat stable antigen
HSC	hemopoietic stem cell

HXM	hypoxanthine-xanthine-mycophenolic acid
Ig	immunoglobulin
IL	interleukin
IRES	internal ribosomal entry site
LFA	leukocyte function association
LIF	leukemia inhibitory factor
LTC	long term culture
LTC-IC	long term culture-initiating cell
LTR	long terminal repeat
M-CSF	macrophage colony stimulating factor
MDR-1	multi-drug resistance-1 protein
MESV	murine embryonic sarcoma virus
MHC	major histocompatibility complex
MIP-1α	macrophage inhibitory protein-1α
MoAb	monoclonal antibody
MoMuLV	Moloney murine leukemia virus
MPSV	myeloproliferative sarcoma virus
MRA-CFU-S	marrow repopulating ability-colony forming unit-spleen
MSCV	murine stem cell virus
NCS	newborn calf serum
NH_4Cl	ammonium chloride
ORF	open reading frame
P	phosphate
PB	peripheral blood
PCMV	PCC4 embryonal carcinoma cell-passaged myeloproliferative sarcoma virus
PCR	polymerase chain reaction
PGK	phosphoglycerate kinase
PI	propidium iodide
RBC	red blood cell
Rh	rhodamine
RNA	ribonucleic acid
R-PE	R-phycoerythrin
RPMI	Roswell Park Memorial Institute
RT	reverse transcription
RU	repopulating unit
SCCM	spleen cell conditioned medium
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SSC	sodium chloride sodium citrate
TE	tris-EDTA
TGF-β	transforming growth factor-β
U	units
VCAM	vascular cellular adhesion molecule
VLA	very late antigen
VSV-G	vesicular stomatitis virus G glycoprotein
W	White spotting mutation
WBC	white blood cell
WGA	wheat germ agglutinin

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CHAPTER 1

INTRODUCTION

1.1 The hemopoietic system

1.1.1 Overview of hemopoiesis

Hemopoiesis is the essential, lifelong process whereby multiple types of highly specialized blood cells are generated. These cells include those responsible for carrying out specific functions such as carbon dioxide and oxygen transport (erythrocytes), blood clotting (platelets), humoral (B lymphocytes) and cellular (T lymphocytes) immunity as well as mounting phagocytic responses to foreign organisms and their products (granulocytes/monocytes/macrophages). In the normal human adult it is estimated that approximately 200 billion erythrocytes (1) and 60 billion neutrophilic leukocytes (2) are produced everyday. This observation has stimulated a great deal of interest in the cells that are ultimately responsible for accommodating this enormous daily output of cells, and in the mechanisms that regulate this process. The cell types mentioned above can be functionally divided into two distinct groups termed myeloid and lymphoid (Figure 1.1).

During normal adult life myeloid cells are produced exclusively within the bone marrow (3) while cells of the lymphoid lineages are produced to varying degrees in the bone marrow, spleen, thymus and lymph nodes. Mature functional end cells and their immediate precursors have a limited lifespan and a limited proliferative capacity and hence are not self-maintaining. Thus, these cells must be continuously replaced from a pool of more primitive proliferating cells. These cells constitute a hierarchy of cells with increasing proliferative potential and wider differentiative capacities.

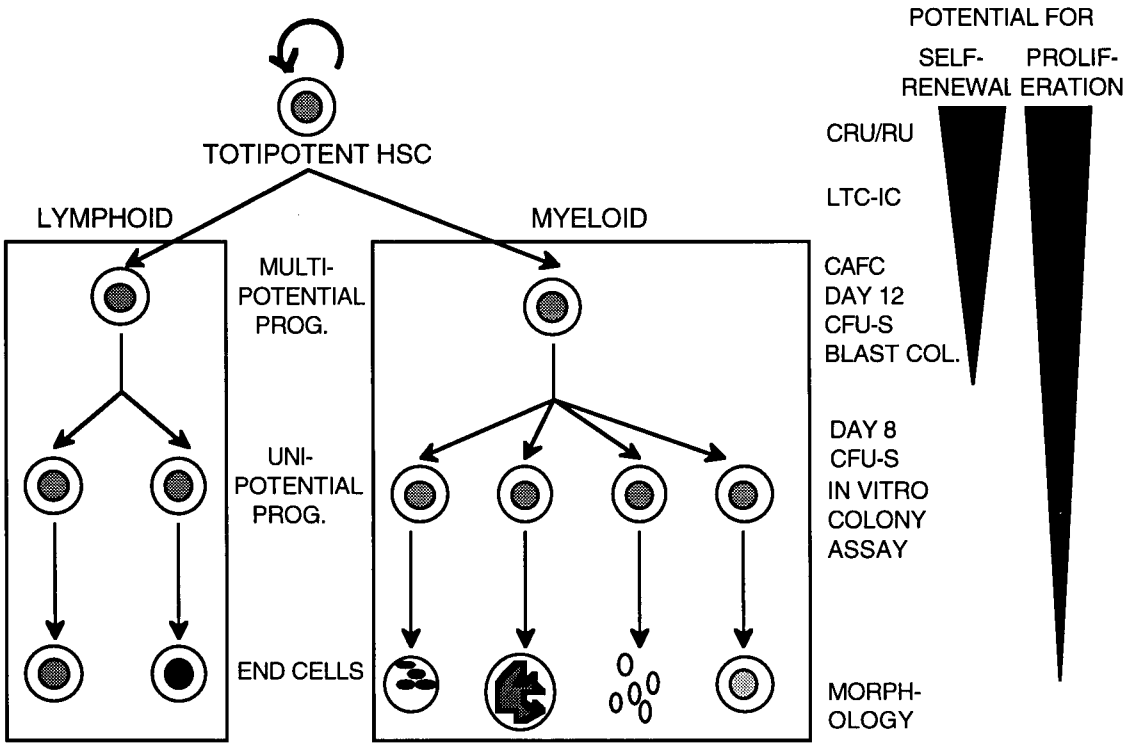

Figure 1.1. Schematic representation of the organization of the hemopoietic system and some of the assays used to evaluate HSCs and various pools of progenitor cells; HSC, hemopoietic stem cell; CRU, competitive repopulating unit assay; RU, repopulating unit assay; LTC-IC, cells with the ability to initiate in vitro long-term cultures; CAFC, in vitro assay for cobblestone forming area cells; day 12 or day 8 CFU-S, colony-forming-unit spleen cells able to produce splenic nodules 12 or 8 days post inoculation respectively; Blast col., in vitro blast colony assay. The relative potential for proliferation and self-renewal of various groups of cells is shown to the right.

Ultimately, all cells of both the myeloid and lymphoid lineages are derived from cells referred to as totipotent stem cells which are estimated to comprise only 0.01% of the total marrow compartment. These cells are operationally defined by their capacity to regenerate and sustain both the myeloid and lymphoid arms of the hemopoietic system for long periods of time following transplant and by their extensive capacity for self-renewal, the process of cellular division resulting in the production of daughter cells which are functionally indistinguishable from the parent cells in terms of their proliferative and differentiative potential. Many important questions remain unanswered regarding the nature and regulation of

totipotent hemopoietic stem cells. These include a basic understanding of their numbers, biological potential, usage over time and the genes encoding extrinsic and/or intrinsic factors which are responsible for the regulation of these biological characteristics. Moreover, with an increasing emphasis on the development of clinical strategies that depend upon the regeneration of HSC numbers (eg. autograft purging and the genetic therapy of inherited hematological disorders) the assessment of the self-renewal potential of HSCs is of particular interest.

The ability to manipulate HSCs genetically has provided a powerful tool to begin to address the above issues. The overall goal of the work presented in this thesis was to develop methodologies to increase the utility of gene transfer for the efficient genetic manipulation and tracking of HSCs and to utilize these procedures to define more clearly the self-renewal potential of HSCs following bone marrow transplant. The following introduction examines the current state of knowledge of the phenotypic and functional properties of HSCs as well as the methods available for their genetic manipulation.

1.2. Assays for early hemopoietic cells.

1.2.1. In vitro clonogenic progenitors

The ability to culture hemopoietic cells in vitro has provided a large amount of information on the cellular organization, the proliferative and differentiative potential, and growth factor requirements of cells at various stages of hemopoietic development. Assays first described by Bradley and Metcalf (4), and Pluznik and Sachs (5) involved the growth of hemopoietic cells in a semi-solid matrix of agar that allowed colonies of hemopoietic cells derived from single cells to be identified and characterized. Subsequent assays for such "clonogenic progenitors" utilized plasma clots or, now most often, methylcellulose to provide semi-solid medium. Such growth medium is typically supplemented with nutrients and growth factors required by the dividing cells and which have been provided historically in part by poorly defined "conditioned" cell medium. Today a large number of hemopoietic

growth factors have been purified and their genes cloned resulting in the availability of pure recombinant factors (reviewed in (6)). The vast majority of clonogenic progenitors detectable in assays of normal bone marrow are of uni- or bipotent potential that are able to give rise to colonies consisting of granulocytes and monocytes/macrophages (colony-forming units granulocyte-macrophage; CFU-GM) (7), pure granulocytes or monocytes/macrophages (CFU-G or CFU-M) (4, 5), erythrocytes (BFU-E and CFU-E) (8), and megakaryocytes (BFU-Mk) (9). More recently assays for cells with B, but not T, lymphoid potential have been described (10). Such cells, while sometimes possessing considerable proliferative potential yielding colonies of several thousands of cells, have limited or no capacity for self-renewal as determined by their inability to generate equivalent secondary colonies in subsequent replatings (11). These cells also appear to be actively cycling under normal steady-state conditions in vivo since they are highly susceptible to killing by cycle-specific cytotoxic drugs such as 5-fluorouracil (5-FU) (12). Such cells, then, are believed to be relatively late in the hemopoietic hierarchy.

Such in vitro assays also enable the identification of earlier progenitors characterized by their ability to produce colonies consisting of multiple lineages (ie. granulocyte / erythrocyte / macrophage / megakaryocyte from CFU-GEMM) (13, 14), of great size (eg. high proliferative potential colony-forming cells, HPP-CFC) (15), and/or cells with a primitive undifferentiated cellular morphology (ie. blast colony forming cells) (16-18). CFU-GEMM and blast colony forming cells are also characterized by a capacity for self-renewal as demonstrated by the ability of some of their clonal progeny to form secondary and less frequently tertiary multi-lineage and blast colonies in replate assays (16, 19).

1.2.2. Colony-forming units-spleen (CFU-S)

The first assay available to study hemopoietic cells shown to possess "stem cell-like" properties was the in vivo spleen colony assay described by Till and McCulloch in 1961 (20). This assay is based on the ability of certain cells (CFU-S,

for colony forming unit-spleen) to home to the spleen and grow to form macroscopic hemopoietic nodules detectable on the surface of the spleen 8-12 days post-transplant. The origin of individual nodules from a single cell (ie. clonal in origin) was first determined by injecting bone marrow cells from mice harboring unique radiation-induced chromosomal abnormalities (21, 22) , and this was later confirmed using unique retroviral integration events as markers (23, 24). Individual spleen colonies detected on day 8 post-transplant usually are restricted in the types of cells they contain (either erythroid or granulocytic but not both (25, 26)) and rarely produce daughter colonies upon subsequent retransplantation (11). Thus, day 8 CFU-S resemble uni-potential in vitro clonogenic progenitors. In addition, a large proportion of day 8 CFU-S are also sensitive to killing by the cycle-active drug 5-FU (27). However, those CFU-S which result in large colonies detected on day 12 are often composed of cells of multi-lineages (20), are more resistant to killing by 5-FU and are often able to generate numerous daughter CFU-S (28). Whether these cells possess the potential to produce cells of the lymphoid lineages remains controversial (29-31).

Although spleen colony-forming cells were once thought to constitute the most primitive hemopoietic compartment since they possess a large capacity for proliferation, are multi-potential and have significant self-renewal ability, recently the use of counterflow centrifugal elutriation has been used to show that day 12 CFU-S can be physically separated from cells with long term repopulating ability (32).

1.2.3. The hemopoietic stem cell

It has been proposed that the most useful, rigorous definition of a hemopoietic stem cell should be based on such a cell having an in vivo capacity for the long term production of all blood cell lineages (33). Evidence for the existence of cells with such characteristics has primarily come from transplantation models. For example, Wu et. al. (22) detected common chromosomal abnormalities in both

the myeloid and lymphoid compartments of myeloablated recipient mice that had received a transplant of marrow cells from donor mice harboring unique radiation induced chromosomal abnormalities. Subsequent evidence suggesting the existence of totipotent HSCs was provided by Nakano et. al. (34) who performed marrow transplants between congenic strains of mice which possessed distinguishable hemoglobin and isoenzyme markers. Moreover, the transplant of retrovirally marked adult day 4 5-FU bone marrow (35) or fetal liver cells (36) into irradiated (35) or genetically anemic [1]W/W^V (23) mice has also demonstrated the existence of lympho-myeloid repopulating stem cells based on the detection of common proviral integrants in cells of both the lymphoid and myeloid compartments in the recipient mice. As described in the subsequent section, hemopoietic stem cells cannot yet be positively identified on the basis of any unique morphological, physical or cell surface characteristics. Thus, rigorous identification of totipotent hemopoietic stem cells relies on functional assays based on the ability of such cells to regenerate and sustain the hemopoietic systems of myeloablated or genetically anemic (ie. W/W^V) recipients. A variety of transplantation strategies have been designed in an effort to quantify hemopoietic stem cells derived from various cell populations. One such approach involves assessing the survival of irradiated recipient mice for 30 days following transplantation with limiting numbers of marrow cells (39-41). However, such an assay is complicated by the potential contribution from residual recipient cells, and the possibilities that such short term radioprotective capacity may in part derive from more mature cell types (42, 43). Moreover, the transplant of insufficient numbers of cells with short-term radioprotective capacity can result in the death of the recipient before cells with long term repopulating capacity can be read out.

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[1]The W locus, located on chromosome 5, encodes the receptor for Steel factor which is a hemopoietic growth factor shown to support the proliferation of both immature and lineage restricted lymphoid and myeloid progenitor cells in combination with other hemopoietic growth factors (37, 38). Mice harboring mutations at the W locus are characterized by an intrinsic defect in primitive hemopoietic stem cells and thus, the hemopoietic systems of these mice can be replaced with normal wild type hemopoietic cells without the use of conditioning regimens such as irradiation.

A second, very powerful approach to the quantification of hemopoietic stem cells has been the use of the competitive repopulation assay first developed by Harrison (44, 45). This assay is based upon comparing the long-term repopulating abilities of two separate populations of hemopoietic cells which are distinguishable on the basis of allelic differences in hemoglobin and Gpi-1 isoenzyme markers. One of the cell populations (the "competitor") consists of a fixed number (usually 1-2 x 10^6 cells) of fresh marrow that serves as a standard for repopulating potential. Varying numbers of a "donor" or "test" source of stem cells are then injected and the mean relative contribution of the two populations to hemopoiesis measured. Repopulating units (RU) are calculated according to the formula RU=%(C)/(100-%), where % is the measured percentage of peripheral blood cells in the recipient with the donor phenotype, and C is the number of fresh competitor marrow cells used/10^5. Each repopulating unit represents the repopulating ability shown by 1 x 10^5 fresh marrow cells from the competitor pool. This method has a number of advantages over 30 day survival assays. Recipient mice are analyzed at long periods post transplant providing a rigorous measure of stem cell function. Moreover, the short term hematological rescue of myeloablated recipient mice is not dependent upon the cell population being tested since life sparing doses of donor cells are provided in the competitor cell population. However, this method cannot be used to assess properties of individual HSCs such as their proliferative capacity since one cannot distinguish between differing proliferative capacities and differing numbers of HSCs.

The Competitive Repopulating Unit assay developed by Szilvassy et. al. combines limiting dilution and competitive repopulating procedures to quantify totipotent repopulating stem cells. In its original form, the CRU assay involves the co-injection of limiting numbers of male test cells along with a fixed number (2 x 10^5) of female competitor cells into myeloablated female recipient mice. The competitor cells are derived from mice which have undergone two previous rounds

of hemopoietic transplantation and thus, are relatively depleted of cells with long-term repopulating capability. The function of the helper cells is to ensure the survival of the myeloablated recipient even when limiting numbers of test cells are transplanted. Recipients demonstrating repopulation by donor HSCs are defined as those with a significant contribution (ie. > 5%) from male cells to both the lymphoid and myeloid compartments as identified by probing DNA extracted from hemopoietic tissues with Y chromosome specific sequences. The frequency of hemopoietic stem cells, or competitive repopulating units (CRU) as they are referred to in this assay, is determined by assessing the proportion of recipient mice which meet the repopulation criteria described above when limiting dilution is reached. The frequency of CRU in a test cell population is subsequently calculated using Poisson statistics according to: CRU frequency = 1 / number of marrow "test" cells that result in 37% of recipient mice being negative for repopulation. The CRU assay has since been modified by the use of normal marrow cells (10^5) as competitor cells (46) and the use of mouse strains that enable the identification of donor versus recipient cells on the basis of allelic differences at a gene encoding a peripheral blood leukocyte cell surface antigen (Ly5.1 or Ly5.2) (46-48) (see Figure 1.2). The ability of this assay to detect totipotent (lympho-myeloid) repopulating stem cells has been demonstrated by the observation of common reconstitution of lymphoid and myeloid tissues by limiting numbers of cells, and by retroviral marking procedures (43, 49). One of the great advantages of the CRU assay is that it can be used to quantify quickly and accurately the frequency of hemopoietic stem cells with long term lympho-myeloid reconstituting ability in any population of test cells (43). Szilvassy and his colleagues have demonstrated that CRU frequencies determined at 10 weeks post transplant were virtually identical to those obtained at later time points suggesting that this assay can be used to obtain accurate estimations of totipotent HSC numbers in little over two months time.

1.2.4. Phenotyping and purification of HSCs

The frequency of repopulating HSCs among adult bone marrow mononuclear cells has been estimated to be 1 in 10^4 to 10^5 (43, 50-53). This low frequency and the fact that the identification of HSCs involves functional assays (ie. are retrospective in nature based upon the long term repopulation of irradiated or genetically anemic recipient mice) have hampered direct analysis of self-renewal and early events associated with HSC commitment and differentiation. Major effort has thus been directed towards purification of cells with stem cell characteristics. Physical, immunological and supravital stains have all been employed alone or in combination as strategies to enrich for HSCs. Physical techniques (velocity sedimentation (54), density gradient separation (55-57) and counterflow centrifugal elutriation (32, 58)) separate cells on the basis of buoyant density and/or cell size. Using these techniques, HSCs have been generally characterized as relatively small, low density cells with an undifferentiated blast cell-like morphology.

With the advent of flow cytometry, the goal of isolating purified populations of HSCs took a major leap forward. HSCs possess medium forward light scatter and low to medium orthogonal light scatter properties indicative of intermediate size and low granularity respectively (59). The development of monoclonal antibodies (MoAb) (60) and the existence of lectins which bind distinct sugar moieties on the cell surface (ie. Wheat Germ Agglutinin; WGA) has enabled the enrichment of purified stem cells candidates on the basis of two distinct strategies; positive and negative selection procedures. Positive selection procedures are based upon the identification and selection of cells which express specific cell surface antigens and/or lectins. MoAbs directed against Ly-6A/E (Sca-1), MHC-class I molecules (31, 48, 53, 59, 61-64), WGA (53, 65-67), Thy-1 and the c-kit receptor have been widely used for the enrichment of stem cells from adult bone marrow. In addition,

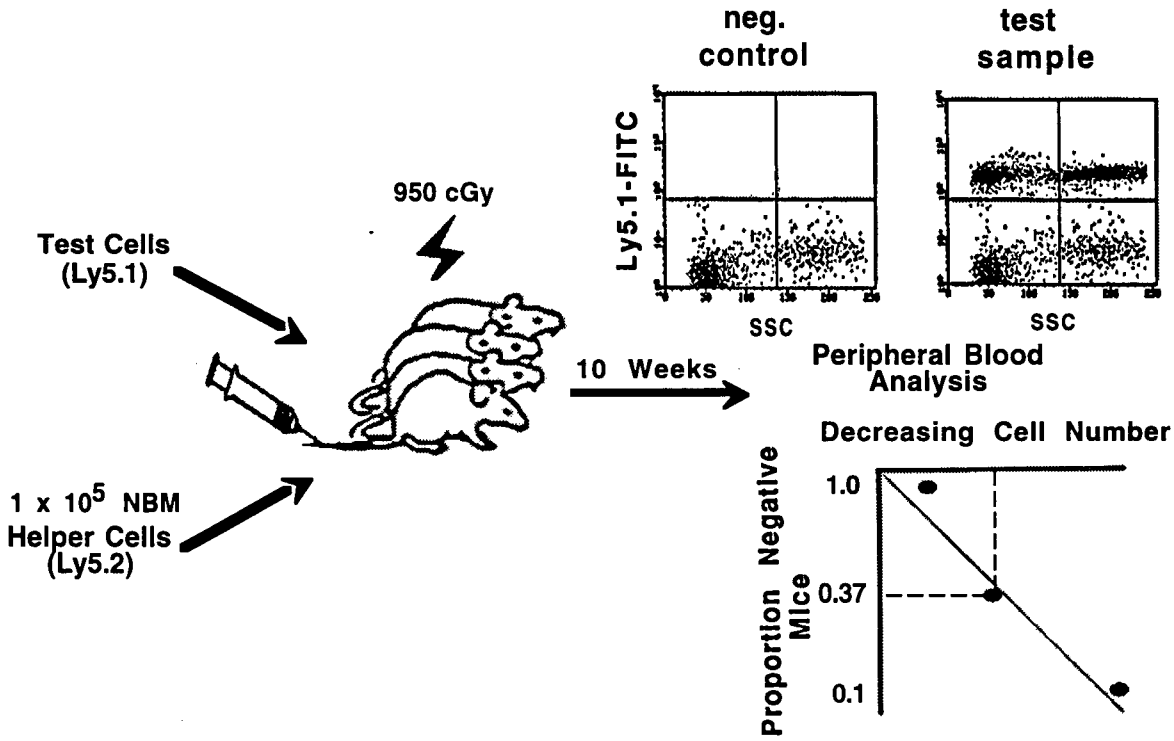

Figure 1.2. Schematic representation of the limiting dilution assay for CRU. Limiting numbers of Ly5.1$^+$ test cells derived from PepC3F1 donor mice are co-injected along with 10^5 Ly5.2 helper cells into B6C3F1 (Ly5.2) recipients following 950cGy of whole body irradiation. Test cells are phenotypically distinguishable from both helper and surviving endogenous host cells on the basis of allelic differences at the Ly5 locus. The proportion of mice showing > 1% Ly5.1$^+$ cells of both lymphoid and myeloid lineages is determined by staining peripheral blood cells with an antibody specifically recognizing the Ly5.1 antigen and analysis by FACS. The frequency of CRU is then calculated using Poisson statistics. NBM: normal bone marrow cells obtained from a unmanipulated control mouse. SSC: side scatter.

MoAb AA4.1 has been used for the isolation of HSCs from fetal liver and the yolk sac of the early mouse embryo (63, 64).

Alternatively, or in addition, negative selection procedures can be used to remove cells which are not of interest. HSCs derived from adult bone marrow have been shown not to express a number of markers characteristic of later lineage restricted cells (68). Thus, marrow cells expressing so called lineage or "Lin" markers can be identified and removed using MoAbs recognizing B220 (B-cells), CD4, CD8, CD3 and CD5 (T-cells), Mac-1 and 15-1.1 (monomyelocytic cells), Gr-1 (myeloid cells), and Ter119 and 10-2.2 (erythroid cells) (31, 53, 64, 67-74). The enrichment of stem cells achieved varies to certain degrees depending upon which sets of MoAbs are employed, but generally, the selection of cells with either the $Sca\text{-}1^{+}Lin^{-}Thy1.1^{lo}$ (31, 48, 75) or $Sca\text{-}1^{+}Lin^{-}WGA^{+}$ (53, 67) cell surface phenotype have resulted in enrichment factors of approximately 500 to 1000-fold. These enrichment factors result in detectable HSC frequencies of 1 in 10 (76) to 1 in 30 (46) stem cell candidates. Although it would seem that HSCs have not yet been purified to homogeneity, one must consider the possibility that the long-term repopulating assay has a lower limit to the number of transplanted HSCs that will reproducibly result in donor reconstitution. This limit may result from the seeding efficiency of HSCs to the marrow, competition from HSCs in the competitor cell population or surviving endogenous HSCs, or ill defined regulatory mechanisms that direct test HSCs into quiescence or to differentiate rather than self-renew upon arrival in the marrow compartment. Thus, the enrichment factor stated above may be an underestimation of HSC frequencies in purified subpopulations.

Differential retention of certain fluorescent dyes such as Rhodamine 123 (Rh123) and Hoechst 33342 has also been employed to separate HSCs from more mature progenitors. The retention of these dyes by HSCs tends to be poor (41, 77-80); in the case of Rh123 this is due to the functioning of a P-glycoprotein pump which actively removes Rh123 from the cell (81). The use of such dyes can enable

an approximate 500-fold enrichment of stem cell candidates. Interestingly, some phenotypic differences between HSCs derived from differing ontological sources have been reported. For example, $Sca^{+}Lin^{-}$ subpopulations enriched for HSCs derived from day 14.5 fetal liver stain with the AA4.1, anti-Mac, and anti-CD45RB antibodies and retain Rhodamine 123 ($Rh123^{bright}$) while adult bone marrow $Sca^{+}Lin^{-}$ cells similarly enriched for HSCs are $AA4.1^{-}$, $Mac\text{-}1^{-}$, $CD45RB^{-}$ and $Rh123^{dull}$.

Despite the fact that impressive advances in the identification and purification of HSCs has been made over the past years, a surface marker specifically expressed on HSCs has not been reported. Moreover, a number of groups have shown that cell surface phenotype is not always an accurate prediction of biological capability. Rebel et. al. demonstrated that although the in vitro culture of $Sca^{+}Lin^{-}WGA^{+}$ cells for four weeks in serum free medium supplemented with steel factor, IL-3, IL-6 and Epo resulted in >1000-fold increase in the number of cells with the starting surface phenotype, the number of repopulating HSCs in these cultures was 1.3-fold lower than input values (46). In addition, Spangrude et. al. demonstrated that following transplantation of recipient mice with 200 $Thy\text{-}1.1^{low}Lin^{-}Sca^{+}Rh\text{-}123^{low}$ marrow cells, cells with this surface phenotype were expanded to approximately 1000-fold over input levels. Despite this considerable increase in the number of cells with the starting surface phenotype, these cells possessed poor reconstituting ability; only 8 of 83 secondary transplant recipients (9.6%) receiving 5, 10, or 20 $Rh\text{-}123^{low}$ cells or 400 $Thy\text{-}1.1^{low}Lin^{neg}Ly\text{-}6A/E^{+}$ cells obtained from primary recipients exhibited donor-derived cells in peripheral blood 12 weeks post transplant (76). Thus, functional assays remain the most reliable and rigorous method to assess for HSC numbers.

1.3. Ontogeny of the murine hemopoietic system

1.3.1. Development of the hemopoietic system

During vertebrate ontogeny, hemopoiesis is in a state of continuous change in terms of the genes which are expressed, the cellular constituents and the site(s) of production. For example, during ontogeny hemopoiesis takes place sequentially in the embryonic yolk sac, paraaortic gonadal mesenephros region (AGM), fetal liver, spleen and finally the adult bone marrow. While the molecular steps that regulate these changes are poorly understood, recent studies have begun to delineate early events and patterns of cellular migration that constitute this developmental program. Recently there has been a great deal of interest in the use of hemopoietic cells derived from developmentally early sources such as fetal liver or umbilical cord blood for use in human bone marrow transplantation. This interest has been stimulated by the findings of several studies (discussed in more detail in section 1.3.2) *hinting* that HSCs derived from ontologically early sources *may* possess a superior regenerative capacity as compared to adult bone marrow HSCs. In Chapter 5 of this thesis data are presented demonstrating and quantifying the superior regenerative capacity of HSCs derived from fetal liver as compared to adult bone marrow following transplant.

Hemopoiesis begins in the embryonic yolk sac at approximately day 7.25. Between days 7.5 and 8.5 the yolk sac is a source of in vitro clonogenic progenitors (82), B lymphoid precursors (83) and day 12 CFU-S (82) with HSCs capable of long term lympho-myeloid repopulating ability detected by day 11 (82, 84). More recently, similar hemopoietic cell populations have been demonstrated to be present within the AGM region with CFU-S activity detectable on day 8 to day 11 (85, 86) and long term repopulating HSCs by day 10 (84). The production of blood cells within the yolk sac and AGM regions begins to decline as the fetal liver takes over as the major site of embryonic hemopoiesis. Hemopoiesis is detectable in murine fetal liver by day 10 of gestation and increases between days 10-13 (11). The fetal liver remains the major site of hemopoiesis until birth whereupon it

decreases rapidly. Erythropoiesis is detectable in the spleen by day 15 although by day 17 granulopoiesis predominates in this organ. Hemopoiesis in the spleen reaches its peak at approximately 4-8 days following birth where it decreases steadily as the marrow begins to take over as the predominant site of hemopoiesis for the remainder of the animal's life. Figure 1.3 shows the contribution of various organs to hemopoiesis during ontological development.

1.3.2. Comparison of stem cells from fetal liver and adult tissues

The relationship between fetal liver and adult bone marrow HSCs remains unclear. Although HSCs from these two differing populations are believed to be ontologically related, that is derived from the same initial pool of primordial stem cells, fetal liver cells display functional characteristics that differ from those found in the adult marrow. Fetal liver day 8 CFU-S cells for example possess a superior self-renewal capacity as compared to their adult bone marrow

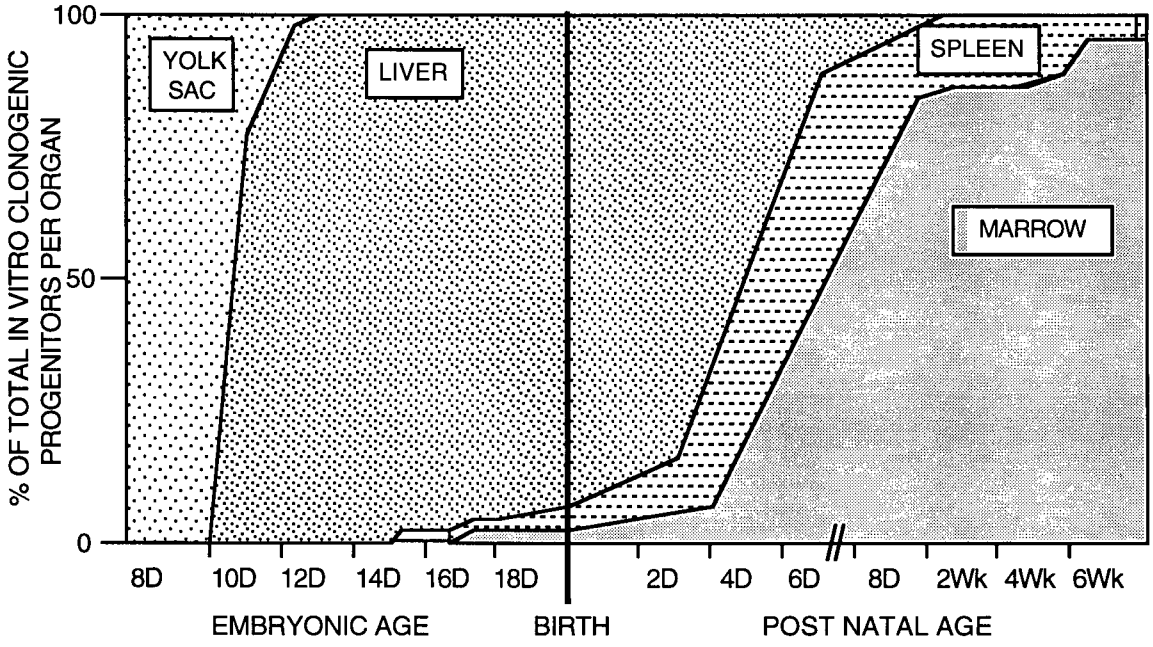

Figure 1.3. Changing sites of hemopoiesis reflected in the relative proportion of in vitro clonogenic progenitors cells found within the embryonic yolk sac, fetal liver, spleen and bone marrow during ontogeny and post natally.

counterparts (87), and fetal liver cells show a greater competitive repopulating ability as compared to adult bone marrow cells in in vivo transplantation assays (88). Rebel et. al. have shown that limiting numbers of fetal liver CRU are able to

produce a greater output of mature blood cells in vivo as compared to adult bone marrow (89). Moreover, it was also demonstrated that when marrow cells from primary recipients of limiting numbers of fetal liver CRU were injected into secondary recipients, a significantly higher percentage of these secondary mice showed donor cell-derived reconstitution of their lymphoid and myeloid compartments as compared to mice that had received marrow cells from primary recipients of similar numbers of adult bone marrow CRU. One possible explanation for the differences in regenerative potential between these two cell populations is that fetal liver CRU possess a greater intrinsically determined probability to undergo self-renewal versus differentiation divisions when proliferating within the adult marrow microenvironment. Vasiri et. al. (90) have recently proposed the theory that the progressive loss of telomeric DNA with each round of cellular division may act as a mitotic clock and therefore be the molecular basis by which fetal liver cells may be able to undergo a greater number of cell divisions prior to undergoing senescence. However, this explanation does not preclude the possibility that fetal liver HSCs may maintain differences in cell cycle times or the number of cells recruited or maintained within the microenvironment of the adult marrow, or express additional intrinsically defined molecules that may direct the cell into favoring self-renewal versus differentiation divisions. Intriguing new evidence of possible intrinsic determinants of self-renewal was recently reported by Sauvageau et. al. in studies of the Homeobox family of transcription factors (91). Engineered overexpression of HOXB4, a gene whose expression is normally restricted to the most primitive adult bone marrow cells, was found to result in up to a 50-fold increase in the regeneration of retrovirally transduced HSCs as compared to marker gene-transduced control cells.

1.4. Properties of hemopoietic stem cells

1.4.1. Cycling status

In contrast to committed clonogenic progenitors and a large proportion of day 8 and 12 CFU-S, the vast majority of long term repopulating HSCs exist in a state of quiescence within the marrow under normal homeostatic conditions as determined by their ability to survive a single injection of the cycle-specific cytotoxic drug 5-FU (92). However, following treatment with 5-FU the most primitive HSCs are recruited into cycle since they become highly sensitive to a second dose of 5-FU given 3-5 days later (93).

1.4.2. Developmental potential and dynamics of HSCs

Although the existence of totipotent HSCs is now a well documented phenomenon, the number of HSCs and their usage in steady state hemopoiesis remains unclear. One theory that has been put forth to explain the dynamics of HSC utilization is the clonal succession model proposed by Kay in 1965 (94). This model proposes that hemopoiesis is maintained by the sequential activation of stem cell clones that proliferate, differentiate and eventually become exhausted. In this model HSCs are akin to fuel for hemopoiesis, continually being activated and "consumed" from a reserve pool of cells. A significant body of data from bone marrow transplantation studies has provided support for this theory. Studies based upon the transplant of various mixtures of retrovirally marked or enzymatically distinguishable adult bone marrow or fetal liver stem cells into either irradiated (24, 95) or genetically anemic W/W^V mice (36, 96) revealed major fluctuations in the contribution of individual HSCs to hemopoiesis within the first six months following transplant. During this period numerous HSC clones were observed to contribute significantly but transiently to hemopoiesis, the span of their contribution lasting on average for only a few months. Further evidence supporting the clonal succession theory was also obtained from large animal models. Abkowitz et. al. provided data demonstrating large clonal fluctuations in cats heterozygous for the X chromosome-linked enzyme glucose-6-phosphate dehydrogenase (G6PD) following transplants of autologous marrow (97). However, the animals used in

these experiments were only analyzed for relatively short periods of time following transplant (ie. 1-5 months or 1-1.5 years for murine and feline recipients respectively). Subsequent studies using retrovirally marked marrow cells sequentially analyzed the clonal contribution to the peripheral blood of murine recipients for longer periods of time (ie. 7-12 months) post transplant (64, 98, 99). Following a period of clonal instability for up to 6 months post transplant, hemopoiesis became dominated by a small number of totipotent stem cell clones that continued to function for long periods of time, often for the remainder of the animal's life. Interestingly, the clonal fluctuations observed in felines transplanted with allogeneic marrow described by Abkowitz et. al. in 1990 (97) were found to stabilize when these same recipients were analyzed at longer times post transplant (3.5-6 years) (100). One explanation for the clonal fluctuation initially observed following transplant is that it is due to the utilization of more "mature" stem cells which possess a limited capacity for proliferation and self-renewal. HSCs are believed to be organized as a hierarchy of cells with decreasing self-renewal and proliferative potential (refer to Figure 1.1) and heterogeneity with regards to both physical and functional properties of HSCs have been documented. Thus, more mature HSCs would quickly become exhausted and eventually be replaced by more primitive HSCs which possess a more long-lived capacity for proliferation and self-renewal. An alternative explanation of HSC behavior following bone marrow transplant has been provided by Jordan and Lemischka (98). The authors proposed that the initial period of clonal fluctuation observed was the result of an expanding pool of totipotent HSCs undergoing stochastic commitment versus self-renewal events. These events are portrayed in Figure 1.4. According to this model, the clonal fluctuation observed within the first six months post transplant (depicted in Figure 1.4A) are the consequence of either stochastic mechanisms or the demands of the radiation-ablated hemopoietic system resulting in the commitment of the HSCs without significant self-renewal. Thus, although this clone initially

contributes to hemopoiesis, without self-renewal it is unable to maintain its numbers and is exhausted. Stem cell clones which are observed to contribute to hemopoiesis at both short and long periods post transplant are the result of commitment decisions which occur in parallel with a significant degree of self-renewal (Figure 1.4B). Alternatively, some clones may undergo a substantial degree of self-renewal shortly after transplant. Such clones, although they would not be observed to contribute to hemopoiesis during the initial period of regeneration would ultimately come to dominate hemopoiesis for lengthy periods of time, possibly for the remainder of the animal's life (Figure 1.4C).

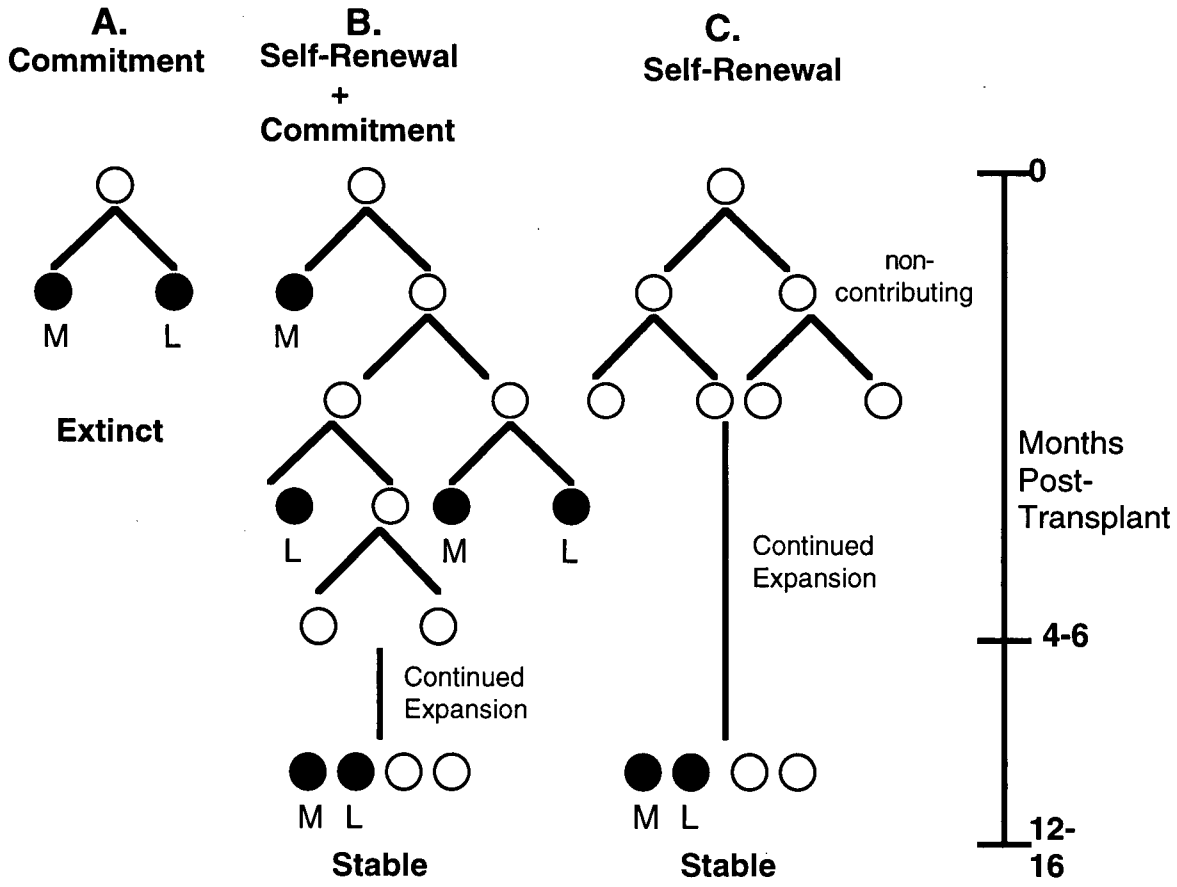

Figure 1.4. Model proposed by Jordan and Lemischka (64) to explain the behavior of HSCs at various time points following bone marrow transplant. Open circles designate totipotent HSCs while filled circles represent cells committed to either myeloid or lymphoid lineages.

In summary, it remains unclear whether the clonal fluctuation initially observed following bone marrow transplant is representative of the utilization of more mature HSCs with limited proliferative and self-renewal capacities, or is the result of stochastic mechanisms acting upon an expanding pool of totipotent HSCs.

1.4.3. Self-renewal and aging of HSCs

Studies using retroviral marking of HSCs have demonstrated convincingly the ability of HSCs to undergo self-renewal both in vitro (101) and in vivo (24, 98, 99) through the detection of identical proviral banding patterns in the bone marrow and/or thymus of more than one primary or secondary recipient. Although the studies mentioned above directly demonstrate that self-renewal of HSCs can occur *it remains unknown to what extent this process can occur* since the magnitude of self-renewal events have not been documented. This has been in part due to the fact that quantitative assays for measuring stem cells with long term in vivo reconstituting ability were not available until relatively recently (43, 45). As previously stated, the requirement for a functional assay when analyzing long term repopulating stem cell function is essential since a number of groups have shown that cell surface phenotype is not an accurate determination of biological capability (46, 76). Moreover, the inefficiency of retroviral marking procedures has made the tracking of individual HSCs in vivo laborious. Thus, methods which increase the utility of recombinant retroviral marking would be advantageous towards studies aimed at more clearly defining the regenerative potential of HSCs. Chapter 3 of this thesis describes the development of methods to enhance the power of retroviral marking procedures.

That HSCs have an unlimited ability to replenish their numbers through the process of self-renewal has been brought into question by a number of studies demonstrating that even a single transplantation procedure can reduce the repopulating ability of bone marrow dramatically (102-109). For example, using the T6 chromosome marker system, Ross et. al. demonstrated that the proportion of

donor cell mitoses in recipients of bone marrow which had been subjected to one round of transplantation was only 10% of that detected when normal bone marrow was used (102). The possibility that the loss in repopulating ability of bone marrow following transplant is due to damage to the recipients microenvironment by the irradiation regimen was excluded since similar results were observed using genetically anemic W/W^V recipient mice. Mauch and Hellman (110) evaluated the long-term consequences of transplanting limiting numbers of hemopoietic cells and demonstrated a direct relationship between donor cell dose and day 8 CFU-S recovery and self-renewal capacity that was not reflected in peripheral blood counts or bone marrow cellularity. Further, the decreased recovery of CFU-S parameters did not change with time after transplantation suggestive of a permanent loss of marrow regenerative capacity. Harrison et. al. have provided data suggesting that the reduction in HSC number and function following transplant is the result of two mechanisms (111). They calculated that transplanted marrow showed a two-fold reduction in stem cell numbers as compared to fresh marrow. Moreover, they found that the average proportion of donor derived cells in recipients of previously transplanted marrow was approximately 15% of that of fresh marrow. Thus, transplanted marrow demonstrated a seven- to eight-fold reduction in repopulating ability as compared to fresh marrow. These results suggest that not only are transplant recipients compromised in terms of the quantity of stem cells which they possess but they are also deficient in terms of the quality of stem cells. One explanation for the loss in the regenerative capacity of bone marrow following transplant is the inability of transplanted stem cells to reconstitute fully the HSC compartment to normal (non-transplant) levels due to a limited capacity for self-renewal. An alternative hypothesis is that transplantation places a high degree of stress on the HSCs to undergo differentiative rather than self-renewal events in order to provide sufficient numbers of functionally mature cells to reconstitute hemopoiesis in the myeloablated recipient. Another possibility is that

HSC regeneration following bone marrow transplant is limited due to the action of negative regulatory feedback mechanisms in vivo that can limit stem cell expansion prematurely even though the HSC compartment is far from being regenerated back to normal levels. This scenario might occur through the production of such factors as MIP-1α or TGF-β that have been shown to decrease the proportion of primitive hemopoietic cells in cycle (112-115).

The observed decline in repopulating capacity following bone marrow transplant raised the question as to whether HSCs within the marrow of old unmanipulated mice might also share such a defect. The hypothesis, called the generation-age hypothesis (116), suggested that an entire lifetime demand on the HSCs might result in a pool of HSCs which were compromised in their regenerative capacity since they were forced to have undergone a greater number of cell division events. However, using the competitive repopulation assay Harrison and his colleagues found no differences in the repopulating ability between the marrow of young and old donors (117). Bone marrow derived from mice 2 to 2.5 years in age competed equally well against the competitor cell fraction as did marrow cells derived from young mice 3 to 6 months of age. Moreover, it was also shown that bone marrow derived from old mice competed equally well in serial transplantation experiments as those derived from young mice. For example, the proportion of donor derived cells in recipients of previously transplanted marrow derived from either young or old donor mice was approximately 10% as compared to fresh, non-transplant marrow. Thus, although data from murine serial transplantation studies have suggested that HSCs may have a finite capacity for self-renewal it seems, using the assays available, that this limit is not reached during the normal life span of the animal.

Although the findings of numerous studies suggest that the capacity of HSCs to undergo self-renewal is not unlimited, *few* data are available on the *extent* to which self-renewal of HSCs from various ontological sources can occur. Such data

would be valuable in light of the crucial role that HSCs play in a number of clinical procedures including autograft purging, the genetic therapy of various heritable hematological disorders, attempts at ex vivo expansion of HSCs as well as the use of alternative sources of transplantable stem cell populations (ie. fetal liver and umbilical cord blood). Chapter 5 of this thesis describes data collected on the regenerative capacity of HSCs derived from adult bone marrow or fetal liver.

1.5. Regulation of hemopoiesis

The process by which a small number of HSCs continuously generate the *appropriate* number of mature blood cells constituting the eight major hemopoietic lineages is a complex, regulated process. Moreover, the entry of mature blood cells into the circulation, their localization to the appropriate tissues as well as their functional activation is also under strict regulation. The known molecules which are responsible for regulating these various aspects of hemopoiesis can generally be divided into two groups: extracellular factors (composed of both humoral factors and cell or matrix-associated factors), and intracellular factors (eg. growth factor receptors and transcription factors). Although there is an abundance of evidence supporting the role of molecules belonging to both of these groups in the regulation of hemopoiesis their relative importance and relationship to one another is, at present, unclear.

1.5.1. Regulation by extracellular factors

1.5.1.1. Hemopoietic growth factors

The most extensively characterized group of extrinsic factors are the hemopoietic growth factors. This group is composed of the hemopoietic colony-stimulating factors (G-CSF, M-CSF, GM-CSF), the interleukins (IL-1 to IL-17), hemopoietic inhibitors (TGF-β and MIP-1α) and "stem cell" factors (Steel factor and flk2/flt3 ligand). These factors are glycoproteins of 10-70 kilodaltons, are able to act at extremely low concentrations (ie. 10^{-6} M), and are produced by a variety of cell

types throughout the body (reviewed in (118) and (38)). To date more than 25 distinct hemopoietic growth factors have been identified and the genes for most have been cloned. These factors have been shown to play a role in the survival, proliferation and differentiation of hemopoietic cells at various stages of development (119). By far, the majority of data that have been collected on the function of these growth factors has been their action on the more mature hemopoietic cell types. Much less data regarding their effects upon HSCs is available.

However, several growth factors have been identified that are involved in the activation and regulation of the proliferation of primitive hemopoietic cells and their progeny. Entry of quiescent blast colony forming cells into the cell cycle is regulated by multiple synergistic factors, including IL-6 (120), granulocyte colony-stimulating factor (G-CSF) (121), IL-11 (122, 123), IL-3 (124), and Steel factor (125). In contrast, factors such as MIP-1α and TGF-β have been shown to inhibit the entry of primitive murine and human progenitors into the cell cycle (113, 126, 127).

In addition to bringing quiescent primitive hemopoietic cells into cycle, Steel factor, the ligand for the c-kit receptor, also plays a role in the proliferation of these cells. Although Steel factor by itself has little effect upon the proliferation of primitive hemopoietic cells, when combined with others factors such as IL-3, GM-CSF, granulocyte colony-stimulating factor (G-CSF), IL-4, IL-1α, IL-11 and IL-12, Steel factor has a potent effect upon the proliferation of both immature and lineage-restricted lymphoid and myeloid cells (128-135). More recently a growth factor whose receptor is expressed on a more primitive subgroup of hemopoietic cells has been described. Using Northern blot and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, expression of the flt3/flk2 receptor was largely confined to primitive hemopoietic cell populations which include cells with long term lympho-myeloid repopulating ability (136, 137). Similarly, in humans, the

flt3/flk2 receptor is expressed on thymic, splenic and bone marrow progenitors positive for expression of the CD34 cell surface antigen, a marker found on primitive hemopoietic cells and endothelial cells (138, 139). The flt3/flk2 ligand (FL) (140, 141) is able to stimulate the proliferation of primitive murine and human hemopoietic progenitor cells when combined with other hemopoietic growth factors, reminiscent of the effects of Steel factor. Combined with Steel factor alone, or Steel factor/IL-3/GM-CSF, FL increases the proportion of $AA4.1^{+}Sca\text{-}1^{+}Lin^{high}$ murine fetal liver cells in cycle as determined by [^{3}H]thymidine incorporation assay(140) and the number of in vitro colonies in clonogenic assays following plating of $Thy^{lo}Sca\text{-}1^{+}Lin^{-}$ stem cell candidates purified from murine bone marrow (142). Furthermore, when combined with IL-7, FL induces proliferation of immature day 14 murine fetal T-cell precursors suggesting a role for FL in lymphoid development (142). Although both Steel factor and FL appear to be important early in hemopoiesis these factors are not essential since mice lacking expression of Steel factor, FL or their receptors, due to natural mutations or gene knockout, are still viable (143, 144). These observations suggest the existence of additional unknown stem cell factors and/or known growth factors able to compensate for the loss of expression of Steel factor/FL or their receptors.

While some growth factors play a role in the development of cells of numerous hemopoietic lineages (ie. IL-3), others appear to be more restricted. For example, erythropoietin, thrombopoietin, G-CSF and M-CSF predominantly act in the development of erythrocytes, platelets, neutrophilic granulocytes and macrophages/monocytes respectively. Moreover, several of the interleukins (ie. IL-2 and IL-7) function predominantly in lymphoid development.

1.5.1.2. The extracellular matrix

The hemopoietic microenvironment is composed of a variety of non-hemopoietic cells which include fibroblasts, adipocytes, endothelial cells and reticular cells which together produce the extracellular matrix. Morphological

studies have demonstrated a close physical association between stromal and blood cells within the marrow cavity (145-147). In addition to producing a variety of growth factors (147) ,the above cell types also produce a number of other proteins which have been hypothesized to play a role in regulating the survival, proliferation and differentiation of hemopoietic cells by facilitating communication between cells via cell surface receptors and through the localized concentration and presentation of sequestered growth factors to hemopoietic cells. Examples of the different types of proteins composing the extracellular matrix include a number of collagens (Types I, III, IV and V), glycoproteins (thrombospondin, fibronectin, hemonectin, laminins and tenascin), and glycosaminoglycans (hyaluronic acid, heparan sulfate, dermatan sulfate and chondroitin sulfate). Glycoproteins such as fibronectin and thrombospondin as well as glycosaminoglycans such as hyaluronic acid and heparin sulfate are thought to function in hemopoiesis by acting upon on primitive hemopoietic progenitors. For example, fibronectin and thrombospondin are adhesive ligands for a variety of human progenitors including CFU-GEMM, BFU-E, CFU-GM and LTC-IC.(148, 149). Moreover, hyaluronic acid, heparin sulfate and chondroitin sulfate are involved in the adhesion of primitive human progenitors to stroma in long term cultures in vitro and are thought to enhance hemopoiesis either by binding both primitive progenitors and growth factors that can stimulate them, by concentrating these growth factors at the site of their cellular receptors, or by enhancing the attachment of primitive cells to the stromal feeder layer (150). Interestingly, an increase in the production of chondroitin sulfate in long term cultures has been correlated with an enhancement of day 10 CFU-S and CFU-GM production (151). In addition, integrins such as LFA-1 and VLA-4 and the CD44 glycoprotein which are expressed on $CD34^+$ human bone marrow cells (152) appear to be important in mediating interactions between primitive hemopoietic progenitors and stroma since the addition of antibodies directed against VLA-4 or CD44 retards lympho- and myelopoiesis in long term cultures (153, 154). Further,

VLA-4 interactions with fibronectin, VCAM-1 and L-selectin have been implicated in mediating the in vivo homing of hemopoietic progenitors to the marrow and spleen and the same interactions may be involved in the mobilization of progenitors from the marrow to the blood (149, 155).

The above data strongly suggest that adhesion molecules and molecules of the extracellular matrix play a role in the regulation of primitive hemopoietic progenitor cells (and possibly totipotent HSCs). These molecules may function by increasing the response of progenitors cells to various hemopoietic growth factors since thrombospondin has been observed to increase the response of the cells to IL-3 and GM-CSF (156).

1.5.2. Regulation by intracellular factors

Signal transduction via growth factors involves the binding of the growth factor to its appropriate receptor followed by dimerization of the receptor and initiation of downstream signaling pathways involving a variety of cytoplasmic intermediates such as protein kinases and phosphatases and their substrates. Growth factors are believed to influence a variety of cellular functions including cell survival, cycling status, proliferation and possibly differentiation. Although many of the downstream molecules involved in these pathways have not yet been identified, several transcription factors are now recognized as key regulators in hemopoiesis. Transcription factors that appear to be involved in the regulation of early hemopoietic cell development and self-renewal have recently been identified through overexpression and gene disruption experiments (157). Disruption of the GATA-2 gene in mice results in a marked reduction in all hemopoietic progenitors (158). Moreover, the overexpression of HOXB4 has been shown to be associated with an increase in the regenerative ability of HSCs following bone marrow transplant (91). Some factors are more restricted in their patterns of expression. The SCL/Tal-1 and Ikaros transcription factors, for example, are expressed in cells of the myeloid/erythroid and lymphoid lineages respectively (159, 160). A number

of transcription factors that appear to be involved in the developmental regulation of specific hemopoietic lineages have recently been identified. The transcription factors MZF1, NFAT and Oct-2/Pax5 are specifically expressed in neutrophils, T cells and B cells respectively, while GATA-1 is necessary for the development of cells of the erythroid, mast and megakaryocyte lineages (157, 161, 162).

Thus, a substantial amount of data exists suggesting that transcription factors play a crucial role in early hemopoietic development. As a result intense research efforts are now being focused upon identifying those genes whose activity is regulated by these transcription factors and which may encode the molecular factors which are responsible for governing the self-renewal, proliferation and commitment of HSCs.

1.6. Genetic manipulation of hemopoietic cells using recombinant retroviruses

Based upon their capacity for highly efficient infection and non-toxic and stable integration into the genome of a wide range of cell types, recombinant retroviruses represent the most attractive vehicle for exogenous gene transfer into mammalian target cells .

The ability to transfer exogenous genes into HSCs using recombinant retroviruses (ie. genetically mark them) has provided significant insight into the proliferative and differentiative potential of totipotent HSCs and has provided direct evidence of the ability of these cells to self-renew. In addition, gene transfer methodologies have provided the means to test critically various genes encoding putative regulatory molecules which may play a role in controlling HSC proliferation, differentiation and/or self-renewal at the molecular level. For example, the transfer and overexpression of a variety of genes encoding hemopoietic growth factors in murine recipients resulted in myeloproliferative disorders similar to those observed in many human leukemia patients suggestive that the dysregulated

expression of hemopoietic growth factors may play a role in human disease via autostimulatory mechanisms (163-167). In addition, these experiments demonstrated conclusively the role of growth factors in hemopoietic development and regulation in vivo, supporting results obtained from in vitro clonogenic progenitor assays. Moreover, gene transfer is central to the newly developed field of human gene therapy. Gene therapy became reality in 1990 when a young girl, born with a defective version of the gene encoding adenosine deaminase (ADA), received injections of her own T cells transduced with a recombinant retrovirus expressing a normal functioning copy of the ADA gene. Since then more than 100 clinical trials aimed at treating diseases ranging from inherited disorders such as cystic fibrosis and Gauchers disease to cancer and Acquired Immune Deficiency Syndrome (AIDS) have been approved in the U.S. alone. Unfortunately, despite this flurry of activity, the results that have been achieved with the current gene transfer methodologies have been rather disappointing. This is due to problems associated with the efficiency of gene transfer protocols, expression of transferred genes as well as a lack of understanding of the growth and survival requirements of human HSCs. Marking studies, the investigation of putative regulatory genes in hemopoietic development and gene therapy share at least one of two major requirements: 1) the efficient and stable transduction of the hemopoietic stem cell and 2) appropriate expression of the transduced genes in the desired cell type of interest. The remainder of this introduction deals with the advances that have been made using recombinant retroviruses as a vector for gene transfer.

1.6.1. The lifecycle of retroviruses

Retroviruses do not exist as distinct genetic elements within their host cells but permanently integrate into the genome in the form of a DNA provirus and thus are stably transmitted from parent cell to progeny cell. However, during the course of their lifecycle, the virus alternates from an RNA to a DNA intermediate. The critical features of the retroviral lifecycle involve the synthesis and packaging of a

genomic RNA copy of the provirus into structural viral proteins; shedding of the intact virion particle from the surface of the host cell; entrance of the virion into the target cell via specific cell surface receptors; reverse transcription of the viral RNA into a double-stranded cDNA copy; integration of the proviral cDNA into the genomic DNA of its host cell; and expression of the viral genes.

In general, all retroviruses possess three distinct open reading frames (ORF) called gag, pol and env. These genes encode polyproteins composed of essential structural proteins such as the matrix (MA), capsid (CA) and nucleocapsid (NC) (gag ORF), the enzymes required for the reverse transcription (RT) of viral RNA into cDNA and integration (IN) of the cDNA into the genome of the host cell (pol ORF), and the surface (SU) and transmembrane (TM) proteins responsible for entrance into its target cell via interaction with specific cell surface receptors (env ORF) (reviewed in (168) and (169)). In the proviral form, the gag, pol and env genes are bounded on either side by long terminal repeats (LTRs) which contain the enhancer and promoter regulatory elements as well as the transcriptional initiation and polyadenylation signals. These elements are responsible for directing the synthesis of both the cellular RNA transcripts for the production of the viral proteins as well as the full length genomic RNA copy of the provirus utilizing the cellular RNA polymerase II enzyme. A schematic diagram of a typical murine retrovirus is shown in Figure 1.5. Packaging of the genomic RNA into virion particles is dependent upon interaction of the viral structural proteins with a specific sequence called ψ which lies downstream of the 5' LTR (170). However, additional sequences present within the downstream gag gene are now also known to be essential for efficient RNA packaging (171, 172). Once surrounded in a core of viral protein, the virion particle buds from the cell obtaining a surrounding envelope composed of cellular membrane studded with viral envelope glycoproteins. The infectious virion particle subsequently gains entrance into a potential target cell through the interaction of the viral envelope proteins with specific cell surface

receptors. Several of the cell surface receptors which retroviruses have exploited to gain entry into target cells have recently been identified. These include receptors for ecotropic murine retroviruses (MCAT) (173, 174), feline immunodeficiency virus (CD9) (175, 176), human and simian immunodeficiency viruses (CD4) (177-179), avian leukosis virus type A (Tval) (180), bovine leukemia virus (Blvr) (181) amphotropic murine retroviruses (Ram-1) (182, 183) and the common receptor for gibbon ape leukemia virus (184), feline leukemia virus group B (185) and simian sarcoma-associated virus (186) (Glvr-1). Interestingly, the MCAT, Glvr-1 and Ram-1 receptors are transport proteins which perform essential housekeeping functions; MCAT serves as a cationic amino acid transporter of arginine, lysine and ornithine (173, 187) while Ram-1 and Glvr-1 are both sodium-dependent phosphate symporters (188). Entry into the target cell results in the transformation of the quiescent enveloped virion into an enzymatically active nucleoprotein complex that begins to reverse transcribe the genomic RNA template into a double stranded cDNA copy (189, 190). Successful integration of the proviral cDNA into the genome of the host cell has been shown to require that the cell be actively cycling (191, 192), with the main barrier to successful retroviral integration being the presence of the nuclear membrane (193). Upon dissolution of the nuclear membrane at prophase of mitosis the viral DNA enters the nucleus and inserts itself stably into the genome of its host through the action of the viral encoded integrase which "randomly" nicks the genomic DNA creating a 4-6 base pair staggered cut and ligating it to the staggered cut viral DNA. A summary of the critical steps in the retroviral lifecycle are shown in Figure 1.6.

1.6.2. Recombinant retroviruses as vectors for gene transduction

At present, several hurdles remain to be overcome for the safe, stable and efficient use of recombinant retroviruses as vectors for gene transfer. These include the ability to produce retroviral stocks free of replication competent wild type virus, the ability to introduce the recombinant retrovirus into the target cell through

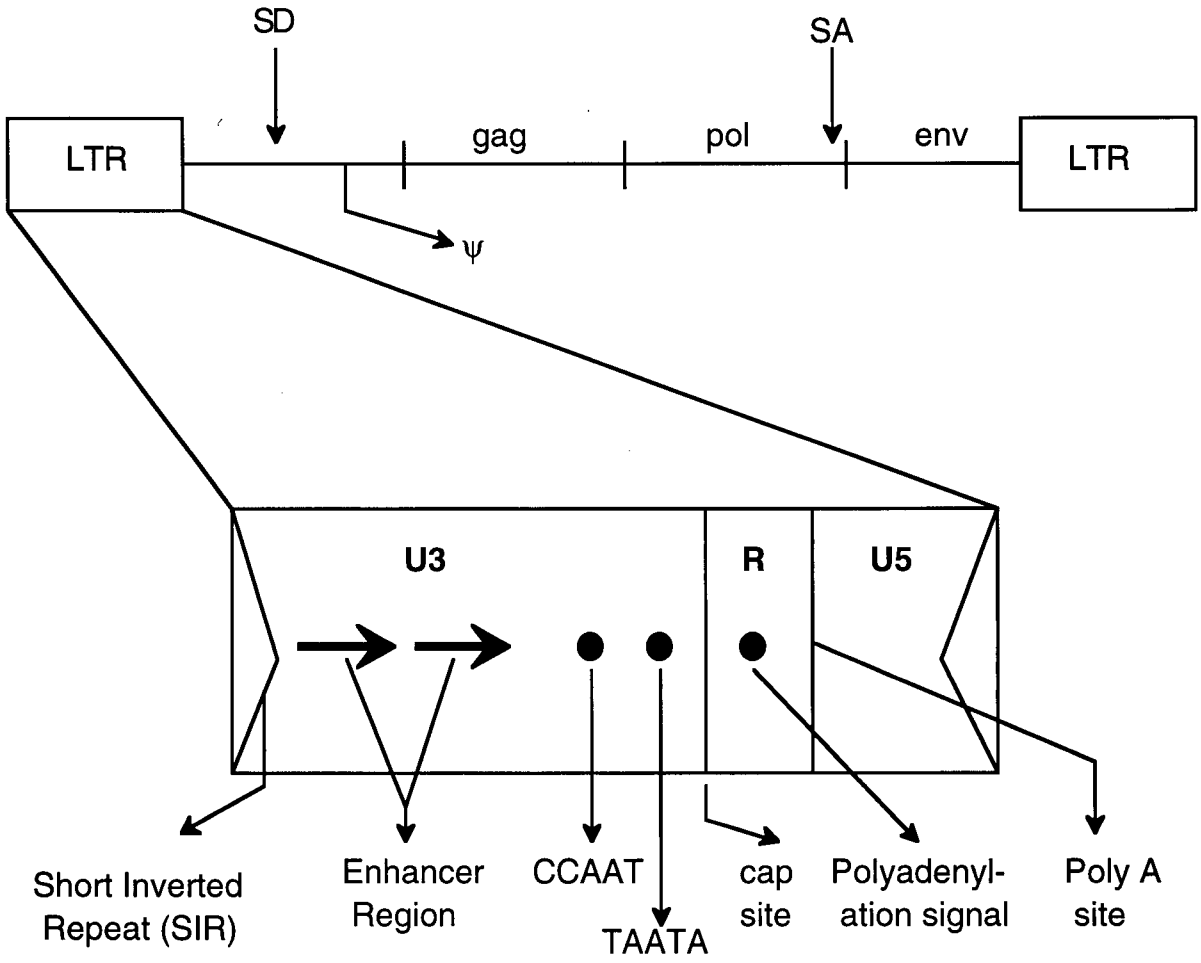

Figure 1.5. Schematic representation of a typical retrovirus showing the gag, pol and env genes, splice acceptor (SA) and splice donor (SD) sites, and the ψ packaging signal. The LTR is composed of three regions; U3, R and U5. U3 contains two direct repeat 72 bp enhancers and the transcriptional start signals. The RNA cap site and polyadenlyation site is located at the beginning and end of the R region respectively.

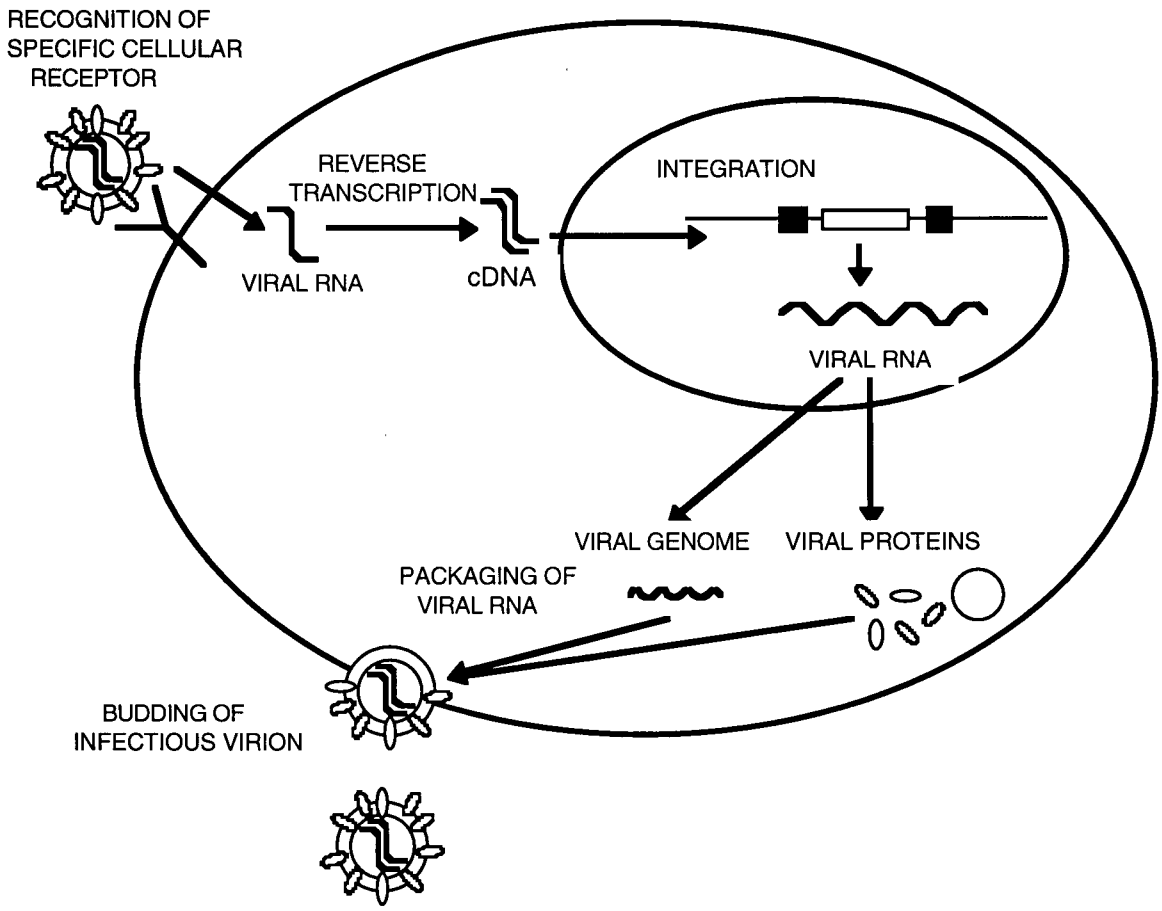

Figure 1.6. Critical features of the retroviral lifecycle. Infectious virions enter a target cell through interaction with specific receptors on the cell surface whereupon the viral RNA is reverse transcribed into a cDNA copy. The viral cDNA undergoes integration into the genome of the host cell and a genomic length RNA molecule is produced and packaged into viral particles composed of viral structural and enzymatic proteins. The virion buds from the surface of the host cell and the cycle repeats itself.

interaction with specific cellular receptors, and the ability to express the transferred gene at high levels in the appropriate cell type following integration into the genome of the host cell.

1.6.2.1. Production of helper-free replication-defective retroviruses

The ability to produce replication incompetent recombinant retroviruses free of any contaminating helper, or replication competent retrovirus is essential to both gene therapy and gene marking studies. The presence of replication competent retrovirus can result in the continuous production of infectious virus particles from target cells producing increasingly numerous and complex marking patterns in

clonal analysis, and has been shown to be associated with lymphocytic leukemia in a non-human primate gene therapy trial (194).

The production of replication-defective retroviruses can be achieved through the replacement of the viral gag, pol and env genes with a particular gene of interest using standard genetic engineering procedures and plasmid constructs. The recombinant retroviral vector is then introduced into a packaging cell line using calcium phosphate precipitation. A packaging cell line is a fibroblast derived line into which has been stably introduced the gag, pol and env genes of a wild type retrovirus and thus, is the source of the essential retroviral proteins. However, these viral sequences are engineered so that although they continuously produce all of the required viral proteins, their RNA is unable to be packaged. Thus, genomic length RNA molecules derived from the *recombinant* retroviral vector combines with the viral proteins and buds from the packaging cells resulting in the release of infectious recombinant retroviral particles capable of one round of infection. Upon entry into a target cell and integration into the host genome, the recombinant retrovirus is unable to undergo further rounds of replication due to the absence of the required viral genes. The provirus is stably propagated in the target cell and the progeny of the target cell and expresses the transferred gene of interest (see Figure 1.7).

The first packaging cells lines (ψ2 (195), ψAM (196) and PA317 (197)) were constructed by deleting approximately 350 nucleotides of the 5' region of the retrovirus which contained the ψ packaging signal (ψ2, ψAM) as well as the 3' LTR and a portion of the 5' LTR (PA317). However, replication competent viruses could be generated through rare recombination events during reverse transcription in which the regions deleted could be corrected for by intact sequences obtained from the recombinant retroviral vector. Today, third generation packaging lines (GP+E86 (198, 199) and ψCRE (200)) are available in which the viral gag, pol and env

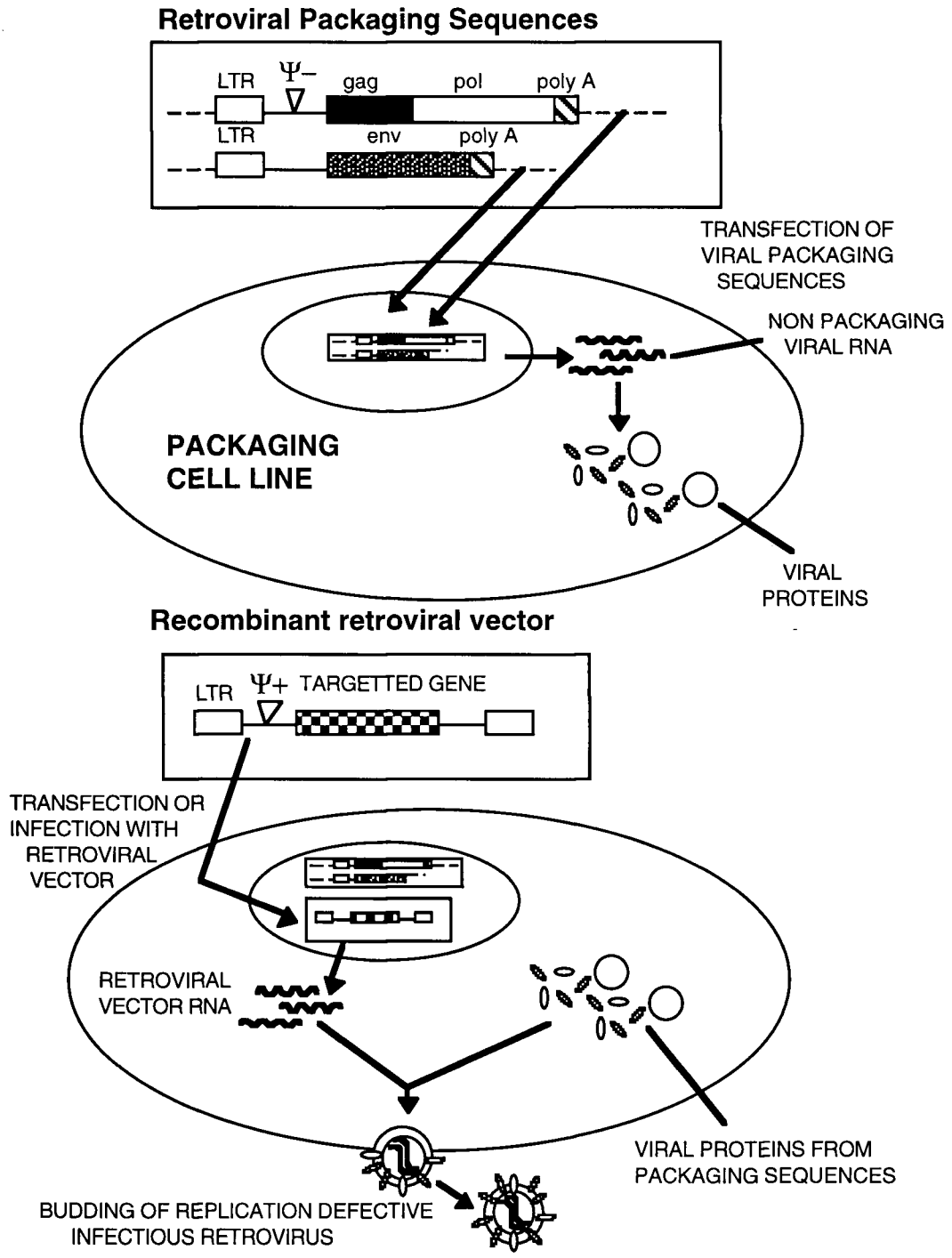

Figure 1.7. Packaging cells lines continually produce the gag, pol and env proteins but viral RNA is unable to be packaged due to deletion of the ψ signal sequence and separation of the gag/pol and env genes onto different plasmids. Introduction of a functional recombinant retroviral vector deleted of viral genes results in the production of genomic length recombinant RNA which are packaged into virion particles and bud from the packaging cell. The release of infectious recombinant retroviral particles are subsequently able to undergo 1 round of infection and integration into a host target cell.

genes are separated onto two separate plasmid constructs with gag and pol on one and env on the other. These lines require three recombinational events for the production of helper virus to occur thus greatly decreasing the likelihood of this event. One drawback of using the lines mentioned above is that the production of stable, high titre viral producer cells can take several weeks. More recently, ecotropic (able to infect mouse and rat cells only) (BOSC) and amphotropic (able to infect mouse, human as well as other cell types)(BING) packaging cell lines derived from a highly transfectable 293T cell line have been produced enabling the rapid, transient production of high titre viral supernatants free of helper virus within 72 hours (201) .

1.6.3. Optimization of retroviral gene transfer

1.6.3.1. Retroviral infection strategies

The efficiency of infection of HSCs is dependent upon a large number of factors including their cycling status, the addition of exogenous hemopoietic growth factors and the method of infection.

As previously mentioned, successful retroviral infection requires that the target cell be in a state of active cycle (191, 192). Unfortunately, the vast majority of HSCs are in a state of quiescence under normal physiologic conditions (93). Following the injection of the cycle specific cytotoxic drug 5-FU, however, a larger proportion of HSCs are actively cycling (93) and are more susceptible to retroviral infection (202, 203).

Although HSCs can survive for a period of time and be susceptible to retroviral transduction in the absence of exogenously added hemopoietic growth factors (204) , the infectability of day 12 CFU-S was found to be greatly increased in the presence of IL-3 (24, 205). Moreover, various combinations of IL-3, IL-6, Steel factor, IL-1 and leukemia inhibitory factor (LIF) were found to enhance retrovirus mediated gene transfer into murine as well as human and non-human

primate HSCs (124, 206-212). In addition to bringing cells into a state of active cell cycle growth factors may also play a role in increasing the number and/or affinity of receptor molecules on target cells (213). Although growth factors increase the efficiency of gene transduction, caution must be used since the culture of marrow cells in the presence of growth factors for extended periods of time in vitro may impair the engrafting ability of the HSCs upon transplantation into irradiated hosts (214).

The method of exposure of the retrovirus to the target marrow cells also has a large effect upon the efficiency of viral transfection. The most successful results using murine bone marrow cells as targets have been achieved by co-culturing the viral producer and marrow cells together (215-217). While early results using a non-human primate model favored the use of filtered viral preparations free of producer cells (218) (219-221), more recent studies have shown that the efficiency of supernatant infection of primitive human hemopoietic cells can be increased through the use of an autologous stromal support layer during the infection procedure (222-224). The stromal layer most likely increases the efficiency of viral infection via the production of various defined and/or undefined growth factors and via cell-cell mediated interaction. Interestingly, Moritz et. al. have reported that the presence of the extracellular matrix molecule fibronectin can increase the efficiency of supernatant infection of committed progenitor cells and primitive cells with long term culture initiating ability (LTC-IC) (225). It has been hypothesized that this molecule is somehow able to bring both the target cell and retrovirus into close proximity thus increasing the probability of retroviral infection. Recently, a method by which retroviral supernatant is allowed to flow through a porous membrane upon which the target cells lie has been described (226). While normal supernatant infection relies upon Brownian motion for virions to come into contact with a target cell (a very inefficient process), the flow-through transduction system overcomes this limitation by "flowing" all virus particles past the target cells resulting in a much

higher probability of contact between virus and cell. This system can lead to high transfection efficiencies even at low viral titre and can be done in the absence of polycations such as protamine sulfate or polybrene which have been historically utilized as methods to increase gene transfer efficiency.

1.6.3.2. Targeting of virions to host cells

The transduction of murine HSCs using ecotropic retroviral vectors carrying a variety of genes (ie. neo^R (23, 35, 204, 205), adenosine deaminase (215-217), β-globin (227) and glucocerebrosidase (228-230)) has, in general, been found to be much more efficient than gene transfer into HSCs from larger animals using amphotropic viruses. One current limitation to retroviral mediated gene transfer into the bone marrow of mice or larger animals is that the desired target cell, the totipotent HSC, is both rare and largely non-cycling (93). Using standard infection protocols Luskey et. al. has estimated the efficiency of gene transfer to murine HSCs to be 20% using Southern blot analysis of DNA obtained from the hemopoietic tissues of recipients transplanted with retrovirally transduced bone marrow at 4 months post transplant as measured by proviral integration and genome copy number analysis (206). Moreover, Einerhand et. al. have estimated the efficiency of gene transfer to marrow repopulating ability-CFU-S (MRA-CFU-S) which is a cell that homes to the marrow of the recipients following transplant and proliferates to produce numerous day 12-CFU-S cells. Thirteen days post transplant the recipient mouse is sacrificed and its bone marrow used to assay for day 12 CFU-S by injection into secondary recipients. Thus, through the analysis of individual day 12 spleen colonies, Einerhand and his colleagues estimated the efficiency of gene transfer to these primitive cells to be 15% (207). The efficiency of gene transfer to HSCs derived from larger animals such as non-human primates (219, 221, 231), felines (232), canines (233-235) or humans (236, 237) appears to be considerably lower. Quantitative PCR performed on DNA obtained from peripheral blood and bone marrow cells at a minimum of one year post transplant

suggested that the efficiency of gene transfer to HSCs from larger animals is only 0.1% to 5%. This low gene transfer efficiency may in part be due to the expression of low levels of the amphotropic receptor Ram-1 (188). Several investigators have attempted to bypass this problem through the use of viral pseudotyping which involves replacing the amphotropic envelope gene with an env gene from a different virus whose receptor is expressed at a much higher density on the desired target cell. Combining the Moloney murine leukemia virus gag and pol genes with the env gene derived from the gibbon ape leukemia virus (238-240) or the vesicular stomatitis virus G glycoprotein (VSV-G) (241, 242) has resulted in an increase in gene transfer to human cell lines, committed human progenitor cells and LTC-IC.

Some investigators have attempted to target retroviral infection to specific cell types by creating a hybrid env gene encoding a portion of the viral env protein and a ligand recognized by a specific receptor on the surface of the target cell. Using this approach, retroviruses have been targeted to specific cells via the erythropoietin receptor (243), the low density lipoprotein receptor (244), epidermal growth factor receptor (245) as well as major histocompatibility class I and class II antigen receptors (246). Although this method does show promise, caution must be used since the fusion of the viral env with a foreign molecule may result in a non-functional envelope protein and thus a non-infectious virus particle.

1.6.3.3. Use of selectable markers to increase the utility of recombinant retroviral vectors

To aid in the identification, enrichment and tracking of transduced target cells a variety of selectable markers have been incorporated into retroviral vectors. The most widely used of these have been genes which confer resistance to toxic compounds such as neomycin and hygromycin. A variety of others such as β-galactosidase and a number of cell surface antigens have also been utilized. Table 1.1 shows the various genes that have been utilized as selectable markers to date.

The ability to identify and select for retrovirally transduced HSCs rapidly and non-toxically could enhance the power of marking studies, efforts to assess the effects of overexpressing putative HSC regulatory molecules and current efforts at human gene therapy. However, at the time that the work described in this thesis was initiated, the use of genes encoding cell surface antigens as selectable markers for the selection of retrovirally transduced in vivo repopulating HSCs had not been reported. As previously mentioned, the work described in Chapter 3 of this thesis describes the novel use of the human cell surface antigen CD24 as a dominant selectable marker in a retroviral vector to enable the efficient selection of transduced murine BM cells including those with totipotent long term repopulating ability.

1.6.3.4. Retroviral vector design

Several factors can influence the performance of any particular retroviral construct including the regulatory elements used to drive expression, the number and size of transcriptional units, the viral backbone used, the direction of transcription and the presence or absence of selectable markers.

Although lineage restricted promoters may be ideal if the transferred gene is to be expressed exclusively in particular cell types (247, 248), viral LTRs have consistently been shown to result in higher levels of gene expression as compared to a variety of internal promoters of viral or cellular origin (249, 250). Unfortunately, viral LTRs such as the Moloney Murine Leukemia Virus (MoMuLV) have been found to be subject to transcriptional shutdown following long periods of time in vivo (215, 251) and in primitive cell types such as embryonic carcinoma (EC) cells (252-254), embryonal stem (ES) cells (255) and primitive hemopoietic cell lines (256) possibly due to methylation of the regulatory elements (257). Several viral mutants have been isolated that are able to express transferred genes at high levels in EC and ES cells. The PCMV (PCC4 embryonal carcinoma cell-passaged myeloproliferative sarcoma virus) (258) and the dl587rev virus (259) possess

Table 1.1. Options in Selectable/Reporter Markers for Retroviral Vectors

Marker Gene	Size in bp*	Methods of Selection	Quantification of Gene Expression at Single Cell Resolution
neomycin^R (260, 261)	800	drug resistance	no
hygromycin^R (262)	1200		
puromycin	700		
methotrexate^R(263, 264)	700		
cytosine deaminase (265)	1600		
MDR-1 (266)	3800		
thymidine kinase (267)	1400		
β-galactosidase (268, 269)	3000	FACS	yes
alkaline phosphatase (270)	4000		
Leu-1 (271)	2300	immuno-based	yes
transferrin receptor (271)	2800	(FACS, panning)	
MDR-1 (272)	3800		
truncated nerve growth factor receptor (273)	1500		
IL-2 receptor (274)	1400		
Heat Stable Antigen (HSA) (275)	228		
mutated murine prion protein (276)	1200		
Green Fluorescence Protein (GFP) (277)	720		
CD24 (278)	240		
Thy-1 (279)	488		

* Approximate size of cDNA encompassing coding region

various deletions and base pair mutations which were found to remove several transcriptional blocks located within the LTR and primer binding sites of the original viruses (280-282). It is the removal of these transcriptional blocks that enables the expression of transferred genes under the control of these regulatory elements in both EC and ES cells. Recently, Hawley et. al. (283) have produced a series of vectors based upon a Murine Stem Cell Virus (MSCV) backbone which combines the LTR from PCMV, the 5' untranslated region from the dl587rev virus, and a number of convenient cloning sites. Data will be presented in this Chapter 4 of this thesis to suggest that the MSCV vectors are able to transcribe transduced genes efficiently in primitive primary hemopoietic stem cell candidates and their progeny for extended periods of time in vivo.

Placing a gene of interest under the regulatory control of an internal promoter downstream of the viral LTR regulatory sequences may result in expression problems due to promoter interference between the viral and internal regulatory elements. Indeed, the expression of internally driven genes has been found to be unpredictable. In some cases efficient expression from the internal promoter was detected (172, 284) while in other cases little or no expression was observed (216, 285-287). As a means to bypass this potential problem self-inactivating or "crippled" vectors can be used. These vectors possess a deletion of a portion of the 3' LTR and are designed such that following infection of the target cell the viral enhancer and promoter regulatory elements are non-functional (288-290).

The number of transcriptional units present within the retroviral vector can also influence the efficiency of transferred gene expression. Although some studies have suggested that superior results are obtained using a simplified vector which contains only one transcriptional unit (215, 291), others have found that the inclusion of an additional gene encoding a dominant selectable marker in the vector can be beneficial (249, 292, 293). However, some studies have reported that

cells selected for on the basis of expression of the selectable marker can result in the suppression of the adjacent gene, most likely due to promoter interference between internal and viral regulatory elements in multi-gene vectors (274, 294). A solution to this problem has been to express both genes from a single regulatory element by linking them together using an internal ribosomal entry site (IRES) element. IRES elements are discrete fragments of DNA (approximately 500-600-bp in size) derived from the 5' untranslated regions of the polio or encephalomyocarditis virus (295, 296). IRES elements allow ribosomes to bind internally on the mRNA rather than at the 5' cap site as normally occurs and thus, enables more than one transcriptional unit to be translated from a single mRNA molecule. These elements have been shown to link up to three separate genes thus allowing the regulation of multiple transcriptional units from a single regulatory element (297, 298).

1.7. Thesis objectives and general strategy

Although recombinant retroviruses currently remain the most efficient method for introducing exogenous genetic material into target cells, the low infection efficiency of HSCs represents a serious hurdle to effective retroviral marking studies and gene therapy. Moreover, although gene transfer methodologies have provided the critical means to test genes encoding putative regulatory molecules which may play a role in controlling HSC proliferation, differentiation and/or self-renewal via overexpression of the transferred gene, at the time that the work described in this thesis was initiated, the direct demonstration that any regulatory element was able to drive high and sustained levels of transferred gene expression in HSCs had not been achieved.

The first objective of this thesis work was to develop procedures that would increase the utility of retroviral gene transfer procedures. These studies focused on determining the feasibility of utilizing a cDNA encoding the human CD24 cell surface antigen as a dominant selectable marker in a retroviral vector to enable the

rapid, efficient and non-toxic identification and selection of retrovirally transduced murine bone marrow cells, including those with totipotent long term in vivo repopulating ability (Chapter 3).

My second objective was to exploit the selection protocol developed in Chapter 3 to demonstrate the regeneration of the hemopoietic systems of myeloablated recipient mice with cells derived exclusively from provirally marked HSCs and to quantify the levels of expression of the transferred CD24 marker gene in various phenotypically defined populations of cells in vivo including candidate HSCs defined by the $Sca^{+}Lin^{-}$ cell surface phenotype. The results of these studies (presented in Chapter 4) demonstrate the usefulness of the CD24 selection procedure to enable effective tracking of the contribution of individual stem cells to the regeneration of the HSC and more mature cell compartments in myeloablated recipient mice following bone marrow transplant, and to quantify transferred gene expression both in vitro and in vivo.

Although retroviral marking studies have provided direct evidence of the existence of totipotent HSCs, and have provided significant insight into the ability of these cells to regenerate the hemopoietic systems of myeloablated transplant recipients (ie. their proliferative and differentiative potential), to date quantitative data assessing the extent to which these cells can regenerate their numbers following transplant (ie. self-renew) is limited. The final objective of this work was to define more clearly the regenerative (self-renewal) capacity of HSCs following bone marrow transplant. The recovery of totipotent long term repopulating stem cell numbers in lethally irradiated recipient mice was assessed as a function of the number and source (adult bone marrow vs. fetal liver) of cells transplanted. In addition, using the CD24 selection procedure (Chapter 3) the contribution of individual HSC clones to the regeneration of the HSC compartment was assessed in some experiments. The results of this work are presented in Chapters 4 and 5.

CHAPTER 2

MATERIAL AND METHODS

2.1. Construction of retroviral vectors, virus production and viral assays

2.1.1. Recombinant retroviral vectors

Experiments discussed in Chapters 3, 4 and 5 utilized a retrovirus derived from the JZen1 retroviral backbone kindly provided by Dr. S. Cory (Walter and Eliza Hall Institute, Melbourne, Australia). The 3' LTR of JZen1 is derived from the myeloproliferative sarcoma virus (MPSV)(167). To construct JZenCD24tkneo, a 310 bp Sal I fragment containing bp 1 to 303 of the published CD24 cDNA sequence (299) and encompassing the entire 240 bp coding region was removed from PAX114 (300) and inserted into the Xho I site of JZentkneo using standard procedures. JZentkneo was constructed by inserting into the Hpa I - Hind III sites of JZen1 a 1092 base pair Sma I- Hind III fragment from pTZ19Rtkneo that contains the neo^R gene linked to a mutant polyoma virus enhancer tandem repeat and Herpes Simplex virus thymidine kinase gene promoter isolated from pMC1neo (301).

MSCVneoIRESCD24, used in experiments presented in Chapter 4, was constructed using the MSCVneoEB vector (283) (kindly provided by Dr. Robert Hawley, Sunnybrook Health Science Center, Toronto, ON) originally derived from the MESC retroviral vector of Grez et. al. (302) and the LN retroviral vectors of Miller et. al. (303). PGKneo was removed from MSCVneoEB by digesting with BglII/BamHI and religating to create MSCV(-). A 948-bp EcoRI/XhoI fragment from a previously described construct (304) encompassing the encephalomyocarditis virus internal ribosomal entry site (IRES) sequence and the 240-bp coding region of the human CD24 cell surface antigen cDNA (299) was ligated into an EcoRI/XhoI digested MSCV(-). Lastly, a 847-bp blunted MluI/SalI fragment derived from pMC1neo (301)

containing a neomycin resistance gene was inserted by blunt end ligation into the EcoRI site of MSCVIRESCD24 to create MSCVneoIRESCD24.

2.1.2. Viral packaging and other cell lines

The ecotropic packaging cell line, GP+E-86 (199), and the amphotropic cell line, GP+AM12 (198), were used to generate helper-free recombinant retrovirus. The cell lines were maintained in HXM medium composed of Dulbecco's Modified Eagles Medium (DMEM; StemCell Technologies, Vancouver, British Columbia), 10% heat-inactivated (55^{o} C for 30 minutes) newborn calf serum (Gibco/BRL Canada; Burlington, Ontario), hypoxanthine (15 mg/ml; Sigma Chemical Co., St. Louis, MO), xanthine (250 mg/ml; Sigma), and mycophenolic acid (25 mg/ml; Sigma). The IL-3-dependent murine hemopoietic cell line Ba/F3 (305) was maintained in RPMI with 10% fetal calf serum and 5% mouse spleen cell conditioned medium (SCCM) (StemCell Technologies). All cells were cultured at 37^{o}C in a humidified atmosphere of 5% CO_2 in air.

2.1.3. Generation of viral producer cell lines

Purified proviral plasmid DNA was introduced into the GP+AM-12 packaging cell line using the calcium phosphate ($CaPO_4$) transfection technique. DNA precipitate was formed by combining 18 µg of purified DNA, 50 µl of 2.5 M $CaCl_2$ and dH_20 up to 0.5 ml, which was slowly added to 0.5 mL of 2 X HBS (50mM Hepes, 3M NaCl, 1.5 mM Na_2HPO_4, pH 7.12) while gently bubbling with air to mix. The solution was allowed to stand at room temperature for 30 minutes and then added dropwise to 9 ml of medium on 2 x 10^5 GP+AM-12 packaging cells plated in a 100 mm tissue culture dish (Beckton Dickinson, Lincoln Park NJ) 24 hours previously. After 24 hours the medium was replaced with fresh medium containing 1 mg/ml (approximately 0.6 mg/ml active compound) of the neomycin analog G418 (Gibco/BRL). Medium was replaced every three days. Medium was placed on dishes of confluent G418^R GP+AM-12 cells and 24 hours later the supernatant was removed, filtered (0.22µm filter, Millipore, Bedford, MA) and overlayed onto 1 x 10^5

GP+E-86 ecotropic viral packaging cells in the presence of 7 μg/ml polybrene (Sigma). Twenty four hours later the medium was removed and replaced with fresh medium containing 1 mg/ml G418.

2.1.4. Viral titering and helper virus assay

Viral titres were determined by assaying medium conditioned by viral producer cell lines for transfer of neomycin resistance to NIH-3T3 cells (American Type Culture Collection [ATCC], Rockville, MD). Medium was placed atop subconfluent 100mm dishes of viral producer cells and 24 hours later the supernatant was removed and filtered. Various dilutions of the viral supernatant were placed in a final volume of 2 mls and placed on top of 2 x 10^5 3T3 cells plated in 60 mm tissue culture dishes (Becton Dickinson) with 7 μg/ml polybrene added. Twenty four hours later the supernatant was replaced with fresh medium containing 1 mg/ml G418. Medium was replaced every three days. $G418^R$ colonies were scored after staining with methylene blue to derive the number of infectious virus particles carrying neo^R generated by the viral producers (colony forming units per ml).

Assay for the presence of helper virus was performed by attempting to serially transfer neo^R to 3T3 cells (195). Confluent dishes of $G418^R$ 3T3 cells were obtained following infection with 5 mls of filtered undiluted viral supernatant followed by G418 selection as described above. Media was changed on confluent dishes of $G418^R$ 3T3 cells and 24 hours later the medium was removed, filtered and placed on top of 2 x 10^5 3T3 cells plated out 24 hours previously. Twenty four hours later the medium was removed and replaced with 5 mls of fresh medium containing 1 mg/ml G418. Colonies were subsequently scored 2-3 weeks later as described above.

2.2. Hemopoietic cell culture and assays

2.2.1. Mice

Mice used in these experiments were 8 to 12 week old (C57Bl/6J x C3H/HeJ)F1 (B6C3F1) male and female mice bred and maintained in the animal facility of the British Columbia Cancer Research Centre from parental strain breeders originally obtained from the Jackson Laboratories (Bar Harbor, MA). Mice used as bone marrow donors in competitive repopulation experiments were 10 to 14 week old male or female (C57Bl/6Ly-Pep3b x C3H/HeJ)F1 (PepC3F1) mice. B6C3F1 and PepC3F1 mice are phenotypically distinguishable on the basis of allelic differences at the Ly5 cell surface antigen locus; B6C3F1 mice are homozygous Ly5.2 and PepC3F1 mice are Ly5.1/Ly5.2 heterozygotes. All animals were housed in micro isolator cages and provided with sterile food and acidified sterile water.

2.2.2. Viral infection of bone marrow cells and cell lines

Bone marrow cells from adult male or female B6C3F1 or PepC3F1 mice injected 4 days previously with 5-fluorouracil in sterile phosphate-buffered saline (5-FU, 150 mg/kg body weight; Hoffman-LaRoche Ltd., Mississauga, Ontario) were flushed from femoral shafts with alpha medium and 5% FCS (StemCell Technologies). For co-culture infections $3x10^6$ marrow cells were incubated on a confluent monolayer of irradiated (1500 cGy X-rays) CD24 viral producer cells for 24 to 48 hours in medium composed of DMEM, 15% FCS, 10 ng/ml human interleukin-6 (IL-6), 6 ng/ml murine IL-3, 100 ng/ml murine Steel factor, and 7 mg/ml polybrene (Sigma). All growth factors were used as diluted supernatants from transfected COS cells prepared in the Terry Fox Laboratory. Cells used for competitive repopulation experiments were incubated for 48 hours in the above medium in the absence of polybrene prior to co-culture with viral producer cells in an effort to enhance gene transfer to the most primitive cells (124, 217). Loosely adherent and non-adherent cells were recovered by gentle agitation and washing of dishes with DMEM and 15% FCS. Cells were pelleted, resuspended in fresh

culture medium and incubated for a further 48 hours at 37°C to allow for expression of the transferred CD24 gene.

The murine cell line Ba/F3 was infected by exposure to filtered viral supernatant from the JZenCD24tkneo ecotropic viral producer cell line. Viral supernatant was supplemented with 7 μg/ml polybrene. Cells were then plated in methylcellulose (described below) in the presence of G418 (1 mg/ml). Independent $G418^R$ colonies were picked and expanded in the presence of G418 to be used as positive controls for FACS and Southern blot analysis.

2.2.3. In vitro clonogenic progenitor assay

Sorted and unsorted bone marrow cells were plated in 35mm petri dishes (Stem Cell Technologies, Vancouver, British Columbia) in 1.1 ml culture mixtures containing 0.8% methylcellulose in alpha medium supplemented with 30% FCS, 1% bovine serum albumin (BSA), 10^{-4} M β-mercaptoethanol, 3 U/ml human urinary erythropoietin (100,000 units/mg), 2% SCCM and 10% agar-stimulated human leukocyte conditioned medium, all of which were obtained from StemCell Technologies Inc.. Cells were plated in the presence or absence of 1.5 mg/ml (approximately 0.9 mg/ml active compound) of G418 (Gibco/BRL) and incubated at 37°C in 5% CO_2. Large single and multi-lineage colonies were scored after 8-14 days incubation according to standard criteria (19).

2.2.4. CFU-S assay

Lethally irradiated B6C3F1 mice (910-950 cGy, 110 cGy/min, ^{137}Cs γ-rays) were injected intravenously with 1 x 10^3 to 1 x 10^4 cells from indicated cell fractions. Twelve days later, animals were sacrificed via cervical dislocation and well isolated macroscopic spleen colonies individually dissected and suspended for flow cytometric and DNA analysis.

2.2.5. Bone marrow transplantation and quantification of CRU

In the experiments described in Chapters 3 and 4, 1 x 10^4 to 4 x 10^5 retrovirally transduced $CD24^+$ selected or unselected bone marrow cells derived

from PepC3F1 (Ly5.1/Ly5.2) donors were intravenously injected into irradiated (137Cs γ-rays, 950 cGy, 110 cGy/min) B6C3F1 (Ly5.2) recipients with or without a lifesparing dose of Ly5.2 competitor bone marrow cells; either 1 x 10^5 marrow cells from a normal mouse or 2 x 10^5 marrow cells from a compromised animal (59). The function of these latter cells is to ensure the short term survival of the recipient following the irradiation procedure. The level of reconstitution of recipients with donor (Ly5.1) cells and expression of the transferred CD24 gene was assessed at 5 to 32 weeks post-transplantation by flow cytometric analysis of peripheral blood samples (50-100 μl) obtained by tail vein puncture.

For the experiments described in Chapter 5 fetal livers were removed from day 14.5 embryos obtained from timed matings of C57Bl/6Ly-Pep3b(Ly5.1) male and C3H/HeJ(Ly5.2) female mice. Cells were suspended in alpha medium containing 5% FCS (StemCell Technologies) by repeated gentle aspiration through 5 ml, 2 ml and 1 ml pipettes followed by 18- and 21- gauge needles. Bone marrow cells from male or female PepC3F1 mice injected 4 days previously with 5-fluorouracil (5-FU; 150 mg/kg body weight) were flushed from femoral shafts with alpha medium and 5% FCS and a single cell suspension similarly obtained. Cells were counted using a standard hemocytometer. Cell survival was >98% as determined by trypan blue exclusion. Fetal liver cells or post 5-FU bone marrow cells from Ly5.1$^+$ donors were injected in combination with a lifesparing dose of 10^5 bone marrow cells from normal 8-12 week old B6C3F1 (Ly5.2$^+$) mice (59) into the tail vein of recipient B6C3F1 (Ly5.2$^+$) mice previously irradiated with 950cGy (110cGy/min, ^{137}Cs γ-rays). CRU were measured by injecting groups of 7 lethally irradiated B6C3F1 (Ly5.2$^+$) recipients in combination with 10^5 syngenic (Ly5.2$^+$) normal bone marrow cells and assessing the recipients 16 weeks later for the presence of Ly5.1$^+$ lymphoid and myeloid cells in their peripheral blood. Recipient mice were considered positive if >1% of each of myeloid and lymphoid peripheral blood cell populations (identified by their unique forward and side scatter profiles,

respectively) demonstrated the donor Ly5.1 cell surface phenotype. CRU frequency was calculated by determining the number of negative recipients as a function of the number of test cells injected and applying Poisson statistics using the method of least likelihood (306, 307).

2.3. Molecular analysis

2.3.1. Southern Blot Analysis

DNA was purified from NaDodSO$_4$/proteinase K-digested cells by phenol/chloroform extraction (308) or using the DNAzol reagent (Canadian Life Technologies, Burlington, Ontario). DNA was dialyzed for 16 hours against 1 x TE (10 mM Tris pH7.5, 1 mM EDTA pH 8.0) buffer and 10-20 μg digested with XbaI, SstI or EcoRI (Gibco/BRL) at 37^{o}C for 12-16 hours. Following ethanol precipitation, DNA was dissolved in 20 μl of 1 x TE buffer and separated on a 0.8% agarose gel. Gels were then treated for 35 minutes with Solution I (0.5M NaOH, 1.5 NaCl) and for 35 minutes with Solution 2 (1M Tris pH 7.0, 2M NaCl). DNA was then transferred to a nylon membrane (Zeta-Probe; Bio-Rad Laboratories, Richmond CA) in 10X SSC by standard blotting methods. Blots were prehybridized at 60^{o}C for 2 hours in 4.4X SSC, 7.5% formamide, 0.75% SDS, 1.5mM EDTA, 0.75% skim milk, 370 mg/ml of salmon sperm DNA. Blots were then hybridized for 20 hours at 60^{o}C under the same conditions as above with the inclusion of 7.5% dextran sulfate (Sigma). Membranes were separately probed using a XhoI/SalI fragment of pMC1neo (301) containing neo^{R} specific sequence. Probes were labeled with ^{32}P-dCTP (3000 Ci/mmol; Amersham) by random priming and purified on a Sephadex-G50 column before hybridization. Membranes were subsequently washed twice at 60^{o}C for 30 minutes each in 0.3X SSC, 0.1% SDS and 1 mg/ml of sodium pyrophosphate. Autoradiography was performed with Kodak XAR-5 film and an intensifying screen at -70^{o}C for 1-10 days. Membranes were stripped for re-probing by boiling in 1% SDS, and washing for 40 minutes. Blots were re-probed with the KpnI/MseI

fragment of pXM(ER)-190 which releases the full length erythropoietin receptor cDNA (kindly provided by A. D'Andrea, Dana-Farber Cancer Institute, Boston, MA) for an internal control for DNA loading. Densitometric analysis was performed using a phospho-imager with ImageQua^{TM} software (Molecular Dynamics, Sunnyvale, CA)

2.3.2. Antibody staining procedures

To analyze CD24 cell surface antigen expression following retroviral infection cells were washed once in alpha medium with 5% FCS, resuspended (1 to 7 x 10^6 cells/ml) in 0.2 to 0.4 ml of medium conditioned by hybridoma 2.4G2 which secretes an anti-mouse IgG Fc receptor antibody (309), and incubated on ice for 30 minutes in an effort to reduce non-specific staining. Cells were then washed once with Hank's balanced salt solution containing 2% FCS (HF). Tetramolecular complexes of monoclonal antibodies were used for the staining procedure (310) by mixing anti-CD24 antibody 32D12 (a generous gift from Dr. S Funderud, Oslo, Norway), anti-R phycoerythrin antibody ID3 and F(ab')$_2$ fragments of the anti-IgG antibody P9 (311) at a 1:2:3 molar ratio. Such tetrameric complexes provide a rapid and flexible means of generating labeled antibody from even small quantities of starting material and are equivalent to directly labeled antibody. These tetrameric antibody complexes were used for staining at a final concentration of 0.8 µg/ml. Cells were incubated on ice for 40 minutes, washed twice with HF, and then stained with R-phycoerythrin (R-PE) at 2 µg/ml. After a further 40 minutes on ice, cells were washed twice with HF and resuspended in HF containing 1 µg/ml of 7-amino actinomycin D (7AAD, Sigma) to distinguish dead cells prior to analysis by flow cytometry using a FACScan cell analyzer (Becton Dickinson and Co., San Jose, CA). Cells used in sorting experiments were stained with propidium iodide (PI, Sigma) to distinguish dead cells. Bone marrow cells cultured alone or on GP+E-86 packaging cells were used as negative controls.

Repopulation of recipient mice with donor-derived cells was assessed by staining peripheral blood cells, bone marrow spleen and thymus cells with an FITC-conjugated anti-Ly5.1 mAb kindly provided by Dr. G. Spangrude (Rocky Mountain Laboratory, Hamilton, MT) and analysis by flow cytometry. Peripheral blood samples were obtained via tail vein puncture and depleted of erythrocytes by incubating them for 10 minutes on ice in the presence of 4 volumes of sterile 1M NH_4Cl solution. Thymic and splenic cells were obtained by teasing these organs apart and passaging the cells through an 21 gauge needle in order to obtain a single cell suspension. Bone marrow cells were flushed from femurs and tibia using an 21 gauge needle and alpha medium and 5% FCS (Stem Cell Technologies). In some experiments phenotypic analysis of Ly5.1 donor derived peripheral blood leukocytes was achieved through double antibody labeling with Ly5.1-FITC in combination with one of Gr-1-PE (from hybridoma RB6-8C5 provided by Dr. G. Spangrude) to identify granulocytes, Mac-1-PE (from hybridoma M1/70, ATCC, Rockville, MD) to identify macrophages, B220-PE (from hybridoma RA3-6B2, Dr. G. Spangrude) to identify B lymphocytes or Ly1-PE (from hybridoma TIB104, ATCC) to identify T lymphocytes as described below. Levels of expression of the transferred CD24 gene in cells from repopulated mice were assessed by staining cells from hemopoietic tissues with anti-CD24 tetrameric antibody complexes and R-PE as described above. CD24 expression among peripheral blood leukocytes was analyzed by staining peripheral blood samples with anti-CD24/R-PE tetramers in combination with FITC labeled Gr-1 to identify granulocytes, Mac-1 for macrophages, Ly-1 for T cells and B220 for B cells. CD24 expression on peripheral blood erythrocytes was assessed by staining peripheral blood samples prior to exposure to NH_4Cl. The expression of the transferred CD24 gene on marrow stem cell candidates defined by the Sca^+Lin^- cell surface phenotype was achieved through multiple antibody labeling of bone marrow cells with anti-CD24/R-PE

tetramers in combination with Gr-1-FITC, Mac-1-FITC, B220-FITC, Ly-1-FITC and Sca-1-Cy-5 (E13-161.7, Dr. G. Spangrude).

2.3.3. FACS sorting

Cells were sorted on a FACStar+ (Beckton Dickinson and Co., San Jose, CA) equipped with a 5W argon and a 30mW helium neon laser. Cells were collected in sterile eppendorf vials in alpha medium with 50% FCS.

CHAPTER 3

SELECTION OF RETROVIRALLY TRANSDUCED HEMOPOIETIC CELLS USING CD24 AS A MARKER OF GENE TRANSFER

The results presented in this Chapter have been described in:
Pawliuk, R., R. Kay, P. M. Lansdorp, and R. K. Humphries. 1994. Selection of retrovirally transduced hematopoietic cells using CD24 as a marker of gene transfer. Blood 84: 2868-2877.
Pawliuk, R., R. Kay, P. M. Lansdorp and R. K. Humphries. 1995. CD24 as a marker gene for the selection and tracking of retrovirally transduced stem cells. In: Molecular Biology of Hemoglobin Switching. Eds. G. Stamatoyannopoulos and A. Nienhuis. Vol. 2 of the Proceedings of the 9th Conference on Hemoglobin Switching, pp. 231-247.

3.1. Introduction

The development of recombinant retroviruses as vectors for gene transfer has provided a powerful tool to address current questions regarding HSC numbers, biological potential, kinetics and regulation. Moreover, recombinant retroviruses have played a pioneering role in the field of gene therapy. However, the power of retroviral gene transfer is currently limited by the poor infection efficiency of HSCs due to their rarity, cycling status and paucity of viral receptors.

To aid in the identification, enrichment and tracking of transduced target cells, a variety of selectable markers have been incorporated into retroviral vectors. The most widely used of these have been intracellular components which confer resistance to toxic compounds such as neomycin (23, 35, 205), hygromycin (262, 312, 313), chloramphenicol (314), methotrexate (216, 234, 263, 315), mycophenolic acid (316), or various chemotherapeutic agents (266, 317-319). However, use of these markers in selection protocols carry disadvantages that include non-specific drug toxicity and difficulties in quantifying expression levels. The bacterial β-galactosidase gene (lacZ) and the human placental alkaline phosphatase gene have also been employed to select transduced cells in vitro (268, 269) and as reporter molecules both in vitro (268, 270, 320, 321) and in vivo (322-325). However, the presence of an endogenous mammalian lysosomal β-galactosidase and problems in achieving adequate levels of expression of the exogenous β-gal gene have limited its effective use.

Genes encoding cell surface antigens have also been utilized as selectable markers of gene transfer to fibroblasts (271, 274, 319) and more recently to human peripheral blood lymphocytes (326). The use of such genes offers several potentially significant advantages including: the rapid and quantitative detection of transferred gene expression in the desired target cell population by flow cytometry; the efficient and non-toxic selection of transduced target cells by FACS or other immuno-based selection techniques; and the tracking of transduced cells and their

progeny both in vitro and in vivo. However, at the time that the work described in this thesis was initiated the applicability of this approach to primitive hemopoietic cells had not yet been demonstrated. Moreover, the genes which had been used as selectable markers are relatively large, leaving limited space in the retroviral vector for other genes of interest.

Recently, cDNAs encoding the human hemopoietic cell surface antigen CD24, and its murine homologue the heat stable antigen (HSA) have been cloned (299, 327). The function of these molecules is not yet resolved although CD24 has been associated with activation and differentiation events in B cells as well as the oxidative burst response in granulocytes (299, 327), and roles for HSA in T cell development and activation have been suggested (328, 329). Both antigens are glycoproteins attached to the outer surface of the plasma membrane by a glycosyl phosphatidylinositol lipid anchor and are expressed on multiple lineages of hemopoietic cells. The mature peptides are only 30-35 amino acids in size with the entire coding region being encompassed within an approximate 240 bp DNA fragment. In addition, the mature CD24 and HSA proteins share only limited sequence homology (57%) with one another and antibodies to CD24 and HSA are not cross reactive (300, 327). These features of small coding size, potential for cell surface expression on multiple hemopoietic lineages and limited homology, suggested to me that HSA and CD24 would be possible candidates for selectable markers of gene transfer to heterologous hemopoietic cells. In the work presented in this Chapter I demonstrate the feasibility of utilizing CD24 for the identification and selection of retrovirally transduced primary cells of the murine hemopoietic system including those with long-term lympho-myeloid repopulating ability.

3.2. RESULTS

3.2.1. The CD24 viral vector

To explore the possible use of CD24 as a selectable cell surface marker, the retroviral vector depicted in Figure 3.1 was constructed. This vector contains the minimal 240 bp CD24 cDNA sequence encompassing the complete coding region under the control of the MPSV long terminal repeat enhancer and promoter regulatory elements. For initial feasibility studies the neomycin resistance gene under the control of the thymidine kinase gene promoter was also included in the vector to aid in viral titering and to provide an independent means to assess gene transfer. A CD24 viral producer was generated using the ecotropic GP+E-86 packaging line and had a titre of ~5 x 10^5 CFU/ml as assessed by neo^R gene transfer to NIH-3T3 cells.

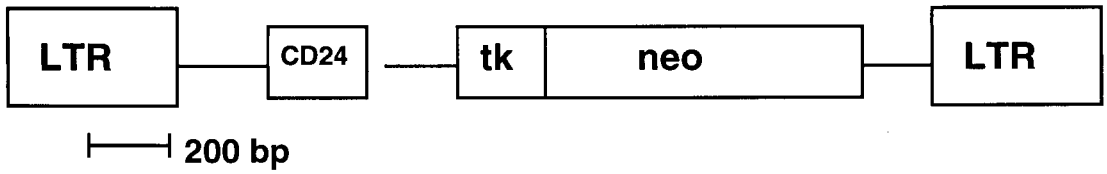

Figure 3.1. Schematic of the JZenCD24tkneo provirus. It incorporates a 240-bp portion of the CD24 cDNA encompassing the complete coding region; a thymidine kinase-neomycin resistance cassette (tkneo) from pMC1neo; and LTR sequences from the MPSV as described in Material and Methods in Chapter 2.

Transfer of the CD24 gene to hemopoietic cells was initially evaluated in IL-3-dependent murine Ba/F3 cells. Approximately 80% of Ba/F3 cells were found to express high levels of surface CD24 antigen after 2 days of co-cultivation with CD24 viral producers and a further 5 days of growth in the absence of G418 selection (Figure 3.2). Similar levels of expression were detected as early as 12 hours post infection (data not shown).

3.2.2. FACS selection of CD24-transduced in vitro clonogenic progenitors and CFU-spleen (CFU-S)

Day 4 5-FU bone marrow cells were co-cultivated with CD24 viral producers for 24 hours and recovered non-adherent cells were cultured for a further 48 hours

to allow expression of the transferred CD24 gene prior to flow cytometric analysis and cell sorting. As shown in a representative FACS profile for one experiment,

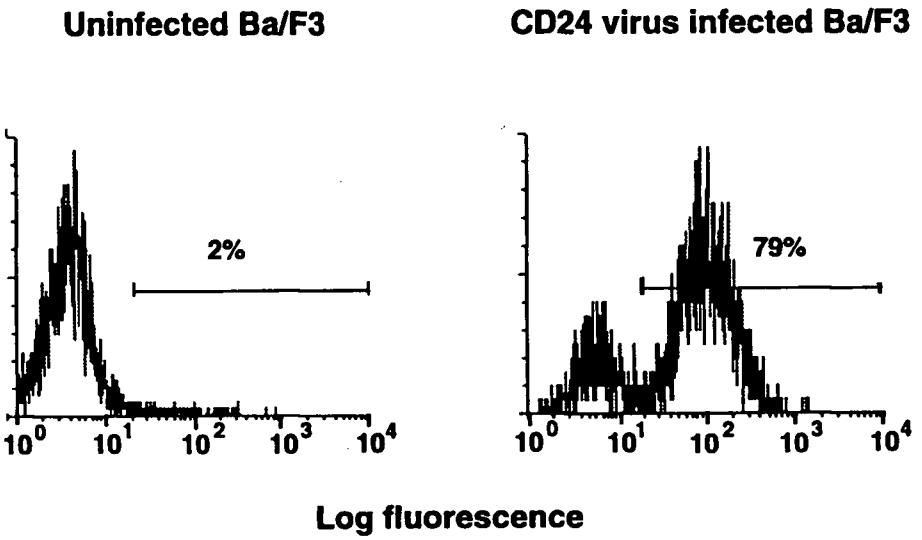

Figure 3.2. Flow cytometric analysis of CD24 expression by Ba/F3 cells previously cocultivated with CD24 viral producer cells and then cultured for 5 days in the absence of G418. Infected and control (uninfected) cells were stained with an anti-CD24-based tetramolecular antibody complex coupled to R-PE as described in Materials and Methods (Chapter 2).

approximately 1/3 of the bone marrow cells recovered after co-cultivation infection without prior growth factor prestimulation were positive for the CD24 cell surface antigen (see Figure 3.3 top panel). In 3 experiments 96±3.6% of in vitro clonogenic progenitors recovered in the $CD24^+$ fraction were G418-resistant compared to 35-69% in the unsorted marrow population (see Figure 3.3 bottom panel). Some G418-resistant progenitors (12-27%) were also detected in the $CD24^-$ fraction, likely as a result of overlap between the $CD24^+$ and $CD24^-$ cell populations and the relatively low sorting threshold chosen.

$CD24^-$ and $CD24^+$ fractions were also assayed for day 12 CFU-S. A summary of findings from 3 experiments are presented in Table 3.1. All spleen colonies derived from the $CD24^+$ fraction (37 of 37 analyzed) were positive both for

proviral DNA sequences and significant levels of CD24 expression above background staining (range 5-79% of cells analyzed). The observation that not all cells within a colony expressed detectable levels of CD24 is likely a result of some admixture of contaminating inter-colony cells as well as differences in the absolute level of CD24 expression on cells within each colony. In contrast, only approximately 50% of spleen colonies derived from the unsorted marrow (13 of 30) showed evidence of gene transfer by Southern blot analysis. Furthermore, only 4 of 13 marked colonies were positive for expression of the transferred CD24 gene. Surprisingly, a significant proportion of spleen colonies derived from the $CD24^-$ fraction (15 of 26) were also found to contain intact provirus although only 3 expressed detectable levels of CD24. Thus retrovirally-transduced day 12 CFU-S can be successfully enriched based on their immediate expression of a transduced CD24 gene. In addition, the differentiating day 12 progeny of the CFU-S thus selected also show maintained expression of the transduced CD24 gene in vivo.

3.2.3. Selection by FACS of CD24-virus-infected CRU

Subsequent experiments were conducted to determine the feasibility of selecting CD24 transduced CRU by FACS. In an effort to facilitate gene transfer to repopulating cells, day 4 5-FU bone marrow cells were prestimulated with growth factors for 48 hours prior to co-culture with CD24 viral producer cells. The CD24 expression profiles of non-adherent cells recovered 48 hours after the co-culture period for 2 experiments are shown in Figure 3.4. Greater than 50% of cells were $CD24^+$ using this infection protocol compared to 33% with no pre-stimulation (see Figure 3.3 top panel). Unsorted and sorted $CD24^-$ or $CD24^+$ cells (Figure 3.4 expt 1) from Ly5.1 donor mice were injected into lethally irradiated Ly5.2 recipients under competitive repopulating conditions. In preliminary studies injection of limiting numbers of bone marrow cells recovered following the sorting procedure revealed a competitive repopulating cell frequency of approximately $1/3 \times 10^4$. Therefore, recipients were transplanted with 10^4 or 4×10^4 unsorted or sorted cells

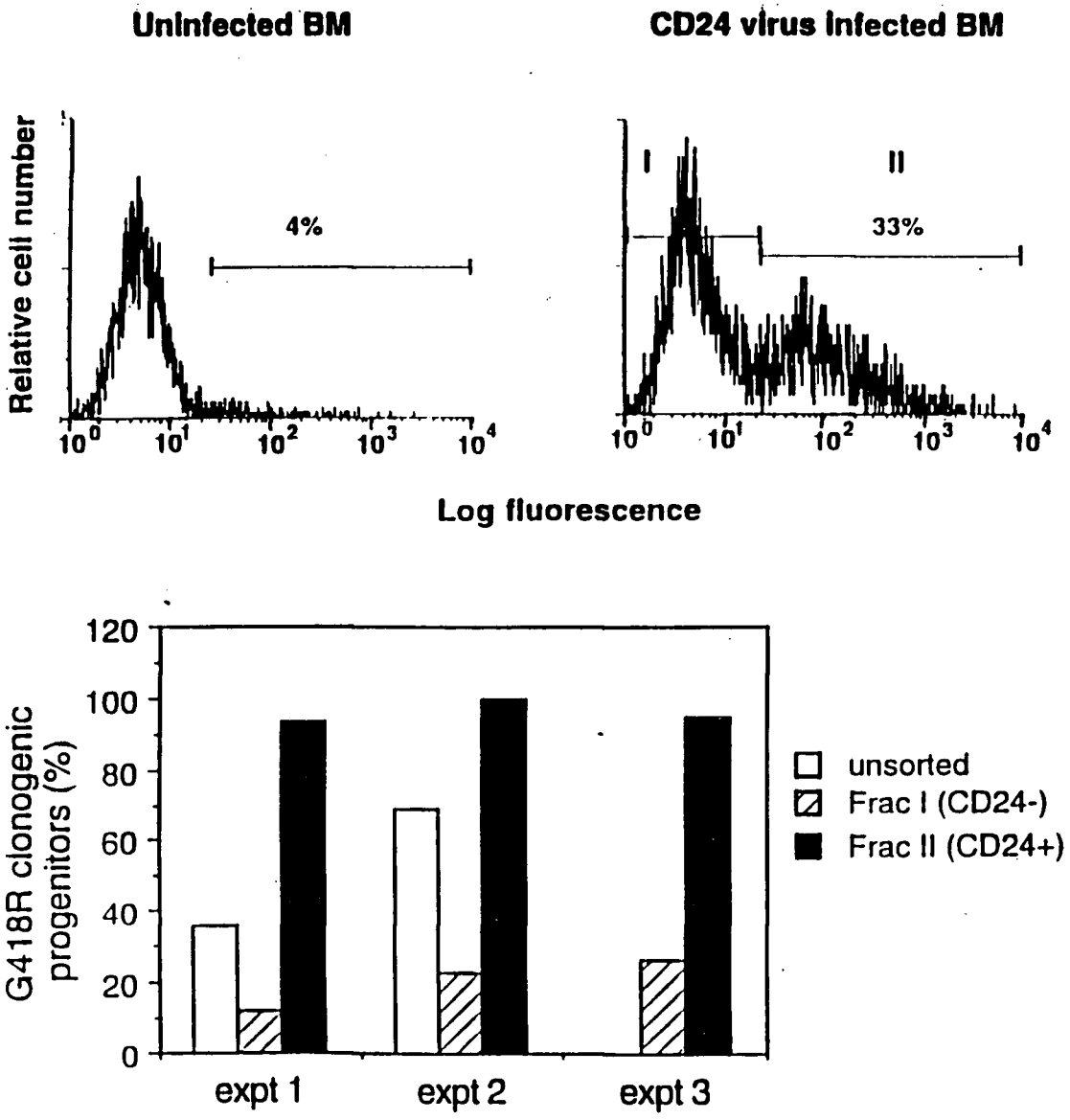

Figure 3.3. FACS selection of CD24 virus-infected in vitro clonogenic progenitors and day 12 CFU-S. (Upper panel) Expression of CD24 on day 4 5-FU BM cells 48 hours after coculture with CD24 viral producers compared to uninfected day 4 5-FU BM cells. Cells were stained with anti-CD24/R-PE tetrameric antibody complexes and analyzed by flow cytometry. Infected cells were sorted into $CD24^-$ (I) and $CD24^+$ (II) fractions by FACS and assayed for in vitro clonogenic progenitors and day 12 CFU-S. (Lower panel) Proportion of G418-resistant in vitro clonogenic progenitors in the $CD24^-$ (I), $CD24^+$ (II) and presort BM populations for three independent experiments. Presort BM cells were not obtained in experiment 3 because of lack of cells. No G418 resistant clonogenic progenitors were observed in uninfected control BM cells. Results of analysis of day 12 CFU-S-derived spleen colonies are presented in Table 3.1.

Table 3.1. Proviral Integration and CD24 Expression on Cells from Individual Spleen Colonies Derived from Sorted and Unsorted Bone Marrow Cells Following CD24 Virus Infection.

Cells Transplanted	Total No. Colonies Analyzed	No. Colonies Positive for CD24 Expression	No. Colonies Positive for Proviral Integration
Unsorted	30	4	13
Frac II (CD24+)	37	37	37
Frac I (CD24-)	26	3	15

Recipient mice were transplanted with 1 x 10^3 to 1 x 10^4 bone marrow cells, which had been co-cultured with CD24 viral producer cells, from one of each of the presort, $CD24^-$ or $CD24^+$ marrow fractions as described in the legend to Fig 3.3. Well isolated spleen colonies were dissected and analyzed for CD24 expression using flow cytometry and for proviral integration using Southern blot analysis. The table represents data accumulated over 3 separate experiments.
* A spleen colony was concluded to be positive for CD24 expression if >5% of the cells bound significant levels of the anti-CD24 tetramolecular antibody complex.

in an effort to minimize the likelihood of $CD24^-$ CRU contributing to the transplant due to contamination of the $CD24^+$ fraction. Based upon the proportion of uninfected control cells found within the $CD24^+$ sorting window (0.7%, Figure 3.4 expt. 1) our calculations predict that for every 2 x 10^4 cells sorted less than 0.005 $CD24^-$ (i.e. contaminating) CRU would be found within the positive sort window due to occurrences such as non-specific binding of the CD24/RPE tetrameric antibody complex.

For recipients transplanted with cells from the $CD24^+$ fraction, 10 of 11 were found to be reconstituted with provirus-containing CRU 5 weeks post-transplantation (see Table 3.2). Expression of CD24 on peripheral blood leukocytes of these mice ranged from 7-24%. In 3 mice, evidence of the same proviral integration fragments in cells of both myeloid and lymphoid tissues suggested gene transfer to a totipotent repopulating cell. Retrovirally-infected CRU were also found in the $CD24^-$ fraction with 8 of 11 mice showing evidence of

proviral marking in either bone marrow and/or thymus DNA. However, none of these 8 recipients showed detectable levels of CD24 expression on peripheral blood leukocytes. Of 5 recipients injected with unsorted bone marrow cells, all 5 were repopulated with retrovirally marked cells but only 2 showed detectable levels of CD24 expression in peripheral blood cells. These results demonstrate that, as for day 12 CFU-S CD24 expression in combination with FACS can be used for the selection of retrovirally transduced CRU whose progeny maintain expression of the transferred CD24 gene for at least 5 weeks post transplantation. These findings were confirmed and extended in a second experiment in which retrovirally infected Ly5.1 bone marrow cells were sorted into $CD24^-$ as well as $CD24^{+low}$, $CD24^{+med}$, and $CD24^{+high}$ fractions 48 hours post infection (see Figure 3.4 expt 2). Limiting numbers (1×10^4) of cells from each of the 4 fractions were then injected into lethally irradiated Ly5.2 recipients under competitive repopulating conditions. Based upon the sorting windows chosen (Figure 3.4 expt 2) our calculations suggest that for every 2×10^4 cells sorted, less than 0.03, 0.01 and 0.0001 $CD24^-$ CRU were sorted, respectively in the $CD24^{+low}$, $CD24^{+med}$, and $CD24^{+high}$ windows, due to occurrences such as non-specific antibody staining. In these experiments, recipients were analyzed for evidence of Ly5.1 donor cell-derived repopulation and CD24 gene expression at 5 and 16 weeks post transplant, as well as for expression of the neo^R gene and evidence of proviral marking at 16 weeks post transplant.

Mice transplanted with $CD24^{+low}$, $CD24^{+med}$, or $CD24^{+high}$ cells all showed significant levels of multi-lineage (i.e., lymphoid and myeloid) Ly5.1 donor-cell-derived repopulation and all recipients of $CD24^+$ selected marrow cells again showed evidence of proviral marking in bone marrow and/or thymus DNA (Figure 3.5). In 6 of these mice (mouse m1, $CD24^{+low}$ fraction; mouse m1, m3 and m4, $CD24^{+med}$ fraction; and mice m1 and m2, $CD24^{+high}$ fraction; Figure 3.5) retroviral marking patterns observed by Southern blot analysis of bone marrow and

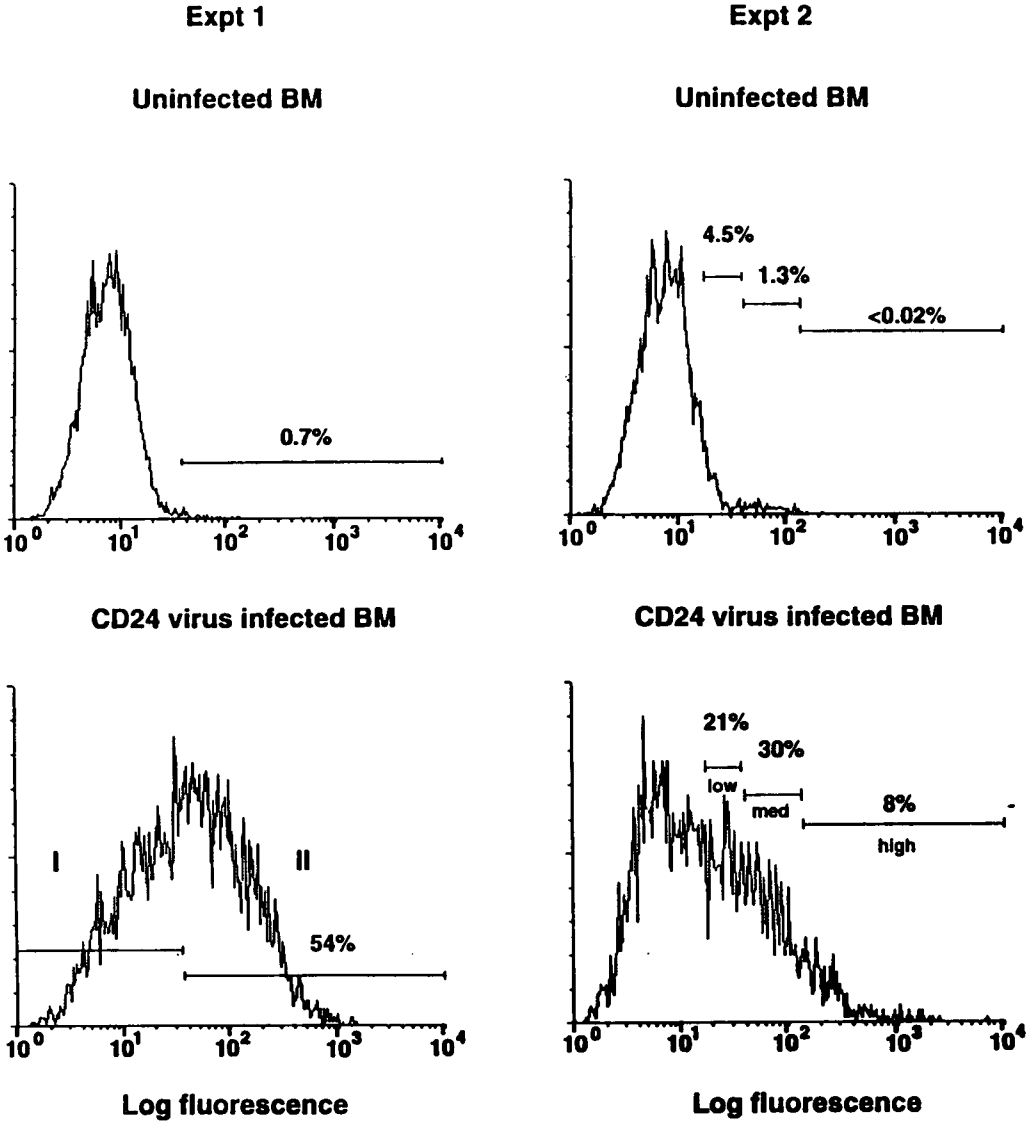

Figure 3.4. Selection of CD24 virus-infected CRU by FACS. The CD24 expression profiles of day 4 5-FU uninfected control marrow cells (top panels) and day 4 5-FU BM cocultured with CD24 viral producer cells (bottom panels) 48 hours postinfection are shown for two independent experiments. Cells were stained with anti-CD24/R-PE tetrameric antibody complexes and analyzed by flow cytometry. In experiment 1, infected cells were sorted into $CD24^-$ (I) and $CD24^+$ (II) fractions and injected into lethally irradiated recipient mice under competitive repopulating conditions at 1 x 10^4 to 4 x 10^4 cells per mouse. Mice were analyzed 5 weeks post transplantation for both CD24 cell surface expression on peripheral blood leukocytes and proviral integration in BM and thymus (results are shown in Table 3.2). For experiment 2, BM cells in presort, $CD24^-$, $CD24^{+low}$, $CD24^{+med}$ and $CD24^{+high}$ expressing fractions were injected into lethally irradiated recipient mice under competitively repopulating conditions at 1 x 10^4 cells per mouse. Mice were analyzed 16 weeks posttransplantation for CD24 cell surface expression on their peripheral blood cells and proviral integration in BM and thymus (results are shown in Table 3.3 and Figure 3.5). Percentages of cells in the sort windows are indicated.

thymus were clearly indicative of gene transfer to a totipotent long-term repopulating cell. The degree of Ly5.1 donor cell repopulation among these mice strongly correlated with the intensity of proviral marking (Figure 3.5), an observation consistent with gene transfer to all repopulating cells in the $CD24^+$ fractions. Although CD24 expression was detected in the majority of recipients of $CD24^+$ selected marrow at 5 weeks post transplant, expression levels were poorly maintained at the later 16 week time point analyzed in this experiment (Figure 3.6). In this case, in only 2 of 8 mice repopulated with cells from the $CD24^+$ fractions (mice m2 and m3, $CD24^{+med}$ cells) were $CD24^+$ peripheral blood (4.3% and 17.1%, respectively) or bone marrow cells (3.4% and 42%, respectively) still present. In addition, $G418^R$ clonogenic progenitors were detected in the marrow of these two mice (3% and 68% respectively). In the other 6 mice, neither $CD24^+$ nor $G418^R$ cells were detected although provirally marked cells were present in the bone marrow and/or thymus of all of these animals (data not shown).

The proportion of recipient mice showing expression of the transferred CD24 gene on peripheral blood leukocytes also decreased with time post transplant in a third experiment in which recipients were transplanted with 1×10^5 bone marrow cells from unsorted or $CD24^-$ or $CD24^+$ sorted fractions. Recipients were assessed for CD24 expression at 8 and 32 weeks post transplant. While the vast majority of recipients transplanted with $CD24^+$ sorted marrow showed expression of the transferred CD24 gene at 8 weeks (14 of 15 mice) only a fraction were found to continue to express CD24 when analyzed at 32 weeks (3 of 13) despite the detection of intact provirus in all mice at this time point. Representative flow cytometric profiles for 1 of these mice are shown in Figure 3.7.

One of 8 mice transplanted with unselected marrow showed the presence of CD24 on peripheral blood leukocytes at 8 weeks post transplant although this mouse was found to be negative for CD24 expression at the later time point. No mice transplanted with $CD24^-$ sorted cells demonstrated CD24 expression at either of

Table 3.2. Proviral Integration and CD24 Expression on Cells from Competitively Repopulated Mice Assessed 5 Weeks Post -Transplantation (Expt. 1).

	Cells Transplanted		
	Unsorted	Sort Fraction I (CD24-)	Sort Fraction II (CD24+)
Reconstitution with Retrovirally Marked Cells*	5/5	8/11	10/11
CD24 Cell Surface Expression in P.B. Cells (>5%)‡	2/5	0/11	10/11

Shown are the number of mice found to be positive for reconstitution with retrovirally marked cells or CD24 cell surface expression on peripheral blood (P.B.) leukocytes over the total analyzed at 5 weeks post transplantation.

* Retroviral marking was assessed by Southern blot analysis of bone marrow and thymus from each transplanted recipient. Blots were separately probed with ^{32}P labeled fragments of the neo^R gene and CD24 cDNA with identical results.

‡ Expression of the CD24 antigen on peripheral blood leukocytes was assessed by staining peripheral blood samples depleted of erythrocytes with anti-CD24/R-PE tetrameric antibody complexes and analysis by flow cytometry.

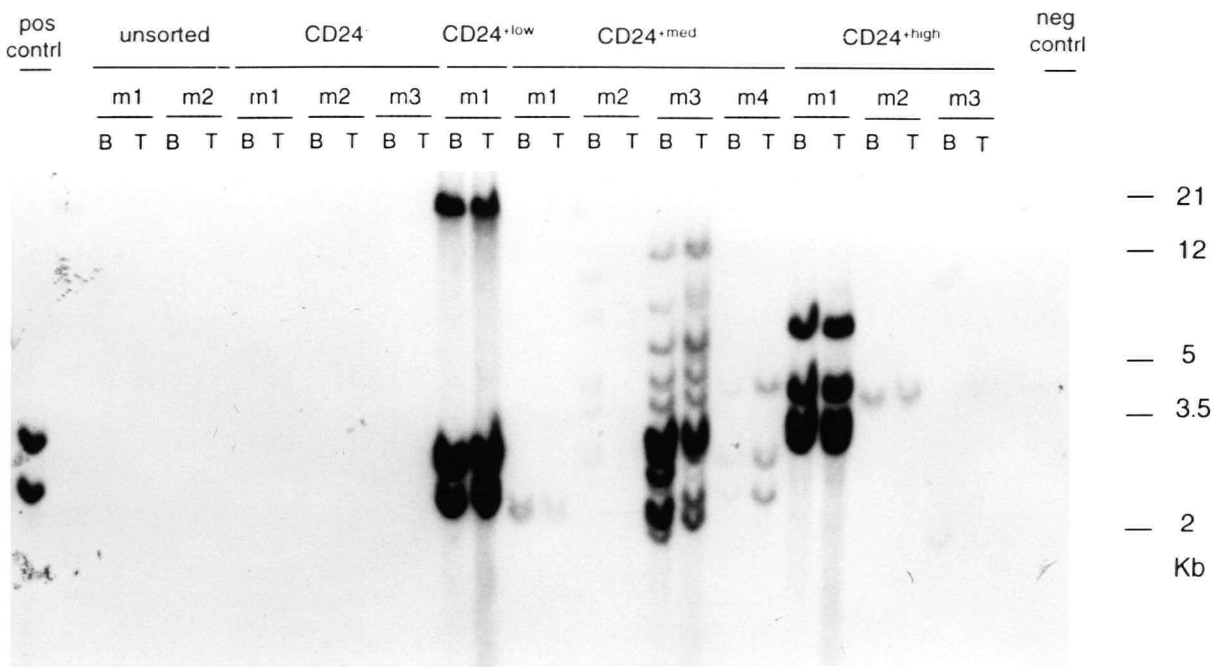

Figure 3.5. Hemopoietic reconstitution from CD24 retrovirus-infected competitive repopulating cells as assessed by Southern blot analysis of proviral integration in BM (B) and thymus (T) 16 weeks posttransplantation. Recipients received 1 x 10^4 cells of CD24 virus-infected marrow from presort, $CD24^-$, $CD24^{+low}$, $CD24^{+med}$, or $CD24^{+high}$ fractions as shown for Expt 2 Figure 3.4. Individual recipient mice are labeled as m1-m4. DNA (20 μg) from each tissue sample was digested with Eco RI, an enzyme that cuts once within the CD24 provirus sequence. Shown are results of a blot probed with a ^{32}P-labeled fragment of the neo^R gene; identical results were observed using the CD24 cDNA as a probe. The positive control represents DNA obtained from a retrovirally infected Ba/F3 clone harboring two copies of provirus.

the time points analyzed in this experiment (see Figure 3.6). Additional analysis of bone marrow and thymus DNA from these mice revealed no gross rearrangements in proviral structure to account for the lack of expression in these recipients. Lack of CD24 expression also did not appear to be a result of promoter interference since $G418^R$ clonogenic progenitors were detected only in those mice showing CD24 expression on peripheral blood leukocytes. The proportion of $G418^R$ clonogenic progenitors tended to correlate with the proportion of $CD24^+$ peripheral blood leukocytes in recipient mice, ranging from 3% to 68%.

3.3. Discussion

In the work presented in this Chapter, I have tested the utility of a cDNA encoding CD24, a human cell surface antigen, for the post-infection selection of hemopoietic cells transduced with a retrovirus containing this cDNA. FACS analysis in combination with functional studies revealed that under the conditions used, Ba/F3 cells, a factor-dependent hemopoietic cell line, as well as primary marrow in vitro clonogenic progenitors, day 12 CFU-S and, most significantly, the earliest cells capable of competitive long-term hemopoietic repopulation all expressed CD24 within 48 hours of termination of the infection procedure. Southern blot analysis of bone marrow and thymus cells from mice competitively repopulated with limiting numbers of selected $CD24^+$ cells demonstrated proviral integration in virtually all animals in which donor cells were detected. Because long-term repopulating cells are such rare cells and have been difficult to purify to homogeneity, it has been difficult to analyze the diversity in properties and behavior of individual cells of this type, particularly following their transplantation in vivo. The ability to obtain, prior to transplant, a population of cells that are 100% provirally marked should now greatly enhance the power of studies aimed at addressing such questions. In addition this type of strategy should be useful as a preclinical model for the development of more effective gene transfer strategies to long-term human repopulating stem cells for use in gene therapy.

The recovery of competitive repopulating cells in the top 8% of CD24 expressing cells provides indirect evidence that the MPSV LTR enhancer and promoter are able to drive high level gene expression in the most primitive hemopoietic cells present in adult marrow tissue. The use of such a selectable marker should be useful for optimizing vectors to achieve high and sustained levels of transferred gene expression in very primitive hemopoietic cells and ultimately for studies aimed at the genetic manipulation of stem cell behavior. An interesting finding of this study was that CD24 expression observed in primitive retrovirally

infected cells at the time of selection was also maintained in their progeny although this did decrease with time after transplantation. The most dramatic evidence of continued CD24 expression was seen at the level of CFU-S; 100% of the retrovirally-infected day 12 CFU-S in the sorted $CD24^+$ fraction gave rise to spleen colonies which were also positive for CD24 expression. In contrast, despite the detection of intact provirus in approximately half of the CFU-S in the initial (unsorted) or $CD24^-$ fraction, only a minority of these yielded colonies of cells expressing detectable levels of CD24. One explanation for this observation is that the expression of the transferred CD24 gene is integration site dependent. Similarly, most mice transplanted with limiting numbers of sorted $CD24^+$ CRU were found to have CD24 expressing peripheral blood leukocytes 5-6 weeks post transplantation (expt. 1), whereas such cells were not observed in animals repopulated with CRU from the $CD24^-$ fraction despite the presence of provirus in the marrow and/or thymus of a number of these. Sustained expression of CD24 in recipients of initially selected $CD24^+$ CRU was also observed 4 months post-transplantation although in a small proportion of mice despite the persistence of retrovirally marked cells in myeloid and/or lymphoid tissues in all. Neither gross rearrangement in proviral structure (as assessed by Southern blot analysis) or promoter interference between the CD24 and neo^R genes could account for the lack of CD24 expression observed in these mice. The loss of CD24 expression in some long term repopulated mice transplanted with $CD24^+$ selected cells is suggestive of a shutdown of exogenous promoter activity *in vivo*, a phenomenon which has been previously reported (215, 251). Such shutdown of promoter activity may be related to the methylation status of the regulatory elements (257, 330, 331). However, others have reported the continued expression of transferred genes such as human CD8 (291) and the human glucocerebrosidase gene (332) for long periods post transplantation. It is important to note, however, that in these studies recipients were purposely transplanted with only one or few stem cells to enable

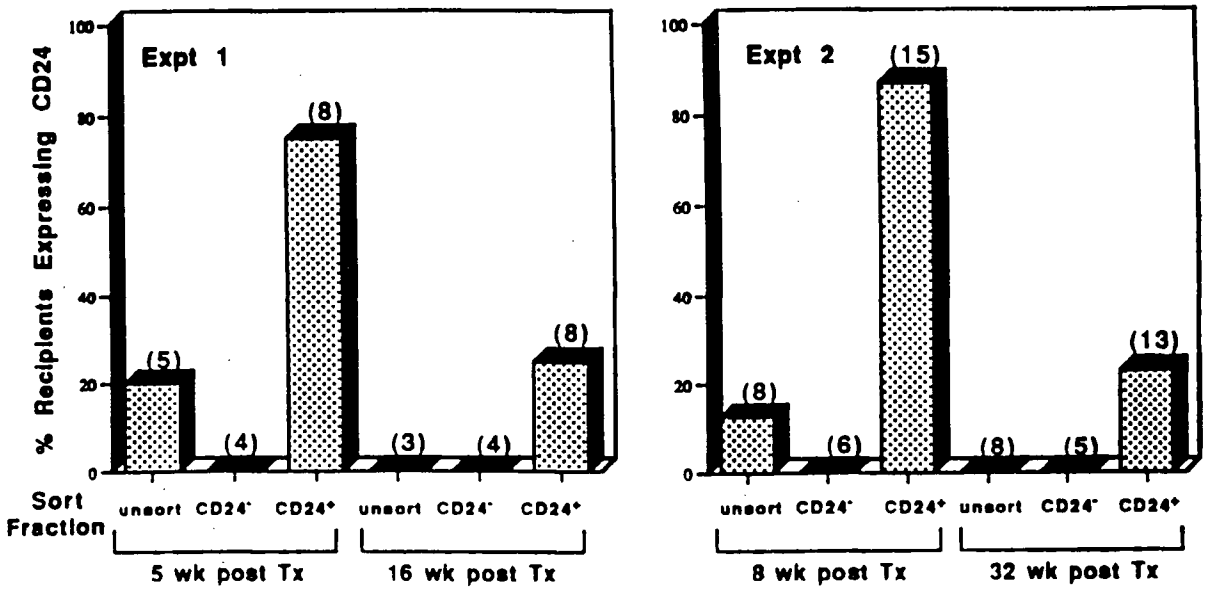

Figure 3.6. Proportion of recipient mice found to be expressing the transferred CD24 gene at early and late time points post transplant. Ly5.2 recipient mice were transplanted with 1 x 10^4 to 1 x 10^5 retrovirally infected Ly5.1 BM cells from the sort fractions indicated. Erythrocyte-depleted peripheral blood samples were tested 5-32 weeks post transplantation with anti-CD24 tetrameric antibody complexes/R-PE and analysis by flow cytometry. Animals were scored positive if > 2% of cells were $CD24^+$. The total number of animals tested is shown in parentheses and the proportion positive for CD24 expression indicated in percent. Repopulation of recipients with Ly5.1 donor-derived cells was assessed by staining with FITC-conjugated Ly5.1 antibody and analysis by flow cytometry.

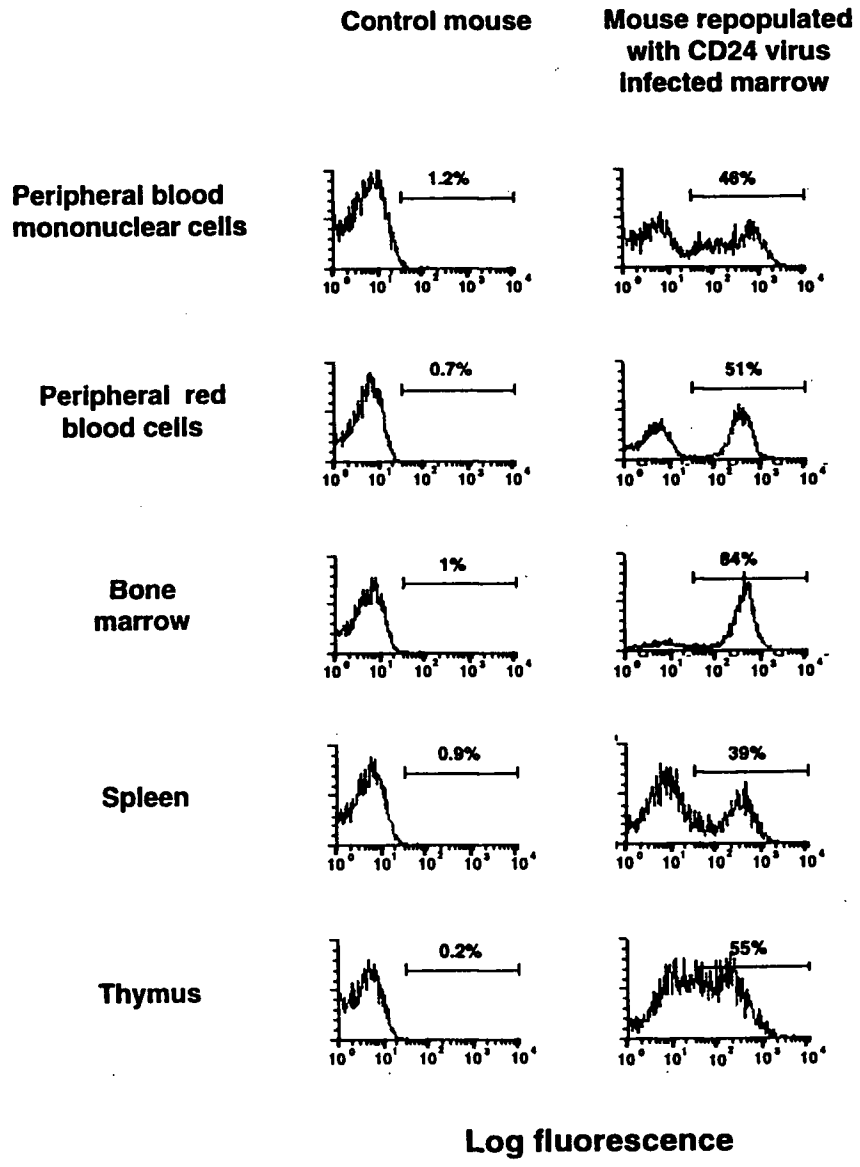

Figure 3.7. Flow cytometric analysis of CD24 expression in the hemopoietic tissues of a mouse repopulated with CD24 retrovirus-infected BM 4 months post transplantation. Cells were stained with anti-CD24/R-PE tetrameric antibody complexes. To assess CD24 expression on red blood cells samples were not depleted of erythrocytes prior to the staining procedure.

the analysis of gene expression at the clonal level whereas in the studies mentioned above recipients were not transplanted at limiting dilution. My results would suggest that at least for LTR controlled gene expression the phenomenon of promoter shutdown may be more widespread than previously appreciated. Moreover, it may be that mice repopulated with limiting numbers of $CD24^+$ selected cells continue to express the transferred CD24 gene but at levels below that which can be detected with FACS. Because of the ease and sensitivity of methods for monitoring transduced CD24 expression in peripheral blood cells, this vector construct should be well suited for further studies of vector modifications that may abrogate promoter shutdown in primitive hemopoietic cells and their long-term progeny.

No gross abnormalities in hemopoiesis were observed in mice expressing high levels of CD24 following transplantation with CD24 virus-infected marrow compared to normal control animals. Further, sustained expression in recipient mice at least 4 months post transplantation demonstrates that the use of such a foreign antigen as a retroviral marker is compatible with long term expression in hemopoietically reconstituted lethally irradiated recipients.

The work presented in this Chapter has demonstrated the feasibility of utilizing the CD24 cell surface antigen as a selectable marker and a reporter molecule in primary hemopoietic cells including the most primitive elements of this system. This technique should prove useful as a method for increasing the power of retroviral marking studies, rapidly testing various retroviral infection protocols and the identification of regulatory elements which optimize gene expression in primitive hemopoietic stem cells or other target cells of interest.

CHAPTER 4

HIGH LEVEL RECONSTITUTION WITH PRESELECTED HEMOPOIETIC CELLS EXPRESSING A TRANSDUCED GENE ENCODING A CELL SURFACE ANTIGEN.

The results presented in this Chapter have been described in:
Pawliuk, R., and R. K. Humphries. High level reconstitution with preselected hemopoietic cells expressing a transduced gene encoding a cell surface antigen. Manuscript in preparation.

4.1. Introduction

Gene therapy represents an attractive strategy for the treatment of various human heritable disorders, cancer and Acquired Immune Deficiency Syndrome (AIDS). Successful gene therapy of hematological disorders requires that two major goals be met: (1) efficient and stable transduction of the hemopoietic stem cell (HSC) and (2) appropriate sustained expression of transduced gene(s) in the cells of interest. Although significant progress has been made towards achieving these goals, efficient high level retroviral gene transfer to long term repopulating stem cells remains a challenge, likely due to a multiplicity of factors including low levels of viral receptors and the largely quiescent nature of HSCs.

To overcome these difficulties, a number of strategies involving the incorporation of selectable marker genes into retroviral vectors have been developed to enable the enrichment of transduced target cells. The most widely used of these have been those which confer resistance to toxic compounds such as neomycin (23, 35, 205) or hygromycin (262, 312). The analysis of murine recipients engrafted with bone marrow cells subjected to drug selection either before (215, 227, 260) or after (266) transplant has shown that the proportion of provirally marked cells in the recipient can be increased using this strategy. More recently, a number of cDNAs encoding cell surface antigens, including the human low affinity nerve growth factor receptor (273, 326), Thy-1 (279), and MDR-1 (multi-drug resistance-1) (272), have been utilized as dominant selectable markers. Richardson et. al. used a cDNA encoding MDR-1 as a selectable marker to demonstrate that preselection of retrovirally transduced midgestational fetal liver cells leads to an increase in the proportion of circulating peripheral blood leukocytes expressing MDR-1 as compared to mice receiving unselected marrow (333). However, despite the potential that these procedures possess, their effectiveness for achieving high level hemopoietic reconstitution with exclusively provirally marked cells has not yet been fully explored. Moreover, despite

increasing interest in genetically manipulating HSC behavior, little is known regarding regulatory elements which maximize the expression of transferred genes in these cells. Thus, methods enabling the efficient selection, as well as the tracking and quantification of transferred gene expression in primitive hemopoietic cells and their progeny for extended periods of time in vivo are required.

I have examined these issues in a system utilizing the human cell surface antigen CD24 as a dominant selectable marker in combination with FACS. In Chapter 3 I demonstrated that retrovirally transduced totipotent in vivo repopulating stem cells could be selected for on the basis of CD24 expression within 48 hours of termination of the infection protocol (278). In the work described in this Chapter, this CD24 selection approach is shown to enable the almost exclusive regeneration of hemopoiesis in myeloablated recipient mice with provirally marked cells. The contribution of individual HSCs to hemopoiesis was analyzed by proviral integration analysis. Moreover, persistent expression of the transferred CD24 gene was observed in a significant proportion of $Sca^{+}Lin^{-}$ bone marrow cells (a subpopulation known to be enriched for cells with long term in vivo repopulating ability), peripheral blood leukocytes, red blood cells, spleen, thymus and whole bone marrow cells for a minimum of 6 months post transplant. Finally, this study reveals intriguing evidence of vector-based differences in the ability to drive persistent expression of transferred genes in vivo.

4.2. Results

4.2.1. Viral vectors and experimental design

In the course of these experiments two vectors based on different viral backbones (Figure 4.1 upper panel) were employed. In both vectors, the minimal 240bp coding region of the CD24 cDNA was placed under the control of the viral LTR regulatory elements. Both vectors also contained a cDNA encoding resistance to the neomycin analog G418. In the JZenCD24tkneo vector (hereafter referred to as JZenCD24) the neomycin resistance cassette is driven from the thymidine

kinase gene promoter, while in MSCVneoIRESCD24 (hereafter referred to as MSCVCD24) both the neomycin resistance and CD24 cDNAs are driven from the viral LTR due to the inclusion of an internal ribosomal entry site derived from the 5' untranslated region of the encephalomyocarditis virus. Both the JZenCD24 and MSCVCD24 viral producers yielded titres of approximately 5 x 10^5 CFU/ml as assessed by transfer of neo^R to NIH-3T3 cells with no helper virus detected in either line. In an effort to facilitate gene transfer to repopulating stem cells, day 4 5-FU BM cells were prestimulated with growth factors for 48 hours prior to 48 hours of co-culture with irradiated viral producer cells. Cells recovered from co-culture were subsequently stained for CD24 expression as detailed in Chapter 2. The expression profiles and sort thresholds for 1 experiment are shown in Figure 4.1 (lower panel). Following FACS selection, 4 x 10^5 $CD24^+$ cells per recipient were transplanted into a total of 14 myeloablated mice (7 recipients per viral construct). This transplant dose was estimated to contain 12 ± 4 competitive repopulating units (CRU) as determined from previous experiments carried out to determine CRU frequencies in the population of cells recovered following the infection and selection procedure (data not shown).

4.2.2. The majority of donor-derived cells in recipients contain intact provirus

Six months post transplant, the average proportion of transplant derived ($Ly5.1^+$) cells in recipients was 58% for bone marrow (range 22% to 68%), 85% for peripheral blood mononuclear cells (range 47% to 95%), and 93% for thymus (range 47% to 100%)(see Table 4.1). Thus, the vast majority of mice showed virtually complete reconstitution of bone marrow and thymus with Ly5.1 donor derived cells (taking into account that approximately 30% of the marrow is composed of differentiating erythroid progenitors and their progeny which do not express the Ly5.1 antigen). Expression of the transduced CD24 gene was detected

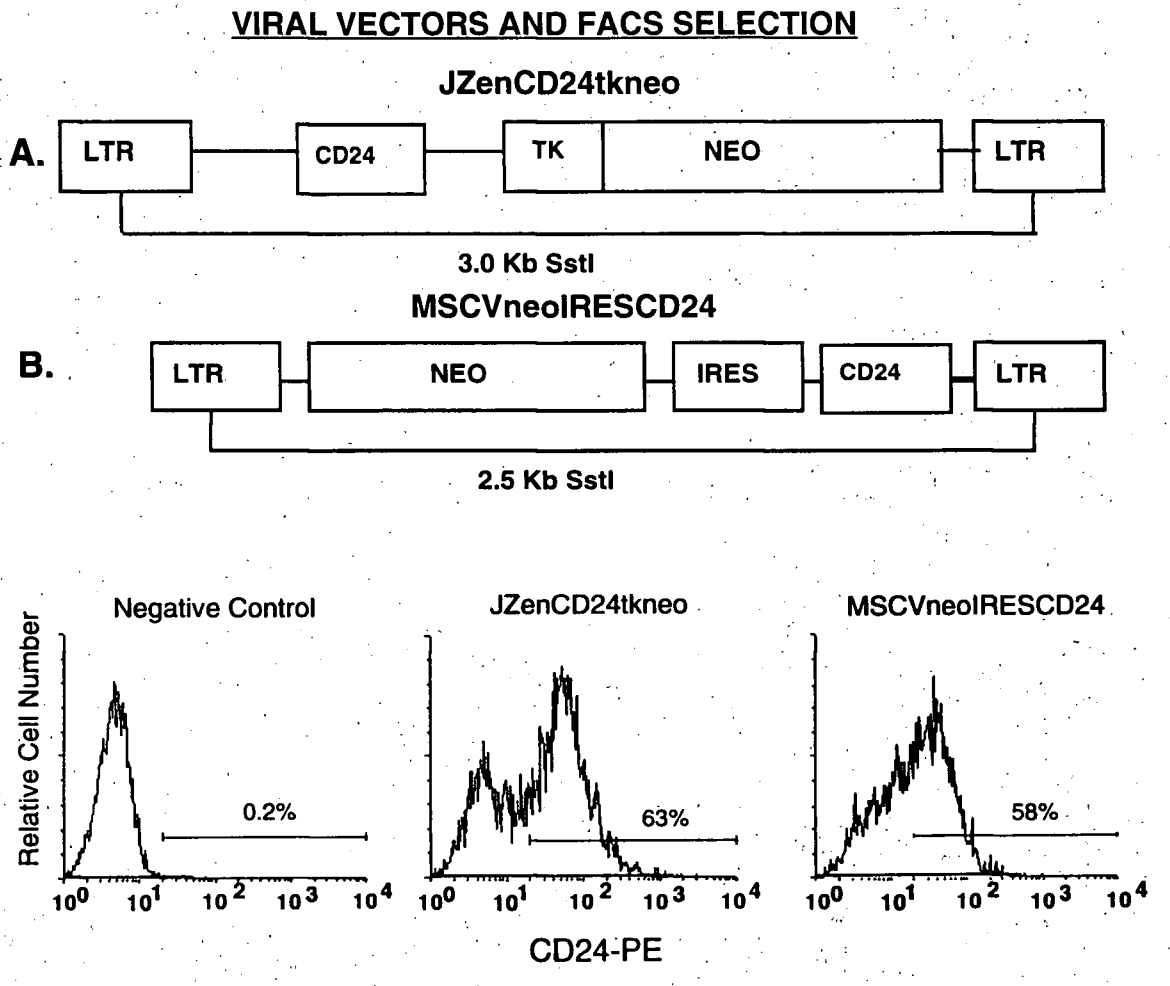

Figure 4.1. Viral vectors used and FACS selection of retrovirally transduced bone marrow cells. (Upper panel) Schematic of the JZenCD24tkneo and MSCVneoIRESCD24 provirus. JZenCD24tkneo incorporates a 240-bp portion of the cDNA encompassing the complete coding region; a thymidine kinase-neomycin resistance cassette (tkneo) from pNC1neo; and LTR sequences from MPSV. MSCVneoIRESCD24 contains the neo gene from pMC1neo; the coding region of the CD24 cDNA; an internal ribosomal entry sequence from the encephalomyocarditis virus; and LTR sequences from MSCV. (Lower panel) Expression of CD24 on day 4 5-FU BM cells 48 hours after co-culture with JZenCD24 or MSCVCD24 viral producers compared to uninfected day 4 5-FU BM cells. Cells were stained with anti-CD24/R-PE tetrameric antibody complexes and analyzed by flow cytometry. $CD24^+$ cells were sorted and transplanted into myeloablated recipient mice at 4×10^5 $CD24^+$ cells per recipient.

on peripheral blood leukocytes, red blood cells and whole bone marrow cells for all recipients and on thymocytes for all but 2 mice. The proportion of transplant derived cells expressing CD24 (ie. Ly5.1$^+$CD24$^+$ cells) varied with the lineage of the cell type tested, and as a function of the virus used (Table 4.1). The greatest proportion of CD24$^+$ cells was observed among BM, peripheral blood leukocytes and red blood cells, with the lowest proportion found in thymus. For example, 74% of whole BM, 90% of red blood cells and 58% of peripheral blood leukocytes in recipient m5, transplanted with MSCVCD24 transduced marrow, were positive for CD24 expression compared to only 18% of thymocytes (Table 4.1). Mice receiving MSCVCD24 transduced BM showed some 6-fold higher proportion of CD24$^+$ cells for all cell types tested as compared to mice transplanted with JZenCD24 infected marrow (Table 4.1). As an independent assessment of gene transfer, the proportion of G418 resistance clonogenic progenitors in the bone marrow of recipients was determined and found to correlate with the proportion of CD24$^+$ cells in total bone marrow (Table 4.1)

Stringent sorting thresholds depicted in Figure 4.1 (< 0.2% of non-transduced control bone marrow cells positive) were chosen in an effort to maximize the probability that only retrovirally transduced stem cells expressing significant levels of CD24 would be isolated. Despite this, on average only a proportion of transplant derived cells were found to express CD24, suggestive of promoter shutdown or the inefficient selection of transduced stem cells. To discriminate between these possibilities Southern blot analysis was carried out to assess the presence of intact provirus in the BM, spleen and thymus of recipient mice. As shown in Figure 4.2, high levels of intact provirus were detectable in the hemopoietic tissues of five representative recipients of JZenCD24 transduced marrow. Comparable results were observed for recipients of MSCVCD24 transduced BM (data not shown). Densitometric analysis of the signal intensities obtained when membranes were sequentially probed with a fragment of the

Table 4.1. Flow Cytometric Analaysis of CD24 Expression in Various Hematopoietic Tissues in Recipients of JZenCD24 or MSCVCD24 Virus-Infected Marrow Assessed 24 Weeks Post-Transplant.

Cells Transplanted	Mouse	Whole BM		Peripheral RBCs	Thymus		Peripheral Blood Leukocytes		% $G418^R$ BM Prog.
		% Ly5.1+	% CD24+/ Ly5.1+ *	% CD24+ †	% Ly5.1+	% CD24+ Ly5.1+	% Ly5.1+	% CD24+/ Ly5.1+	
JZenCD24	m1	64	8	7	98	0	93	4	6
	m2	66	18	3	98	1	94	2	13
	m3	58	16	6	98	3	93	4	11
	m4	57	5	4	96	0	82	4	3
	m5	66	12	2	99	3	91	2	5
	m6	67	21	15	99	1	90	8	24
	m7	67	25	13	99	7	92	10	34
		62 ± 2	15 ± 3	7 ± 3	98 ± 0.5	2 ± 1	91 ± 2	5 ± 1	14 ± 6
MSCVCD24	m1	29	52	25	99	8	70	14	56
	m2	68	85	44	98	21	81	33	90
	m3	22	41	23	31	10	47	28	40
	m4	64	30	29	93	15	92	5	64
	m5	61	74	90	100	18	95	58	87
	m6	66	88	41	99	20	90	29	90
		52 ± 10	62 ± 11	42 ± 13	87 ± 14	15 ± 4	79 ± 9	28 ± 9	71± 10

Repopulation of recipients with Ly5.1 donor-derived cells was assessed by staining erythrocyte-depleted peripheral blood samples with FITC-conjugated Ly5.1 antibody and analysis by flow cytometry. Samples were also tested for expression of the transferred CD24 gene by staining cells with anti-CD24 tetrameric antibody complexes/R-PE and analysis by flow cytometry. CD24 expression on bone marrow stem cell candidates was achieved by combining anti-CD24 tetrameric antibody complexes with a cocktail of antibodies recognizing lineage specific antigens as explained in Chapter 2. The proportion of G418R clonogenic progenitors in BM was determined as described in Material and Methods.

* The expression of CD24 in various hemopoietic tissues is given as the proportion of cells with the $Ly5.1^+$ cell surface phenotype expressing CD24.

†Ly5.1 is not expressed on red blood cells (RBCs). Therefore, the % $CD24^+$ values given for RBC represents the % of total RBCs.

neomycin resistance gene to detect recombinant provirus and the erythropoietin receptor gene as an endogenous control showed that the average proviral copy number per haploid genome was 2.6 (range 1.2 to 4.2) and 2.5 (range 0.6 to 6.5) for recipients of JZenCD24 and MSCVCD24 infected BM respectively. These findings are consistent with high level regeneration with provirally marked cells and suggest that CD24 expression in only a fraction of transplant derived ($Ly5.1^+$) cells is most likely the result of down regulation of the transferred CD24 gene rather than the absence or gross alteration of recombinant provirus.

To further assess the degree of reconstitution with provirally marked cells, femoral BM from primary recipients was transplanted into secondary recipients to generate day 12 CFU-S. Of 19 and 20 individual spleen colonies generated from the BM of recipients of JZenCD24 and MSCVCD24 infected marrow, respectively, all were found to contain intact provirus. This finding strongly suggests that at the time of sacrifice essentially all hemopoiesis was derived from retrovirally transduced stem cells. Integration analysis of hemopoietic tissues of primary transplant recipients and of day 12 spleen colonies generated from these mice are presented in Figure 4.3. Two of the selected mice manifested different patterns of clonal reconstitution. Analysis of splenic and thymic DNA obtained from a primary recipient of JZenCD24 transduced marrow (top panel) shows a complex pattern of proviral marking indicative of polyclonal reconstitution. The analysis of day 12 spleen colonies generated from the BM of this animal is consistent with at least 4 clonal integration patterns being detected and hence, is suggestive of multiple HSCs actively contributing to hemopoiesis at the time of sacrifice. In contrast, the analysis of BM and thymic DNA obtained from a recipient of MSCVCD24 transduced marrow showed a pattern of proviral marking consistent with monoclonal reconstitution (Figure 4.3, bottom panel). The identical pattern of proviral marking among all day 12 spleen colonies generated from the BM of this mouse further supports this conclusion.

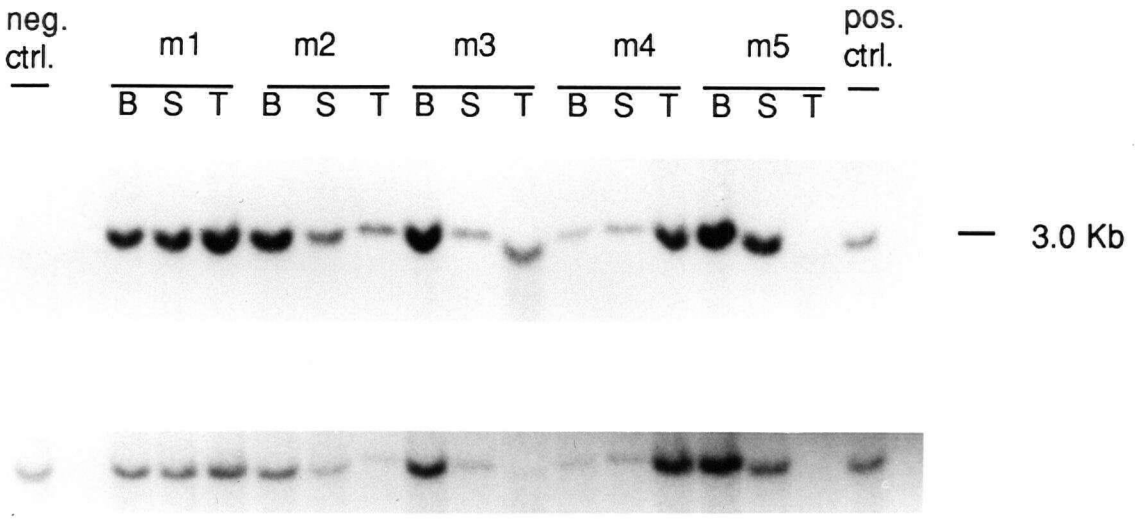

Figure 4.2. Detection of high levels of intact provirus in the bone marrow (B), spleen (S), and thymus (T) of recipient mice transplanted with 4 x 10^5 $CD24^+$ selected, JZenCD24 virus infected bone marrow cells at 24 weeks post transplant by Southern blot analysis. Individual recipient mice are labeled as m1-m5. DNA (10 μg) from each tissue sample was digested with Sst I, an enzyme which cuts once within each proviral LTR. Shown are the results of a blot probed with a ^{32}P-labeled fragment of the neo^R gene (top) and a fragment of the erythropoietin receptor gene as an endogenous control for DNA loading (bottom). The positive control represents DNA obtained from a retrovirally infected Ba/F3 clone harboring two copies of provirus.

Thus, despite the transplant of multiple transduced HSCs, long term hemopoiesis was associated with monoclonal stem cell activity. These differences in the pattern of hemopoietic reconstitution, however, were not related to the viral vector used.

BM from primary recipients of $CD24^+$ selected cells was also transplanted into secondary myeloablated recipients in an effort to assess the regeneration of transduced long term repopulating stem cells. Secondary recipients were sacrificed 5 months post transplant and DNA from BM and thymus analyzed by Southern blot. The presence of recombinant provirus was detected in both BM and thymus of all secondary recipients (Figure 4.3) demonstrating that the femoral marrow of the primary recipient had been regenerated with HSCs that were retrovirally transduced. Moreover, in several instances the pattern of proviral marking observed in the hemopoietic tissues of secondary recipients was identical to that observed among day 12 spleen colonies. For example, an identical pattern of proviral marking was observed between mouse D and spleen colonies 2 and 5 (top panel); mouse A BM with spleen colonies 6-8 (top panel); and mice F-J with colonies 1-9 (bottom panel). This observation demonstrates that hemopoiesis in the primary recipients was being sustained by totipotent cells with the capacity for long term hemopoietic reconstitution. In addition Figure 4.3 provides clear evidence of self-renewal of transduced HSCs within the femoral marrow of the primary recipient. The most striking evidence of self-renewal is seen among secondary recipients shown in the bottom panel. All 5 mice (MSCVCD24 F-J) show the same pattern of proviral marking in BM and thymic DNA suggesting that the HSCs responsible for regenerating these mice were derived from a stem cell that had undergone a minimum of 4 self-renewal division events in the femoral marrow of the primary recipient.

Together, these results demonstrate the utility of the CD24 selection approach to enable the regeneration of the hemopoietic systems of myeloablated recipient mice to very high levels with provirally marked cells, and as a means to

track the self-renewal of individual HSCs and their contribution to hemopoiesis following transplant.

4.2.3. Gene transfer to and expression of CD24 among Sca^+Lin^- bone marrow stem cell candidates

To obtain more direct evidence of expression of the transferred CD24 gene in primitive hemopoietic cells, CD24 expression was analyzed in the Sca^+Lin^- subpopulation of BM, a population known to be enriched for cells with long term repopulating ability. Analysis was carried out 48 hours following termination of the infection procedure, and in reconstituted mice 6 months post transplant. Antibody staining performed 48 hours after retroviral infection showed that both the MSCVCD24 and JZenCD24 vectors were able to drive high levels of CD24 expression in the majority of Sca^+Lin^- cells (75% vs. 66% respectively) (Figure 4.4). No difference in the absolute level of CD24 expression among cells infected with either virus was detected.

At 6 months post transplant, the proportion of regenerated Sca^+Lin^- cells expressing the transferred CD24 gene varied as a function of the vector used. While 81% (range 53%-97%) of the regenerated Sca^+Lin^- cells in recipients of MSCVCD24 transduced marrow were positive for CD24 expression, only 22% (range 12%-45%) of $CD24^+$ Sca^+Lin^- cells were detected in recipients of JZenCD24 transduced marrow (Figure 4.5). These results contrast with the essentially complete reconstitution with transduced cells observed in the BM for both vectors. The expression of CD24 was also detected in secondary transplant recipients, although again, vector related differences in the proportion of Sca^+Lin^- cells expressing CD24 were detected. The average proportion of Sca^+Lin^- cells expressing CD24 was 59% (range 39%-84%), and 8% (range 0%-27%) for recipients of MSCVCD24 and JZenCD24 infected marrow respectively.

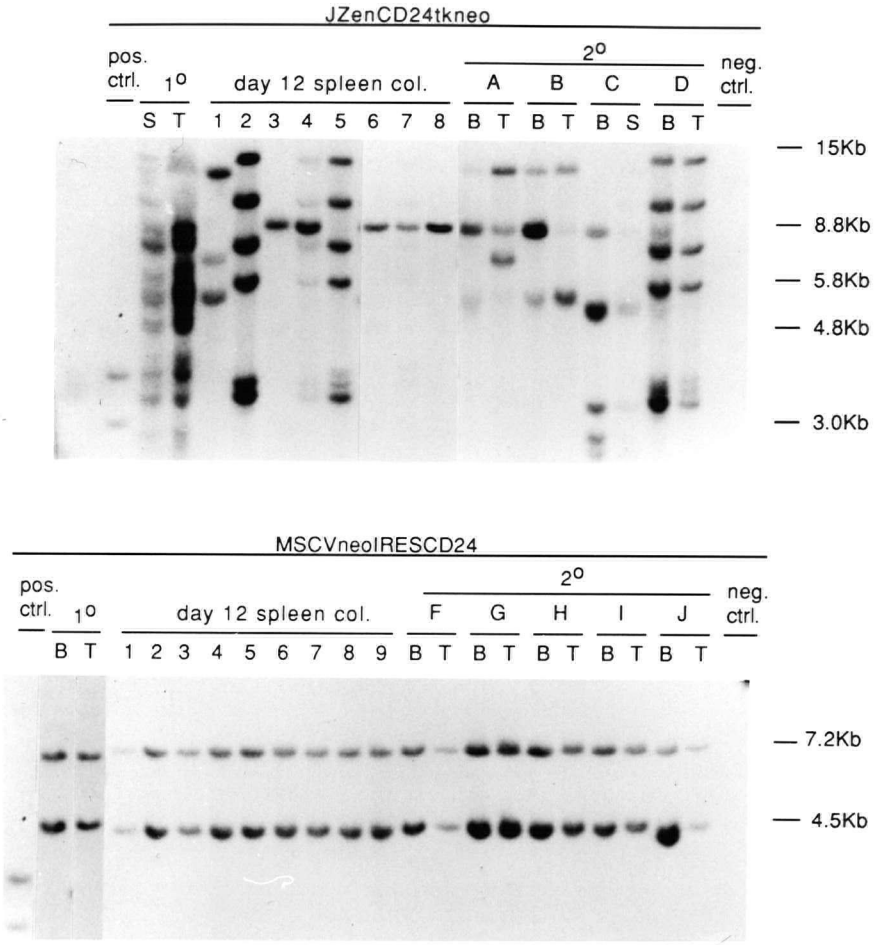

Figure 4.3. Assessment of proviral integration in bone marrow (B), spleen (S), and/or thymus (T) in primary or secondary recipients and day 12 spleen colonies generated from the femoral marrow of selected primary recipients by Southern blot analysis. Primary recipients received 4 x 10^5 $CD24^+$ selected JZenCD24 or MSCVCD24 infected bone marrow cells and were sacrificed at 24 weeks post transplant. Femoral bone marrow from selected primary mice were used as a source of donor cells for the generation of day 12 spleen colonies and for transplant into secondary recipients. For the analysis of day 12 spleen colonies lethally irradiated recipient mice were transplanted with 1 x 10^5 femoral bone marrow cells. Twelve days later animals were sacrificed via cervical dislocation and individual spleen colonies dissected and DNA purified for Southern blot analysis. Lethally irradiated secondary transplant recipients received 2 x 10^6 femoral bone marrow cells. Mice were sacrificed 5 months later and DNA extracted from bone marrow, spleen and thymus. Individual recipient mice are labeled as 1^o or 2^o (A-J) while individual day 12 spleen colonies are numerically labeled (1-9). DNA (10 μg) was digested with EcoRI, an enzyme which cuts once within the proviral sequence. Shown are the results of two separate blots (top; JZenCD24; bottom; MSCVCD24) probed with a ^{32}P labeled fragment of the neo^R gene. The positive control is DNA obtained from a retrovirally infected Ba/F3 clone harboring two copies of the JZenCD24tkneo provirus. The negative control is DNA obtained from the spleen of a normal unmanipulated control mouse.

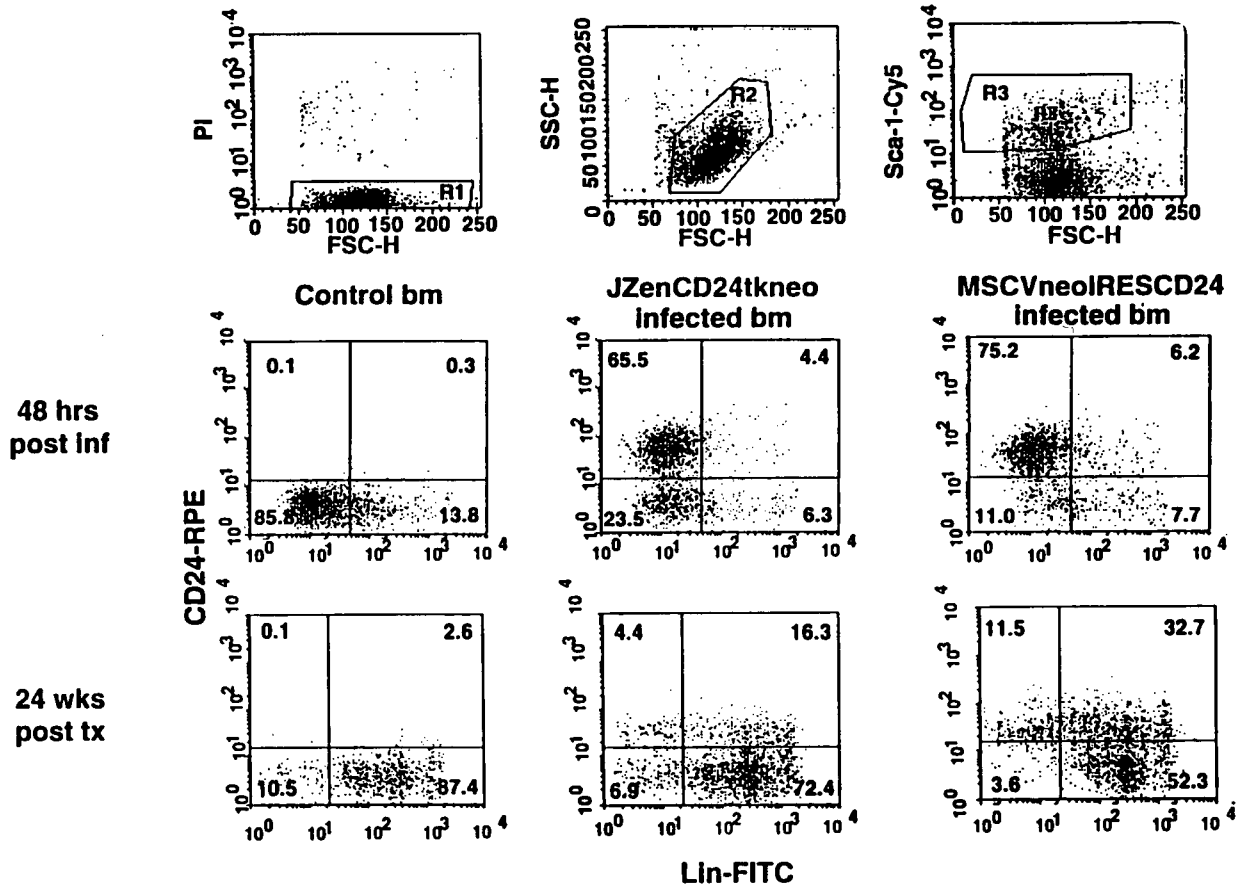

Figure 4.4. Expression of the transferred CD24 gene in primary marrow stem cells candidates as defined by the $Sca^{+}Lin^{-}$ cell surface phenotype. Uninfected control BM and JZenCD24 or MSCVCD24 virus infected marrow were analyzed at 48 hours post infection with viral producers or at 24 weeks post transplant into myeloablated recipient mice. Cells were stained with Sca-1-Cy5, anti-CD24 tetrameric/R-PE and a cocktail of antibodies recognizing lineage specific antigens including Gr-1-FITC and Mac-1-FITC for granulocytes/macrophages, Ly-1-FITC for T lymphocytes and B220-FITC for B lymphocytes. Cells were gated on the basis of uptake of propidium iodide, forward and side scatter profiles, and expression of Sca-1 (top panels) and expression of CD24 on the gated population assessed (lower panels).

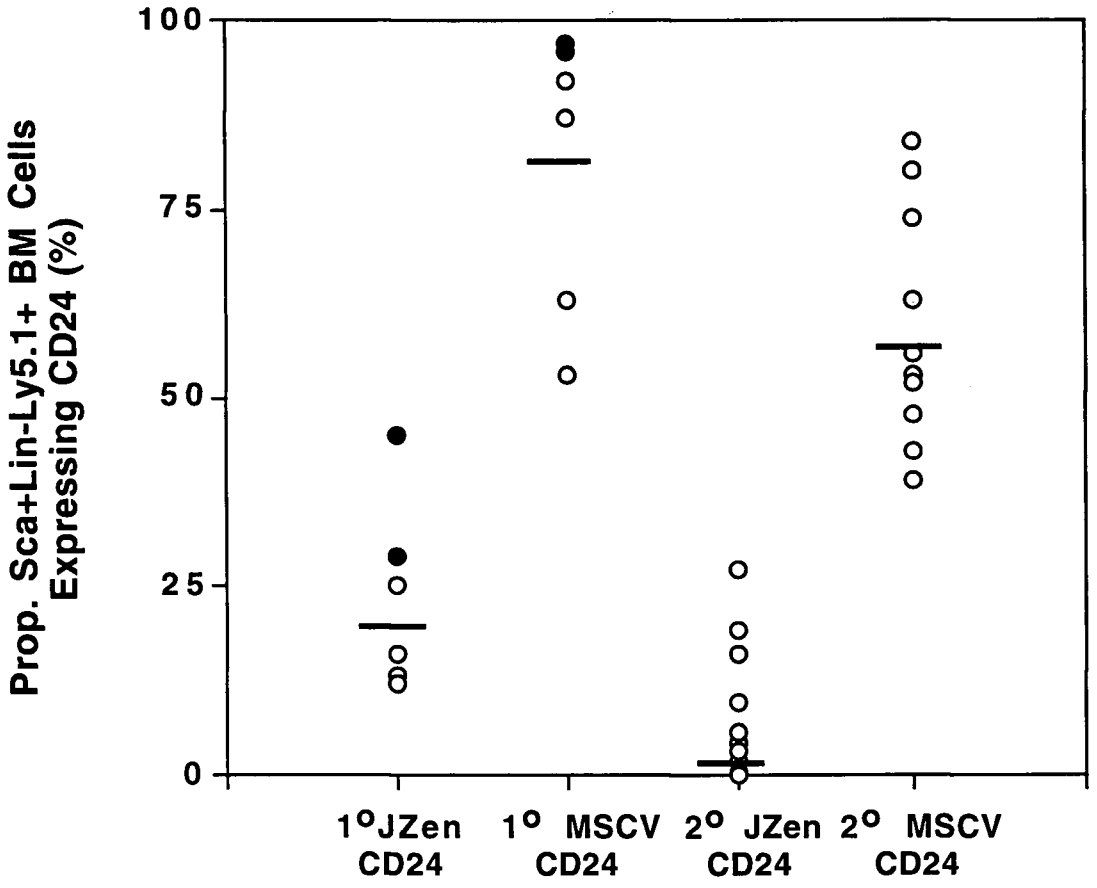

Figure 4.5. Shows the proportion of stem cell candidates defined by the $Sca^{+}Lin^{-}$ cell surface phenotype positive for CD24 expression at 24 weeks post transplant in recipients of JZenCD24 or MSCVCD24 $CD24^{+}$ selected bone marrow. Femoral bone marrow cells from individual recipient mice were stained with Sca-1-Cy5, anti-CD24 tetrameric/R-PE antibody complexes and a combination of anti-Gr-1-FITC, anti-Mac-1-FITC, anti-Ly-1-FITC and B220-FITC recognizing granulocytes/macrophages, T and B lymphocytes and analyzed by flow cytometry. Mice represented by solid circles were used as marrow donors into secondary recipients. Horizontal lines show the average proportion of $Sca^{+}Lin^{-}$ $CD24^{+}$ stem cell candidates in primary and secondary recipients of JZen or MSCV transduced bone marrow.

4.3. Discussion

A key finding of the work described in this Chapter was that preselection of transduced cells based upon the transfer and expression of a cDNA encoding the CD24 cell surface antigen enabled the reconstitution of recipient mice almost exclusively with provirally marked cells. Despite the relatively small transplant dose, reconstitution of recipients with transplant derived ($Ly5.1^+$) cells was essentially complete (Table 4.1). Southern blot analysis of DNA from BM, spleen and thymus revealed the presence of high levels of recombinant provirus (Figure 4.2), with proviral copy numbers per haploid genome >1 in almost all mice. Moreover, the finding that all day 12 spleen colonies contained intact provirus (Figure 4.3) further supports the conclusion that all HSCs contributing to hemopoiesis in these animals were transduced.

While recipient mice were transplanted with numerous transduced HSCs, the number of stem cells found to contribute to long term hemopoiesis was found to vary. In some, hemopoietic reconstitution was essentially monoclonal while in others a number of independent clones contributed to hemopoiesis. These differences suggest a stochastic phenomenon attributable to either seeding efficiencies that are < 100% and/or decisions relating to self-renewal versus differentiation of the stem cell upon arrival in the marrow. It should be possible to explore these issues in more detail using the CD24 selection approach to provide a population of HSCs in which all are retrovirally marked.

The recovery of long term repopulating stem cells within the $CD24^+$ selected fraction indicates that both the MPSV (JZen) and MSCV LTR regulatory elements can drive significant levels of gene expression immediately post infection. This is further supported by the detection of CD24 expression on the majority of Sca^+Lin^- subpopulation of cells following the infection protocol (Figure 4.4). Moreover, CD24 expression persisted on regenerated Sca^+Lin^- cells in both primary and secondary transplant recipients (Figure 4.5). While both JZen and MSCV based vectors gave

similar absolute levels of expression and proportions of transduced cells following infection, MSCV proved superior to JZen in transplant recipients despite the detection of roughly similar levels of intact provirus. This was also evident in the levels of CD24 expression observed among more mature cells in the BM, thymus and periphery (Table 4.1). These data suggest that the JZen LTR regulatory elements are more susceptible to suppression/shutdown (215, 251), perhaps due to the methylation of the promoter and/or enhancer elements (257, 331). The MSCV vector (283) combines the LTR from PCMV (PCC4 embryonal carcinoma cell-passaged myeloproliferative sarcoma virus) (258) and the 5' untranslated region of the dl587rev virus (259), both viruses isolated as mutants able to express transferred genes in embryonic carcinoma (EC) and embryonic stem (ES) cell lines. These retroviral mutants possess various deletions and base pair changes believed to remove potential blocks to transcription located within the LTR and primer binding sites of the original viruses (252, 280-282, 334). My studies provide strong evidence that these changes may similarly have important consequences for the expression of transduced genes in HSCs and their progeny in vivo. Differences in the expression of the transferred CD24 gene was also detected among cells of different lineages. The proportion of $CD24^+$ thymocytes was significantly lower than that observed among cells of either the myeloid (bone marrow) or erythroid lineage (Table 4.1). Further studies using markers such as CD24 may facilitate the identification of regulatory elements to further increase the levels and persistence of transferred gene expression in hemopoietic stem cells or other target cells of interest.

In conclusion, these studies demonstrate the feasibility of using the CD24 cell surface antigen in combination with FACS to enable virtually complete regeneration of the hemopoietic systems of myeloablated recipient mice with provirally marked cells. This approach provides a powerful strategy to track the behavior of individual HSCs in vivo, and as a method to detect and quantify levels

and persistence of gene expression in various phenotypically defined populations of cells in vivo. Efforts to compare the levels of expression of gene expression from alternative retroviral vectors such as MFG, and to extend this approach to the human setting are currently in progress .

CHAPTER 5

EVIDENCE OF BOTH ONTOGENY AND TRANSPLANT DOSE REGULATED EXPANSION OF HEMOPOIETIC STEM CELLS IN VIVO

The results presented in this Chapter have been described in:
Pawliuk, R., C. J. Eaves and R. K. Humphries. 1996. Evidence of both ontogeny and transplant dose regulated expansion of hematopoietic stem cells in vivo. Blood in press.

5.1. Introduction

There is an increasing emphasis on the development of clinical strategies that depend on the regenerative potential of transplantable HSCs. These include efforts directed at autograft purging, gene transfer (including attempts to select transduced cell populations) ex vivo expansion and the use of alternative sources of transplantable hemopoietic cell populations (such as fetal liver and umbilical cord blood). At the same time, the molecular mechanisms that define the regenerative potential of HSC, or that regulate its expression, particularly in vivo, remain poorly understood. Serial transplantation studies have provided evidence that the self-renewal capacity of HSCs may be finite or at least subject to exhaustion (105, 107, 109). In addition, comparative studies of the proliferative activities of fetal liver and adult bone marrow cells have shown that, under the same conditions, these may display differences suggesting intrinsically determined differences in regenerative potential. (87, 88, 335). However, none of these studies has relied on the use of direct measurements of HSC numbers, at least in part because quantitative assays for cells with long-term repopulating ability have only recently become available (43, 45). The critical importance of such assays is further underscored by a growing body of evidence indicating that the cell surface phenotype is not necessarily a reliable indicator of retained stem cell function, nor is the presence of terminal cells a reliable reflection of the size of the HSC compartment (53, 76, 111). Thus, parameters that may affect the kinetics and ultimate extent of regeneration of the HSC compartment obtained after the transplantation of different numbers or sources of hemopoietic cells have not been defined. To address these questions, I used a limiting dilution assay for cells with long-term competitive repopulating ability to measure HSC regeneration in myeloablated recipients of different numbers of HSCs from adult bone marrow or fetal liver.

5.2. Results

5.2.1. Overall experimental design

The purpose of these experiments was to examine and compare the kinetics of recovery of every level of hemopoietic cell development in myeloablated recipients as a function of both the CRU content and the origin of the cells transplanted. Donor cells were obtained from either day 14.5 fetal livers or the bone marrow of adult mice injected 4 days previously with 150 mg/kg of 5-FU and groups of mice then injected with a range of cell numbers estimated to contain 1000, 100 or 10 CRU, ie. 10%, 1% and 0.1% of the total marrow CRU content of an average untreated adult mouse (43) or 90%, 9% or 0.9% of the CRU content of a single day 14.5 fetal liver (335). All mice also received 10^5 bone marrow cells from an unmanipulated adult Ly5.2 $B6C3F_1$ donor containing an estimated competing graft of 10 CRU (53). Recipients of these grafts were sacrificed 8-12 months following transplantation for assessment of the test graft ($Ly5.1^+$)-derived contribution both to their reconstituted peripheral blood cells as well as their marrow CFC, CFU-S and CRU populations.

5.2.2. Kinetics of reconstitution of the terminal compartments

Flow cytometric analysis of mature cells in the peripheral blood 8 months post transplant revealed extensive reconstitution of both lymphoid and myeloid compartments with $Ly5.1^+$ test cells for all transplant groups (see Table 5.1). The proportion of $Ly5.1^+$ cells contributing to both of these mature compartments was highly consistent between experiments for each transplant dose and made up nearly all ($\geq$ 80%) of the cells in recipients of fetal liver cells and in recipients of all but the lowest transplant dose of adult bone marrow cells (containing 10 CRU). In these, the proportion of peripheral blood cells that were $Ly5.1^+$ was slightly lower, albeit still approximately 50% of the total, reflecting their origin from an equivalent proportion (also ~50%) of all the CRU transplanted.

Table 5.1. Proportion of Ly5.1$^+$ Peripheral Blood Cells from Primary Recipients Transplanted with Varying Numbers of Ly5.1$^+$ Syngenic Adult Bone Marrow- or Fetal Liver-Derived CRU

Source and Transplant Dose	Estimated No. CRU Transplanted*	% Ly5.1$^+$ PB cells	
		Expt. 1	Expt. 2
BM 2×10^6	1000	91 ± 2 (5)	91 ± 2 (3)
BM 2×10^5	100	77 ± 4 (5)	81 ± 3 (3)
BM 2×10^4	10	42 ± 7 (13)	59 ± 8 (13)
FL 1.7×10^7	1000	91 ± 1 (5)	92 ± 0 (2)
FL 1.7×10^6	100	94 ± 0 (3)	89 ± 1 (2)
FL 1.7×10^5	10	79 ± 4 (5)	82 ± 4 (2)

Values shown are the mean ± SD (number of mice analyzed) of the proportion of Ly5.1$^+$ cells in the circulating WBC populations present in primary transplant recipients analyzed 8 months post transplantation in two individual experiments.
* The estimation of number of CRU transplanted is derived from a control value of 1/2000 (95% confidence interval: 1 in 1300 to 1 in 5700) for adult 5-FU BM (43) and 1/17000 (95% confidence interval: 1 in 11500 to 1 in 26,000) for fetal liver (335).

5.2.3. Reconstitution of the marrow

Two mice per transplant group were chosen for further analysis of the level of test transplant-derived cells in the marrow 8 months post-transplant. In an effort to detect the maximum levels of reconstitution attained by this source, recipients in which at least 80% of the peripheral blood cells were Ly5.1$^+$ were selected. In addition, the bone marrow cells of one of the recipients were also stained with the Ly5.1 antibody and upon FACS analysis were found, like the blood, to contain >70% Ly5.1$^+$ (ie. test transplant-derived) cells (data not shown).

The total marrow cellularity and CFC numbers in all pairs of primary recipients, irrespective of the number or origin of the cells initially transplanted, had regenerated to levels equivalent to those found in unmanipulated age-matched control mice (Table 5.2). In contrast, for day 12 CFU-S, this was true only for the recipients of the highest transplant dose of fetal liver cells. For recipients of

Table 5.2. Regeneration of Cellularity, Clonogenic Progenitor and Day 12 CFU-S Content in Femoral Marrow of Mice Transplanted with Various Numbers of Adult BM or Fetal Liver CRU (8 Months Post Transplant)

Transplant Source	Estimated No. CRU Transplanted	Total nucleated cells/femur (±SD)	CFU C/femur x 10^2 (±SD)	Day 12 CFU-S/femur x 10^2 (±SD)
Adult BM	10	$2.1 \pm 0.7 \times 10^7$	382 ± 61.0	10.1 ± 2.4
	100	$2.3 \pm 0.1 \times 10^7$	504 ± 101	16.1 ± 1.8
	1000	$2.5 \pm 0.5 \times 10^7$	475 ± 160	17 ± 1.5
Fetal Liver	10	$2.1 \pm 0.5 \times 10^7$	330 ± 98.7	11.8 ± 2.4
	100	$2.3 \pm 0 \times 10^7$	393 ± 17.9	14.3 ± 1.0
	1000	$2.4 \pm 0.7 \times 10^7$	394 ± 37.4	21.6 ± 1.0
Normal Control		$2.3 \pm 0.1 \times 10^7$	476 ± 138	24.4 ± 2.6

Bone marrow cells from primary recipients (2 mice per group) 8 months post transplant were counted using a standard hemocytometer and plated in methylcellulose at 2.7×10^4 cells/ml and colonies scored 12 days later. Regeneration of day 12 CFU-S was assessed by transplanting 7.5×10^4 - 1×10^5 marrow cells from pooled marrow from two primary recipients into 5 irradiated (950cGy) B6C3F1 mice/group. Animals were sacrificed 12 days later and macroscopic spleen colonies counted. Normal control animals were 6-8 month old unmanipulated B6C3F1 mice.
See Table 5.1. for calculated CRU frequencies in unmanipulated adult bone marrow and fetal liver.
For each experiment 2 mice showing the highest proportion of $Ly5.1^+$ cells in the peripheral blood were chosen as donors for secondary transplantation. The average proportion of $Ly5.1^+$ cells in the peripheral blood of chosen mice for each group was; adult marrow 10 CRU: 81%, 100 CRU: 85%,1000 CRU: 94%, fetal liver 10 CRU: 82%, 100 CRU: 94%, 1000 CRU: 92%.

all other sources or numbers of test cells, recovery of day 12 CFU-S numbers was incomplete ranging from 45% to 85% of normal values (Table 5.2). Although there was a trend towards a greater recovery of this more primitive compartment in recipients of higher initial transplant doses, the actual differences between the groups were not statistically significant ($p < 0.05$).

5.2.4. Reconstitution of the marrow CRU compartment

To compare the accompanying level of regeneration of Ly5.1$^+$ donor-derived stem cells in the marrow of these same pairs of primary recipients, CRU frequencies and hence numbers were also determined. The presence of Ly5.1$^+$ myeloid and lymphoid cells in the peripheral blood of the secondary CRU assay recipients was evaluated after 16 weeks and used to derive the CRU numbers shown in Figure 5.1. In most cases, the Ly5.1$^+$ CRU population had regenerated to a level corresponding to only a small proportion of the normal CRU population and the levels achieved correlated positively with the original dose of Ly5.1$^+$ CRU used to reconstitute the primary recipients. Interestingly, the level of CRU regeneration was consistently higher ($p < 0.01$ to < 0.1) for fetal liver transplants as compared to marrow transplants containing the same original number of CRU. Thus, on a per input CRU basis, CRU amplification in the fetal liver transplants was 10X more effective than with primary bone marrow transplants and, only in primary recipients of 1000 CRU of fetal liver origin, did the transplanted CRU recover to a normal sized population within 8 months post-transplant.

Effects of the transplant dose as well as the tissue origin are more dramatically revealed by using the same data to calculate the extent of Ly5.1$^+$ CRU amplification in each experimental situation tested. The results of such calculations are shown in Table 5.3. It can be seen that for both fetal liver and adult bone marrow transplants, CRU expansion was inversely related to the initial number of CRU transplanted although the extent of CRU expansion for input fetal

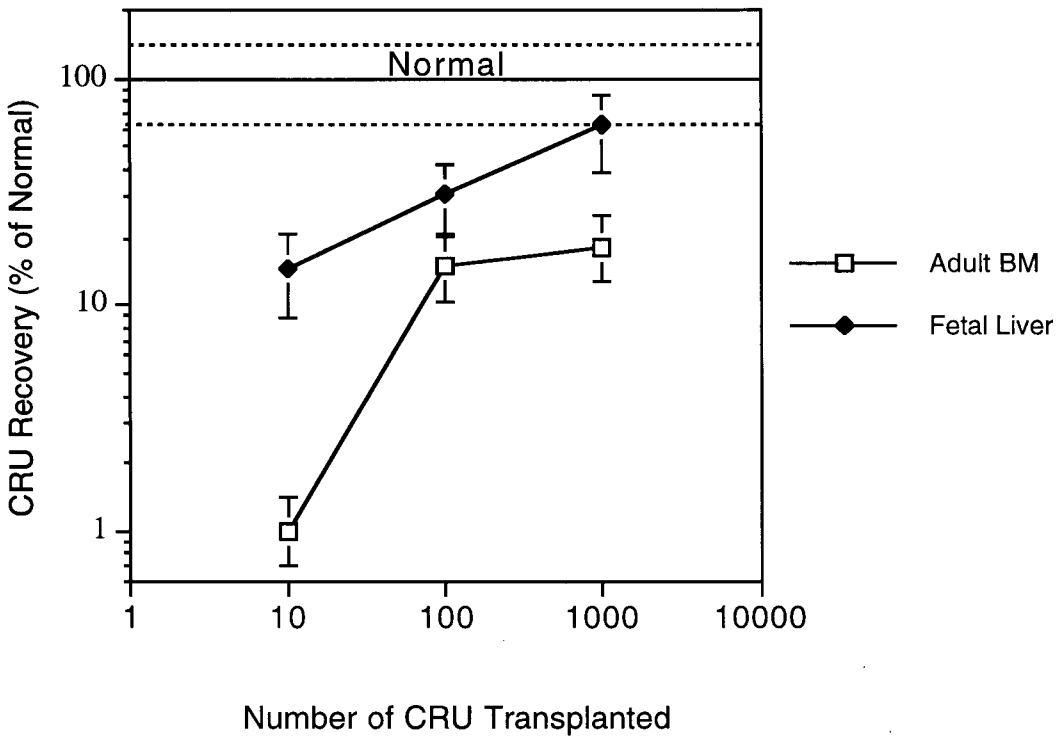

Figure 5.1. Regeneration of Ly5.1$^+$ donor-derived cells following the transplantation into secondary recipients of bone marrow cells from primary recipients originally transplanted with 10, 100 or 1000 fetal liver or adult bone marrow CRU 8 months previously. CRU numbers were determined by measuring the frequency of CRU by limiting dilution analysis in secondary recipients and calculating the number of CRU in the marrow of primary transplant recipients as compared to an unmanipulated control mouse based upon the assumption that an average adult mouse has approximately 200 million marrow cells.
For each transplant dose CRU regeneration by fetal liver was significantly greater than adult bone marrow; 10 CRU: $p < 0.01$, 100 CRU: $p < 0.1$, 1000 CRU: $p < 0.01$.
For adult bone marrow the 10 CRU transplant dose is significantly lower than both the 100 and 1000 CRU dose at $p < 0.01$. For fetal liver the 10 CRU dose is significantly lower than both the 100 and 1000 CRU dose; 100 CRU: $p < 0.1$, 1000 CRU: $p < 0.01$. The 100 CRU dose is significantly lower than the 1000 CRU dose at $p < 0.1$.
The estimation of the number of CRU transplanted is derived from a control value of 1/2000 (95% confidence interval: 1 in 1300 to 1 in 5700) for adult 5-FU bone marrow (43) and 1/17,000 (95% confidence interval: 1 in 11,500 to 1 in 26,000) for fetal liver (335).

Table 5.3. Expansion of Donor-Derived CRU in Primary Recipients of Fetal Liver or Adult Bone Marrow Cells

Transplant Source	Estimated No. CRU Transplanted *	Estimated No. Bone Marrow CRU Post Expansion (range ±SEM)†	Fold Expansion
Adult bm	10	100 (75-150)	10
	100	1500 (1000-2100)	15
	1000	1800 (1300-2500)	2
Fetal Liver	10	1500 (1000-2100)	148
	100	3100 (2300-4200)	31
	1000	6200 (4500-8500)	6
Untransplanted Control	-	10000 (6300-14800)	-

*See Table 5.1 for calculated CRU frequencies in unmanipulated adult bone marrow and fetal liver.
†Results are expressed as the number of CRU per mouse based on the estimate that 2 x 10^7 femoral marrow cells constitutes approximately 10% of the total hemopoietic population of the mouse.

liver CRU was greater than that of their counterparts in bone marrow. The maximum CRU expansion observed was 150-fold for fetal liver CRU by comparison to a maximum CRU expansion of only 15-fold for adult bone marrow transplants.

5.2.5. Regenerative ability of a single CRU assessed using retroviral marking

To gain further insight into CRU regeneration I used retroviral marking for clonal analysis of self-renewal and lympho-myeloid reconstitution. 5-FU-treated Ly5.1 marrow cells were co-cultured with cells producing a retrovirus containing the coding region of the human CD24 cell surface antigen and were then stained with an anti-CD24 antibody and $CD24^+$ cells selected by FACS 48 hours post-infection as described in Chapter 2; Material and Methods. 10^5 of these $Ly5.1^+$ cells (estimated to contain 3.0 ± 1.0 CRU) were transplanted into $Ly5.2^+$ recipients.

All of 13 such recipients showed detectable levels of Ly5.1$^+$ (donor cell-derived) repopulation with values ranging from 3-32% Ly5.1$^+$ peripheral blood leukocytes (10/13 myeloid/lymphoid repopulation; 3/13 lymphoid-restricted repopulation). The hemopoietic tissues of all of these mice also showed the presence of intact provirus at 11 months post transplant but in only 3 was CD24 expression detectable in the peripheral blood leukocytes (where values of 2-20% CD24$^+$ were measured). One of these recipients showing 32% donor-derived Ly5.1$^+$ cells, of which 63% were CD24$^+$, was chosen for further study to quantify the clonal regeneration of Ly5.1$^+$ CRU. Southern blot analysis of the bone marrow, spleen and thymus of this mouse showed identical proviral banding patterns and band intensities in all of these tissues consistent with the repopulation of this primary recipient by a single transduced totipotent repopulating stem cell. When the marrow cells of this mouse were then assayed for their content of Ly5.1$^+$ CRU, 0.2 $\pm$ 0.05 Ly5.1$^+$ CRU per 10^5 marrow cells or 50 Ly5.1$^+$ CRU per femur were detected. This represents 3.7% of the CRU population in the femur of a normal adult B6C3F$_1$ mouse. Seventeen of the 19 secondary transplant recipients who showed the presence of Ly5.1$^+$ peripheral blood cells were also positive for CD24 expression, with values ranging from 1.5% to 14.2% CD24$^+$ peripheral blood leukocytes. Strikingly, the same proviral banding pattern seen in the primary animal was also observed exclusively in the hemopoietic tissues of all of the secondary recipients who were reconstituted with Ly5.1$^+$ cells (Figure 5.2). This observation indicates a 370-fold amplification of the original transduced CRU during the period of 11 months after it was transplanted into the primary recipient. This result provides formal evidence of CRU self-renewal in vivo and extends the previous findings indicating the extent to which this can occur.

5.3. Discussion

Previous studies have indicated that the capacity of most primitive hemopoietic cells capable of regenerating the entire system cannot be maintained

on serial transfer. In this Chapter I showed that this may be at least partially attributable to a common failure of the CRU compartment to be fully regenerated

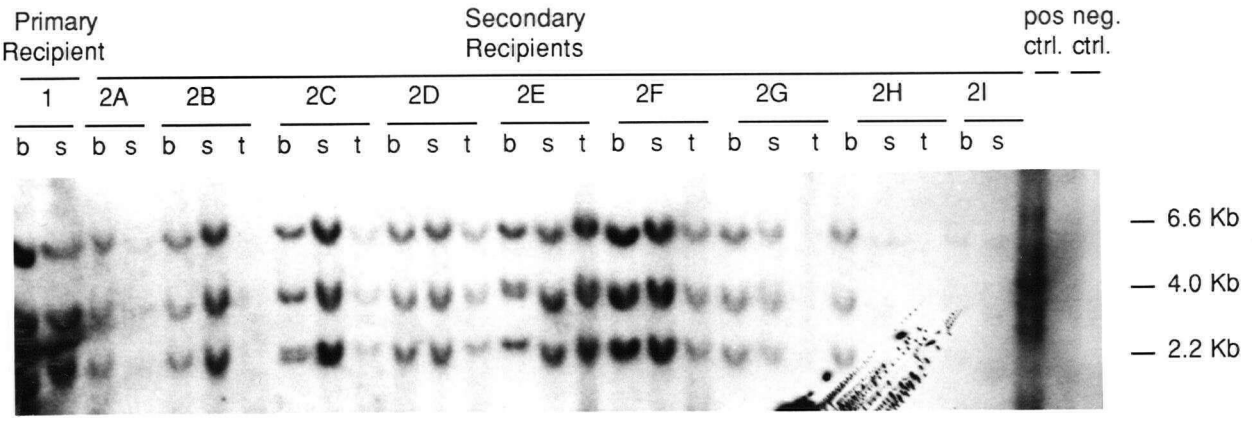

Figure 5.2. Demonstration of CD24 provirus in bone marrow (B), spleen (S), and thymus (T) DNA from primary and secondary transplant recipients. Recipients received 10^5 $CD24^+$ selected cells in combination with 10^5 normal marrow competitor cells. Individual mice are labeled as 1 for the primary recipient (sacrificed at 11 months post-transplant), or 2A-I for secondary recipients (sacrificed at 13 weeks post-transplant). DNA (15 μg) from each tissue sample was digested with Eco RI, an enzyme that cuts once within the CD24 proviral sequence. Shown are results of a blot probed with a ^{32}P-labeled fragment of the neo^R gene. The positive control represents DNA obtained from CD24 viral producer cells which contain > 10 proviral copies.

even 8 months post-transplant in spite of, indeed perhaps because of, a complete recovery of later cell types including day 12 CFU-S as well as cells detectable as CFC. Interestingly, there was, nevertheless, a quantitative relationship between the extent of amplification seen in donor CRU numbers and both the size of the initial transplant and its ontological source.

It has been suggested by several authors that transplantable stem cells may fail to regenerate the stem cell compartment to normal (non-transplant) levels because of an inherently limited capacity for self-renewal (103, 105, 109). According to such a model, the absolute extent of CRU amplification would be anticipated to decrease as the number of stem cells transplanted was decreased since the number of stem cells with the highest self-renewal potential would also decrease proportionately. On the other hand, it is possible that the transplantation of smaller numbers of marrow cells might place a higher "stress" on the system resulting in the production of stimuli that could favor differentiation rather than self-renewal responses. Evidence of decreased stem cell regeneration under conditions that support their proliferation both in vitro (336), in utero (11) and after bone marrow transplantation (102, 103, 105, 109, 117) have been reported. However, such studies do not necessarily measure the capacities of the cells tested but rather their response under a given set of molecularly undefined and poorly understood environmental conditions. The present studies, which have used a quantitative assay to provide a direct measurement of the size of the regenerated totipotent, transplantable stem cell compartment indicate that a higher degree of stem cell amplification is obtained following the transplant of smaller numbers of stem cells as compared to the transplant of larger numbers of stem cells, even though this is insufficient to achieve a comparable level of regeneration of the stem cell population by comparison to its size in unperturbed animals (Table 5.3 and Figure 5.1). Thus, the extent to which either fetal liver or adult bone marrow stem cells express their full regenerative potential varies according to how many of them (and/or accompanying marrow cells) are transplanted and this decreases with innoculum size. A possible explanation for this finding would be the activation of negative feedback regulatory mechanisms in vivo that can limit stem cell expansion prematurely, perhaps via the production by mature hemopoietic cells of such factors as MIP-1α and TGF-ß that may selectively decrease the proportion of

primitive hemopoietic cells in cycle (112-115, 337) such factors might thus attenuate or even terminate CRU expansion even though the numbers of these cells might still be significantly below the "normal" level. It is interesting to speculate that this effect might be promoted by the co-transplantation of large numbers of mature hemopoietic cells or their immediate precursors. Such a possibility is consistent with the observation that the maximum degree of CRU expansion was observed with the smallest transplant dose. Accordingly, it would be anticipated that stem cell expansion might be enhanced when purified stem cells are transplanted to reduce the number of mature cells present during the initial stages of engraftment.

It is important to note that in these experiments only the regeneration of donor Ly5.1$^+$ CRU were quantified. My results clearly show that the transplanted Ly5.1$^+$ CRU were unable to reconstitute the CRU compartment of primary transplant recipients to levels found in normal adult mice. However, the extent to which 10 Ly5.2$^+$ CRU present in the competitor cell population or those surviving in the host might have contributed to regeneration of the total CRU compartment is not known. It seems highly unlikely that a large reserve of inactive Ly5.2$^+$ CRU would have been present in the primary recipients since the vast majority of all the cells in the marrow and the peripheral blood of these mice were Ly5.1$^+$.

Results from many previous studies have indicated that the regenerative behavior of hemopoietic cells in a transplant setting is a function of their ontological state. Thus, over 20 years ago, it was found that a transplant of fetal liver cells would outcompete adult bone marrow following their combined transplantation into recipient mice (88). Similarly, 10 years later, it was shown that day 8 CFU-S derived from fetal liver possess a greater capacity for self-renewal as compared to their adult bone marrow counterparts (87). Recently, Rebel et. al. (335) have shown that limiting numbers of fetal liver CRU are able to produce a greater output of mature blood cells in vivo as compared to adult bone marrow. Moreover, when

marrow cells from primary recipients of limiting numbers of fetal liver CRU were injected into secondary recipients, a significantly higher percentage of these mice showed donor-derived reconstitution of their lymphoid and myeloid compartments as compared to mice that had received marrow cells from primary recipients of similar numbers of adult bone marrow CRU. These results are thus also highly suggestive of a greater regenerative capability of fetal liver HSC by comparison to adult bone marrow. However, because of their design, none of these studies could discriminate between quantitative differences in the self-renewal of transplantable HSC and possible differences in the extent of proliferation achieved by their more differentiated progeny. By addressing this question here, it has been possible to establish that fetal liver CRU are indeed superior to their adult bone marrow counterparts both in terms of the relative and absolute numbers of CRU they will regenerate under similar conditions.

A possible explanation for these differences is that fetal liver CRU possess a greater intrinsically regulated probability for self-renewal when stimulated to divide in the microenvironment of the post-transplant myeloablated mouse. Intriguing evidence of candidate genes that may be involved in the intrinsic control of self-renewal was recently reported by Sauvageau et. al. from studies of the effects of Hox genes on hemopoiesis in vivo (91). These showed that overexpression of HOXB4, whose expression is normally restricted to the most primitive adult bone marrow cells in the adult (91) resulted in a 50-fold increase in the regeneration of transduced CRU as compared to neo-transduced control cells. However, the possibility that the genes expressed within fetal liver and adult bone marrow HSC influence this self-renewal probability does not preclude other mechanisms that might influence the observed differences in the rates of fetal liver and adult marrow CRU amplification. For example, these may also exhibit differences in the time required to transit one complete cell cycle (338) or in the proportion of cells

recruited or maintained within the microenvironment of the marrow of the adult mouse.

The use of recombinant retroviruses as genetic tags has been used extensively to track the proliferative and differentiative behavior of individual stem cell clones both in vitro (49) and in vivo (98, 99). In the present study I utilized a recombinant retrovirus containing the coding region of the human CD24 cell surface antigen to confirm the totipotentiality of the CRU detected using the CRU assay, and to aid in the verification and quantification of the degree of expansion exhibited by individual CRU in vivo. Only one proviral banding pattern was detected in bone marrow and thymic DNA in the primary and all secondary transplant recipients (Figure 5.2), highly suggestive that the regeneration of the stem cell compartment in the primary recipient was monoclonal in nature. This clone regenerated the CRU compartment to 3.7% of normal level, comparable to that observed in recipients of 10 adult bone marrow CRU. This represents a CRU expansion of 370-fold, which was higher than that observed for any transplant dose of fetal liver or adult bone marrow (Table 5.3). Differences in estimates of CRU expansion at the level of the whole population versus individual clones likely reflect heterogeneity amongst the regenerative activity displayed by biologically equivalent individual HSC responding to proliferative stimuli (19, 89, 99, 101, 339). However, at the same time the present results confirm the ability of totipotent repopulating stem cells to undergo self-renewal divisions during clonal expansion in vivo and demonstrate the enormous regenerative potential that some stem cells may therefore possess.

The results of this study have important implications for bone marrow transplantation efforts. A sufficiently quantitatively or qualitatively impaired pool of hemopoietic stem cells could influence the longevity of a patient's graft, and further, might reduce tolerance to cytotoxic agents or other circumstances which would impose a proliferative demand on the stem cell pool. This study thus highlights the

importance of optimizing stem cell numbers in bone marrow transplants and suggests potential consequences of transplanting different numbers or sources of stem cells in protocols seeking to rescue, or, alternatively, to genetically modify the marrow. These studies also set the stage for attempts to enhance CRU regeneration post-transplant by the administration of exogenous agents or the expression of transduced intracellular factors that may enhance the apparent regenerative potential of stem cells expressed under a defined experimental condition in vivo.

CHAPTER 6

DISCUSSION

The use of recombinant retroviruses to transfer exogenous genes into HSCs has provided significant insight into the organization and regulation of the hemopoietic system and has played a central role in the emerging field of gene therapy. However, many important questions remain unanswered regarding the nature and regulation of totipotent HSCs, including a basic understanding of their usage over time, their potential for self-renewal and proliferation, and the genes encoding extracellular and/or intracellular factors which are responsible for regulating these biological characteristics. Although the ability to genetically manipulate HSCs using recombinant retroviruses provides a powerful tool to begin to address these issues, the poor infection efficiency to HSCs remains an obstacle. The overall goal of the work presented in this thesis was to develop methodologies to enhance the utility of current gene transfer protocols for the efficient genetic manipulation and tracking of HSCs and to utilize these procedures to more clearly define the self-renewal potential of HSCs following bone marrow transplant.

Work described in Chapter 3 demonstrates the use of the CD24 cell surface antigen as a dominant selectable marker in a recombinant retroviral vector in combination with FACS to enable the rapid and non-toxic selection of retrovirally transduced murine bone marrow cells including in vitro clonogenic progenitors, day 12 CFU-S and totipotent repopulating stem cells. The presence of totipotent repopulating stem cells within the $CD24^+$ fraction (Chapters 3 and 4) demonstrates that the MPSV and MSCV LTR regulatory elements are able to drive high-level gene expression in the most primitive hemopoietic cells present in adult marrow tissue. The ability of these elements to drive gene expression in primitive hemopoietic cells was also directly confirmed in studies described in Chapter 4 with the analysis of CD24 expression among a Sca^+Lin^- subpopulation of bone marrow cells. These observations have a number of important implications. The

ability to achieve high and sustained levels of transferred gene expression in primitive hemopoietic cells is essential for studies aimed at testing, through overexpression, genes encoding molecules which may be involved in regulating the survival, mobility, cycling, proliferation, differentiation, or self-renewal of HSCs. Moreover, the ability to obtain a population of cells in which 100% are provirally marked should enhance the detection of phenotypes which may be associated with the overexpression of potential putative stem cell regulatory molecules. The overexpression of HoxB4 has been shown to increase the regenerative ability of HSCs following bone marrow transplant (91). Additional molecules which would be of interest to test using this procedure include the ligand for the flt3/flk2 receptor, since the expression of this receptor is restricted to primitive hemopoietic cells (137), and VLA-4 whose interactions with VCAM-1, fibronectin and L-selectin have been implicated in mediating the in vivo homing of primitive hemopoietic progenitors to the marrow and spleen (155).

One intriguing finding described in Chapter 4 of this thesis was the detection of vector related differences in the persistence of expression of the transferred CD24 gene following extended periods of time in vivo. A greater proportion of $CD24^+$ lymphocytes, granulocytes/macrophages, erythrocytes and Sca^+Lin^- stem cell candidates were observed in the recipients of MSCVCD24 transduced bone marrow as compared to JZenCD24 transduced marrow despite the detection of roughly equivalent levels of intact recombinant provirus. One explanation for this is shutdown of the viral (JZen) LTR regulatory elements in vivo, a phenomenon which has been previously reported (215, 251), and which may be the result of methylation of the promoter and/or enhancer elements (257, 331). In addition, differences in the expression of the transferred CD24 gene were detected among cells of different lineages. The proportion of $CD24^+$ thymocytes was significantly lower than that observed among cells of either the myeloid (bone marrow) or erythroid lineage (Chapter 4). Together, these results suggest that despite efforts to

construct/identify vectors able to drive high and persistent levels of gene expression in all which contain recombinant provirus (irrespective of cell lineage), this goal has not yet been realized. Nevertheless, the effectiveness of CD24 as a means to rapidly quantify the levels of transferred gene expression attainable from different vectors suggests that this marker would be useful in efforts to identify regulatory elements that maximize the levels and persistence of transferred gene expression in HSCs or any other target cell of interest. Recently, a novel vector called MFG (Richard Mulligan, Whitehead Institute for Biomedical Research, M.I.T., Cambridge, MA) has been shown to provide higher levels of transferred gene expression than Moloney murine leukemia virus based vectors (340). In addition, Dr. Donald Kohn and his colleagues (Childrens Hospital, Los Angeles, CA) are currently attempting to solve the problem of promoter shutdown in vivo by identifying and removing potential methylation sites present in vectors based on the Moloney murine leukemia virus. It would be interesting to use CD24 to quantify and compare the levels and persistence of gene expression obtainable from these vectors as compared to JZen and MSCV.

CD24 would also be of use in efforts to rapidly screen alternative retroviral infection protocols involving the use of newly discovered hemopoietic growth factors (eg. flt3/flk2 ligand), or newly developed strategies such as those involving the use of fibronectin (described in detail on page 37-38) or the "flow-through" viral transduction system (described on page 38). As an example of this, Dr. Craig Jordan (Somatix, Alameda, CA) is currently using CD24 as a marker of gene transfer in combination with FACS in an effort to correlate the cycling status of primitive human hemopoietic cells with the efficiency of gene transfer (personal communication).

The selection approach described and utilized in Chapters 3 and 4 has now been extended to the human setting. Using retroviral vectors containing HSA, the murine homologue of CD24, in combination with FACS, Conneally et. al. has

demonstrated the ability to enrich for retrovirally transduced primitive human hemopoietic cells, including LTC-IC (275). Efforts to determine whether transduced cells capable of repopulating immunodeficient mice can be enriched for using this technique are currently underway. In collaborative efforts with Dr. Stefan Karlssons' lab (National Institutes of Health, Bethesda, MD) the selection approach developed in Chapter 3 has been applied to test the therapeutic potential of vectors aimed at the genetic therapy of Gauchers disease (a human autosomal recessive lysosomal storage disorder caused by a deficiency of the enzyme glucocerebrosidase). A retroviral vector containing the cDNA encoding HSA as a selectable marker in combination with a therapeutic glucocerebrosidase (GC) cDNA was constructed and used to infect transformed B cell lines from Gaucher patients. While retrovirally transduced, unselected B cells showed a slight increase in the level of GC enzyme as compared to untransduced B cells, FACS selection of HSA^+ B cells enhanced GC enzyme levels to more than 5-fold over untransduced cell levels, and even to levels more than 2-fold over those in normal non-Gaucher cells (341). Such strategies based on this approach may enable the implantation of transduced cells to animals or patients, although the use of cell surface antigens as markers for human gene therapy remains controversial. Nevertheless, this approach should prove useful in preclinical studies (eg. using non-human primates or immunocompromised mice) aimed at optimizing the therapeutic potential of vectors for use in human gene therapy trials. The observation that the CD24 cell surface antigen can be expressed on red blood cells suggests potential for this molecule as a selectable marker in vectors aimed at the genetic treatment of sickle cell anemia and thalassemias. In collaborative efforts with Dr. Philippe LeBoulch (M.I.T., Boston, MA), Dr. Connie Eaves (Terry Fox Laboratory, Vancouver, B.C.) and Dr. Ronald Nagel (Albert Einstein College of Medicine, Bronx, NY) CD24 is currently being employed in retroviral constructs containing a human δ/β-globin

fusion gene designed for anti-sickling properties with the goal of testing these in a β^S (sickle) transgenic mouse model.

The low efficiency of gene transfer to HSCs has made efforts to track the utilization, proliferation and self-renewal of individual HSCs using retroviral marking techniques extremely laborious. The ability to obtain, prior to transplant, a population of cells in which 100% are provirally marked would increase the power and ease of retroviral marking studies aimed at addressing these issues. Results presented in Chapter 4 demonstrate that the CD24 selection approach can enable the isolation of a population of hemopoietic target cells in which virtually 100% are provirally marked and thus, allows one to track the behavior of all HSCs transplanted. The power of this approach to track individual HSCs is demonstrated in Figure 4.3. The transplant of exclusively provirally marked HSCs enabled the determination of whether hemopoiesis was polyclonal (top panel) or monoclonal (bottom panel), and also allowed the accurate determination of the number and relative contribution of each HSC to hemopoiesis in the primary recipient at the time of sacrifice. The dynamics of HSC utilization under homeostatic conditions remains unresolved. One approach to addressing this question would be to sequentially sample the peripheral blood of mice transplanted with retrovirally transduced HSCs obtained following the CD24 selection procedure. Studies using sequential analysis of peripheral blood to track individual HSCs in mice transplanted with retrovirally transduced (non-selected) marrow have been described (98, 99). Alternatively, a procedure has been described whereby femoral marrow can be sequentially obtained from a mouse by inserting a needle into the knee joint of an anesthetized mouse (342). Sufficient quantities of DNA for Southern blot analysis is obtained by using the extracted marrow to generate day 12 CFU-S in secondary recipient mice. The use of this procedure in combination with the CD24 selection approach would enable the contribution of individual HSCs to hemopoiesis to be tracked, theoretically, for the entire life of the recipient

mouse and should provide insight into the number and persistence of HSCs contributing to hemopoiesis under homeostatic conditions.

The results of transplant studies presented in Chapter 5 reveal the inability of HSC numbers to recover to normal levels following the transplant of even large numbers of adult bone marrow or fetal liver stem cells. These results may have relevance to clinical bone marrow transplantation studies. Although clinical bone marrow transplantation has been performed for only 20 to 25 years, today it constitutes an major element of therapy for the treatment of numerous forms of cancer as well as heritable hematological disorders. Indeed, over 6,000 autologous bone marrow transplants were performed in North America in 1994 alone (personal communication: International Bone Marrow Transplant Registry). Might the poor recover of stem cell numbers following bone marrow transplant be a contributing factor to marrow failure? It will be important to monitor the recipients of bone marrow grafts for long periods to attempt to determine whether poor stem cell recovery plays a role in bone marrow failure and subsequent death of the patient. Moreover, the poor recovery of HSC numbers following bone marrow transplant should be taken into consideration when attempting human gene therapy, which may require the selection of transduced stem cells, as well as the transplant of fetal liver or umbilical cord blood HSCs since both may require the transplant of modest numbers of cells into the patient.

The ability to transplant HSCs in which 100% are provirally marked provided intriguing insight into the regenerative capacity of HSCs following bone marrow transplant (Chapter 5). Proviral integration analysis of mice receiving retrovirally transduced, $CD24^+$ selected, bone marrow cells provided evidence for a >300-fold clonal amplification of a single transduced stem cell. A number of questions are raised by this finding. Do all HSCs possess such a capacity for regeneration? If so, what factors or circumstances will enable this potential to be fully realized following transplant? These findings set the stage for attempts to maximize the recovery of

HSCs numbers following bone marrow transplant through the administration of exogenous agents such as hemopoietic growth factors or the expression of transduced genes encoding intracellular factors. It would be interestingly to determine whether the administration of the "stem cell" factors such as Steel factor and/or FL following bone marrow transplant may increase the level of regeneration of stem cell numbers. Alternatively, such an experimental strategy could be utilized to test the role that particular genes, found to be expressed in primitive hemopoietic cells, may play in regulating the self-renewal or commitment of these cells by overexpression using recombinant retroviral vectors combined with the use of a dominant selectable marker gene such as CD24.

In summary these studies have provided methods which enhance the utility of current gene transfer protocols and have for the first time provided quantitative data regarding the regeneration of HSC numbers following the transplant of various numbers of HSCs from various ontological sources. These studies set the stage for future experiments designed to provide further insight into the behavior and biological potential of HSCs as well as studies aimed at developing improved vectors for use in gene therapy.

CHAPTER 7

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