GENETIC MODIFICATION OF HUMAN HEMATOPOIETIC STEM CELLS

by

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ABSTRACT

Human hematopoiesis originates in a population of stem cells with transplantable lympho-myeloid reconstituting potential but an *in vivo* method for quantitating these cells has not been available. In this study an assay was developed that allows human cord blood (CB) cells with in-vivo repopulating potential to be quantitated. It is based on the ability of immunodeficient mice to be engrafted by intravenously injected human hematopoietic cells and uses limiting dilution analysis to measure the frequency of human cells that produce both lymphoid and myeloid cells in the marrow of the recipient mice. The frequency of human competitive repopulating units (CRU) was shown to be ~1 per $6x10^5$ light density CB cells, 1 per 900 $CD34^+CD38^-$ CB cells and 1 per 18,000 $CD34^+CD38^+$ CB cells. In addition, it was demonstrated that under selected culture conditions, a significant expansion of both CRU and Long-Term Culture-Initiating Cells (LTC-IC) could be obtained. The ability to reliably transfer genes into hematopoietic stem cells remains an important but elusive goal. To date the power of recombinant retroviral gene transfer has been severely compromised by the low efficiency of retroviral infection. A series of experiments was undertaken to develop improved, clinically applicable, gene transfer conditions. These focused on the use of retroviral supernatant and the use of fibronectin coated dishes. The applicability of this protocol was tested by infecting CB cells capable of repopulating immunodeficient mice. The gene transfer efficiency as determined by G418-resistance to CRU and LTC-IC was 17 ± 3% and 17 ± 8 respectively. There was a significant correlation between the gene transfer to LTC-IC and CRU, however there was no correlation between gene transfer to CFC and LTC-IC or CFC and CRU. To further optimize the utility of recombinant retroviruses, the murine heat stable antigen (HSA) a cell surface antigen was developed as dominant selectable marker in a retroviral vector

to enable the identification and selection of retrovirally marked human hematopoietic cells. Using this strategy, virtually pure populations of transduced hematopoietic cells including LTC-IC could be specifically isolated on the basis of their ability to express the transferred HSA gene.

Taken together these studies provide a means to quantitate human in vivo repopulating cells and describe culture conditions that allows their modest expansion. The results indicate the utility of the NOD/SCID model for optimizing gene transfer to human repopulating cells. Additionally these studies have provided procedures which will allow purification of genetically modified cells ex vivo and the subsequent tracking of infected hematopoietic cells following transplantation.

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Abbreviations

BFU	burst-forming unit
BIT	Bovine serum albumin, insulin and transferrin
BM	bone marrow
CB	cord blood
CFC	colony forming cells
CFU-C	colony-forming units-culture
CFU-S	colony-forming unit-spleen
CRU	competitive repopulating unit
CSF	colony stimulating factor
CY5	cyanine-5-succinimidyl
DMEM	Dulbecco's modified Eagle's medium
dpc	days post coitum
Epo	Erythropoietin
FACS	Fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorscein isothiocyanate
FL	flk-2/flt-3 ligand
GF	growth factor
HFN	Hank's balanced salt solution with 2% fetal calf serum and 0.02% sodium azide
HIV	Human Immunodeficiency Virus
HSA	Heat Stable Antigen
HPC	hematopoietic progenitor cells
HSC	hematopoietic stem cells
IL	interleukin
LTC	long-term culture
LTC-IC	long-term culture-initiating cell
LTR	long terminal repeats
MoAbs	monoclonal antibodies
MDR-1	multi-drug resistance gene
NOD/SCID	nonobese diabetic-*scid/scid*
NK	natural killer
PBL	peripheral blood lymphocytes
PBPC	peripheral blood progenitor cells
PCR	Polymerase chain reaction
PDGF	platelet derived growth factor
PE	phycoerythrin
PI	propidium iodide
SCID	Severe combined immunodeficient
SF	steel factor
Tpo	thrombopoietin
YS	yolk sac

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Chapter 1 Introduction

1.1 Overview of hematopoiesis

The main focus of this thesis was the development of strategies to improve retroviral gene transfer efficiencies into human hematopoietic stem cells (HSC). Somatic gene therapy approaches are being used for the treatment of classical genetic diseases and increasingly for the development of novel approaches for the treatment of malignant disease. In addition, the introduction of new genetic material into hematopoietic cells represents a powerful approach for analyzing the activity of genes regulating hematopoietic differentiation and regulation. To achieve either goal, reproducible and reliable gene transfer efficiency is a prerequisite. The target cells for many applications of gene transfer are HSC with long-term engrafting potential. Even today, our understanding of how to measure and manipulate these cells limits the successful clinical exploitation of gene transfer-based therapies. It is with this perspective that the following review of current knowledge of the quantitation and regulation of HSCs has been written.

Hematopoiesis is a complex developmental process. It involves the differentiation of at least 12 distinct cell lineages from a small population of pluripotent stem cells and these changes occur over many cell generations. Thus, hematopoiesis may be considered as a hierarchical process in which primitive pluripotent stem cells give rise to differently regulated, lineage-committed progenitor cells, which ultimately produce the various types of mature end cells that circulate in the peripheral blood. The retention of a hematopoietic-committed but lineage-unrestricted state and vast proliferative potential through successive cell divisions is referred to as hematopoietic stem cell self-renewal. Conversely, hematopoietic stem cell differentiation can then be envisioned to include any change that contributes to an irreversible

reduction in one or more of these potentialities. However, these terms (self-renewal and differentiation) remain necessarily vague as the nature, multiplicity and potential interrelationships of different mechanisms that may be involved in irreversibly restricting hematopoietic stem cell functions are still largely unknown. Under normal conditions the numbers of differentiated cells in the blood remain relatively constant, however under conditions of stress or increased demand, (e.g., hemorrhage, or following chemotherapy), rapid changes in cell number are observed, which is followed by a return to normal levels when the stress is relieved. These features reflect the existence *in vivo* of complex feedback control mechanisms that operate throughout the hematopoietic hierarchy. Although much progress has been made in identifying a variety of hematopoietic growth factors/cytokines that can singly or in combination influence various properties of primitive hematopoietic stem cells e.g., self-renewal, cell cycle progression or differentiation, the genetic mechanisms that are responsible for stem cell maintenance, activation and lineage commitment are largely unknown.

1.1.2 Development of the hematopoietic system

For over 25 years it had widely been accepted that the extraembryonic yolk sac (YS) where the first differentiated hematopoietic cells are detected, was the source of the founder HSCs for the fetal liver and subsequently the adult bone marrow (BM) (Moore and Metcalf, 1970). However studies using avian and amphibian embryos have demonstrated that an independent source of hematopoietic activity functions within the embryo proper (Dieterlen-Lievre, 1987). In these species it has been demonstrated that the YS-derived hematopoiesis is transitory and does not supply the adult with full hematopoietic potential (Zon, 1995). In the early ontogeny

of the mouse, hematopoiesis is similarly initiated within the extraembryonic mesoderm of the YS with primitive hematopoiesis and committed hematopoietic progenitors detected in the YS as early as 7-8.5 days post coitum (dpc) (Medvinsky et al., 1993). However definitive evidence that from 8-9 dpc the intraembryonic para-aortic splanchnopleura also contains progenitor cells capable of differentiating into both myeloid and lymphoid lineages has also being obtained in the last few years (Godin et al., 1995). In experiments that utilized a whole organ culture system, HSCs capable of long term repopulation of the entire hematopoietic system were not found in the intraembryonic aorta-gonads-mesonephros (AGM) region until the beginning of day 10. By 11 dpc, long-term repopulating-HSC activity is found in both the YS and the fetal liver (Medvinsky and Dzierzak, 1996). It thus appears likely that HSC appearing in the YS and in other tissues of the embryo at day 11 are the result of dissemination of long-term repopulating-HSCs from both the YS and the AGM (Delassus and Cumano, 1996). Following colonization of the fetal liver by the AGM HSCs, further expansion and maturation is thought to occur. This includes changes in HSC phenotype (Sanchez et al., 1996). Thereafter, there is seeding of the developing lymphoid organs and the marrow. In the adult, the proliferation and differentiation of hematopoietic cells continues in these latter sites. While comparative human developmental data is limited, in 5-week human embryos a dense population of $CD34^+$ cells (exhibiting a similar anatomic location as the murine intraembryonic AGM region) has been identified. These cells are associated with the ventral endothelium of the aorta and have been shown to have multi-lineage clonogenic potential suggesting that the human species may also have an intraembryonic source of definitive hematopoiesis (Tavian et al., 1996).

It has been known for many years that the hematopoietic cell differentiation process changes during ontogeny. This involves changes not only in the expression of a variety of lineage-specific genes (e.g., in developing erythroid cells (Fantoni et al., 1981), T cells (Ikuta et al., 1990) and B cells (Hardy and Hayakawa, 1991; Li et al., 1993)), but also in the surface markers expressed on these cells or on their more primitive pluripotent precursors (Terstappen et al., 1991; Huang and Terstappen, 1994; Traycoff et al., 1994; Morrison et al., 1995; Rebel et al., 1996b). In addition to changes in phenotype, several studies have documented ontogeny-related functional differences between populations of primitive hematopoietic cells (Harrison, 1983; Hogge et al., 1996). For example in transplantation experiments using fetal liver as a source of hematopoietic cells, both stem cells and early hematopoietic cells demonstrate higher levels of self-renewal and greater proliferative potential than their adult BM counterparts (Micklem et al., 1972; Zucali, 1982; Rebel et al., 1996a). Explanations for such differences include the possibility that fetal liver HSC may have an intrinsically higher probability of self-renewal than their adult BM counterparts. Other potential mechanisms involve differences in cell cycle transit time, as suggested by Schofield et al (1970) or differences in homing mechanisms. Similarly, *in vitro* and *in vivo* studies of umbilical cord blood (CB) have demonstrated a higher replating potential and proliferative potential than equivalent progenitors in adult BM (Lu et al., 1993b; Vormoor et al., 1994). In addition, differences in turnover have been demonstrated in cytokine-stimulated cultures of PKH26 fluorescence in human fetal liver, CB and adult BM, with fetal liver demonstrating the highest turnover rate (Lansdorp et al., 1993). Recently, profound differences in the types of growth factors that elicit proliferation (and/or differentiation) responses of both lineage-restricted progenitors and their more primitive precursors from embryonic and adult tissues have been

discerned in studies of cells from both murine and human sources (Rebel and Lansdorp, 1996; Nakano et al., 1996; Miller et al., 1997; 1998 et al., 2001). These ontogeny-related changes have more recently assumed increased importance with the recent introduction of the use of CB as a clinical resource.

1.1.3 Purification, quantitation and characterization of hematopoietic stem cells

Much of the information concerning the biology and functional characterization of HSCs has been derived from studies of their behavior both *in vivo* and *in vitro*. The use of indirect functional assays (described below) and the results of these studies, which have indicated that the frequency of HSC is low in both human and murine cell populations, has greatly spurred efforts to develop methods for obtaining pure populations of HSCs as these would greatly facilitate their molecular and functional characterization. The purification of primitive HSC both from murine and human sources would not, however, have been possible without the prior development of functional assays to quantitate specific subsets of progenitors present in heterogeneous cell populations. Therefore the field of cell purification and HSC quantitation are inextricably linked.

A number of strategies, including physical techniques, immunological methods and supravital staining have been developed to enrich for HSCs. Those based on physical characteristics separate cells on the basis of size and/or buoyant density (Worton et al., 1969a; Haskill et al., 1970; Frickhofen et al., 1982; Sutherland et al., 1989). Using these techniques, HSCs have generally been characterized as relatively small, low density cells with an undifferentiated blast cell-like morphology (Ploemacher and Brons, 1989; Jones et al., 1990). With the introduction of flow cytometry, light scatter properties of cell populations were

found to reflect cell size (forward light scatter) and granularity (orthogonal light scatter) (Van Den Engh and Visser, 1979). HSCs were found to have medium to high forward scatter (which is in agreement with earlier density separation studies) and low to medium orthogonal light scatter properties (Szilvassy et al., 1989b; Sutherland et al., 1989).

The main tools of immunological purification methods are monoclonal antibodies (MoAbs) which specifically bind to cell surface molecules on the target cells. The use of MoAbs has enabled the enrichment of purified stem cells on the basis of both positive and negative selection procedures. Positive selection procedures involve the selection of cells that express specific cell surface antigens. Negative selection procedures are used to remove cells which are not of interest. To date there is no unique MoAb that detects only HSCs and therefore they are identified by the presence or absence of a combination of surface markers. Results of a number of studies, largely derived from reconstitution assays (described below), show that the following surface phenotype is usually associated with murine HSCs: they express high levels of Ly-6A/E (Sca-1) (Spangrude et al., 1988) and H-2K antigens (Szilvassy and Cory, 1993) and Wheat Germ-Agglutinin-binding activity (Rebel et al., 1994), low levels of c-kit receptor (Katayama et al., 1993) and Thy-1 (Spangrude et al., 1988). In addition, they are largely negative for lineage markers associated with terminal differentiation (lin^-), such as B220, CD3, CD4 and CD8 which detect lymphocytes, Mac-1 and GR-1 which detect myeloid cells, and TER 119 which is expressed on erythrocytes (Spangrude et al., 1988).

The phenotypic characterization and purification of human repopulating HSCs has been hampered by a lack of direct assays for these cells, however the finding that clinical transplants of $CD34^+$ enriched BM or peripheral blood progenitor cells (PBPC) cells give timely hematopoietic reconstitution in patients undergoing autologous transplantation

suggests that human cells with repopulating activity are $CD34^+$ (Berenson et al., 1991; Shpall et al., 1994). CD34 is a surface glycophosphoprotein which is detectable on the majority of CFC and LTC-IC but not on more differentiated cells (Krause et al., 1996). The extent of CD34 expression on hematopoietic cells progressively decreases as the cells differentiate (Civin, 1992). As a result, there are detectable differences between the expression of CD34 on LTC-IC and CFC (Sutherland et al., 1989). The endothelial form of the CD34 antigen has been shown to bind L-selectin, the lymphocyte homing receptor, (Baumhueter et al., 1993) and thus it has been suggested that it may play a role in stem/progenitor cell localization /adhesion in the BM (reviewed in (Krause et al., 1996)). $CD34^+$ cells are heterogeneous both functionally and in terms of other markers they express, and as in the murine system, the use of combinations of other cell surface markers have been used to obtain cell fractions that are more highly enriched for primitive cells. Thus the fraction of human hematopoietic cells that express high levels of CD34 and are also Thy-1^+ (Baum et al., 1992; Craig et al., 1993; Murray et al., 1995), c-kit^{low} (Briddell et al., 1992; Kawashima et al., 1996), HLA-DR^- (Sutherland et al., 1989; Srour et al., 1991), $CD71^-$ (Lansdorp and Dragowska, 1992; Mayani et al., 1993b), $CD45RA^-$ (Lansdorp et al., 1990), and lin^- (Murray et al., 1995), are highly enriched in primitive cells. The use of various combinations of these antibodies has been found to allow the recognition and isolation of 0.01-0.1% of hematopoietic cells derived from normal human BM, peripheral blood or CB and yields populations of cells that are enriched in LTC-IC up to 500-to 1000-fold (Sauvageau et al., 1994; Petzer et al., 1996a). CD38, an antigen, which is absent on 1-10% of $CD34^+$ cells, has also proven useful in refining the phenotype of primitive hematopoietic cells (Bazan, 1990; Issaad et al., 1993; Sauvageau et al., 1994; Verfaillie et al., 1990; Rusten et al., 1994; Hao et al., 1995; Hao et al., 1996). CD38 is a

45kDa transmembrane protein that appears to play an important but not yet fully understood function in the regulation of lymphocytes. Analysis of the extracellular portion of CD38 indicates that it may allow attachment to the extracellular matrix (reviewed in (Shubinsky and Schlesinger, 1997)). LTC-IC are found within both $CD34^+$ $CD38^-$ and $CD34^+$ $CD38^+$ populations (Sauvageau et al., 1994; Hao et al., 1995). It has been shown, however, that the $CD34^+$ $CD38^-$ population is more enriched for cells that generate myeloid progeny for prolonged periods of time in LTC-IC assays as compared to the $CD34^+$ $CD38^+$ population (Hao et al., 1996). In addition, the $CD34^+$ $CD38^-$ population contains cells that can individually generate lymphoid and myeloid progeny (Berardi et al., 1997). More recently, the development of *in vivo* immunodeficient models of human hematopoiesis have allowed questions regarding the phenotype of human *in vivo* repopulating cells to be addressed, as described in Chapters 3 and 6 (Bhatia et al., 1997b; Conneally et al., 1997).

1.1.3.1 Colony-forming unit-spleen (CFU-S)

The concept that hematopoiesis originates throughout adult life from a population of pluripotent stem cells came initially from two lines of evidence. As early as 1951, Dameshek (Dameshek, 1951) noted that patients with myeloproliferative disorders affecting predominantly one blood cell lineage often showed enhanced activity in the marrow of other lineages and from these observations correctly deduced that this was due to the occurrence of an initial lesion in a hematopoietic cell with multilineage potential. A few years later, more direct evidence for the existence of pluripotent hematopoietic cells was provided by the finding that mice treated with lethal doses of irradiation developed BM failure and that this failure could be reversed by the injection of unirradiated BM cells (Ford et al., 1956). It was

later shown that these animals were restored in all hemato-lymphoid cell types by cells of BM donor origin (Micklem and Loutit, 1966). These studies set the stage for the subsequent discovery by Till and McCulloch (Till and McCulloch, 1961) of the ability of a rare subset of cells in such transplanted BM cell suspensions to form macroscopically visible nodules in the spleen of lethally irradiated mice. These cells are designated as colony-forming unit-spleen (CFU-S). Individual colonies were found to contain multiple lineages of differentiating cells including myeloid, erythroid and megakaryocytic cells. Later, a common origin of CFU-S and cells that have lymphoid potential was also obtained (Wu et al., 1968), although evidence of lymphoid cells within spleen colonies remained controversial for many years until more definitive methods for their detection became available (Lepault et al., 1993). The clonal nature of CFU-S was formally established in experiments involving the injection of mixtures of cells carrying different radiation-induced chromosomal markers (Becker et al., 1963).

Perhaps an even more important contribution from these pioneering studies was the demonstration of a linear relationship between the number of cells injected and the number of spleen colonies obtained (Till and McCulloch, 1961). This allowed the CFU-S assay to be established not only as a method for detecting multipotent hematopoietic cells, but also for quantifying their numbers in variously manipulated cell suspensions, a step that made their subsequent characterization possible. Such studies revealed extensive heterogeneity amongst CFU-S with respect to many of their phenotypic properties (Worton et al., 1969a; Mulder and Visser, 1987; Bertoncello et al., 1985; Ploemacher and Brons, 1988b). Eventually conclusive evidence was obtained to indicate that the time required to generate a macroscopically visible spleen colony could be linked to the differentiation potential of the cell from which the colony arose, its capacity to generate daughter CFU-S, and its ability to rescue recipients from

radiation-induced death (Hodgson and Bradley, 1979; Magli et al., 1982; Ploemacher and Brons, 1988a). The observed heterogeneity in CFU-S led to the development of the idea that a pre-CFU-S cell existed (Bertoncello et al., 1988; Ploemacher and Brons, 1988c). The further development of various types of quantitative progenitor assays and a comparison of the properties of the cells they detect, as reviewed briefly below, has thus played a major role in contributing to our current understanding of primitive hematopoietic cell behavior.

1.1.3.2 Long-term repopulating cells

Cell separation studies demonstrated that most CFU-S could be physically separated from cells with marrow repopulating activity (Ploemacher and Brons, 1989; Jones et al., 1990), although it was also shown that some mouse cells with long-term *in vivo* lympho-myeloid reconstituting potential could be detected as CFU-S (Dumenil et al., 1989; Wolf et al., 1993). Several assays have been proposed over the last decade that provide better estimates of cells with long-term repopulation potential. These cells can be defined functionally by expression of their ability to generate and sustain multi-lineage hematopoiesis after transplantation into a lethally irradiated or genetically compromised hosts (W/W^V) for an extended period of time. The existence of single cells with such potential were confirmed by using transplants of hematopoietic cells which were genetically marked by retroviral infection (Williams et al., 1984; Dick et al., 1985). In addition retrovirally marked cells from the initial transplanted mice could be shown to reconstitute additional generations of recipients (Keller et al., 1985; Lemischka et al., 1986) demonstrating the extensive proliferative potential of these cells and their ability to undergo self-renewal. In mice, an assay that makes use of limiting dilution analysis has been developed to allow both adult and fetal hematopoietic stem cells with

lympho-myeloid activity to be specifically identified and quantitated in histocompatible but genetically distinguishable recipients (Harrison, 1980; Szilvassy et al., 1990; Rebel et al., 1994; Harrison et al., 1993). To maximize the efficiency of detection of transplantable cells with these properties, the recipient mice are pretreated with a myelotoxic conditioning regimen and then transplanted with a minimal number of additional BM cells that is just sufficient to ensure the survival of the mice independent of the stem cell content of the test transplant they receive. The coinjected cells also serve to provide a basal level of competition to the stem cell(s) in the test transplant and this helps to improve the specificity of the assay. Accordingly, the cells detected using this procedure have been called competitive repopulating units (CRU) (Szilvassy et al., 1990). The ability to individually quantitate and manipulate HSC has made it possible to address many questions regarding properties, purification and regulation of transplantable HSC in the mouse (Szilvassy et al., 1989b; Szilvassy and Cory, 1993; Rebel et al., 1994; Miller et al., 1997; Pawliuk et al., 1996; Rebel et al., 1996b; Spangrude et al., 1988).

The existence of clonal human HSC with similar proliferative and multi-lineage differentiation potentialities was also first inferred from indirect evidence. This was provided by the demonstration of clonal hematopoiesis in women with a variety of disorders, including acute myeloid leukemia, chronic myeloid leukemia and other myeloproliferative disorders who were in addition heterozygous at the X-linked glucose-6-phosphate-dehydrogenase gene locus (Prchal et al., 1978; Martin et al., 1980; Raskind and Fialkow, 1987). Subsequently, a similar approach using other X-linked loci revealed the presence of monoclonal, or oligoclonal populations of lymphoid and myeloid cells of donor (female) origin in occasional recipients of allogeneic marrow transplants (Turhan et al., 1989) indicating the presence of a cell with both

lymphoid and myeloid reconstituting potential in normal adult marrow. Until recently, an *in vivo* assay to quantify human cells with these properties has not been available, however, with the observation that engraftment of primitive human hematopoietic cells can be obtained across species barriers (Zanjani et al., 1994; Dick, 1996) this has become an area of active investigation. Because of the need to be able to quantitate cells capable of *in vivo* reconstitution and to determine whether such cells can be maintained and infected with retroviruses the development of such an assay became one of the goals of this thesis (further described below and in Chapter 3).

1.1.3.3 Immunodeficient models of human hematopoiesis

Mice homozygous for the severe combined immunodeficiency (SCID) mutation initially described by Bosma et al (1983) lack functional B and T cells but have normal natural killer cell and myeloid function. Subsequent analysis of the defects in these mice have shown that they have an abnormality in the V(D)J recombination process necessary for both immunoglobulin and T- cell receptor gene rearrangement (Schuler et al., 1986; Malynn et al., 1988) and also have impaired DNA double-strand break repair (Fulop and Phillips, 1990). Subsequent studies identified a gene on human chromosome 8q11 which had the ability to complement the SCID aberration (Kirchgessner et al., 1995). In independent studies, SCID cells were shown to have a reduction in the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) protein and kinase activity which maps to the same location of chromosome 8 (Kirchgessner et al., 1995; Blunt et al., 1995; Peterson et al., 1995). More recently a mutation in cDNA isolated from cells of SCID mice has been identified which results in the truncation of the DNA-PKcs protein (Blunt et al., 1996; Danska et al., 1996; Araki et al., 1997).

As early as 1988 the characteristics of the SCID defect were exploited to develop models of human hematopoiesis and immunological development (McCune et al., 1988; Mosier et al., 1988; Kamel-Reid and Dick, 1988). In the intervening period these 3 approaches have been further developed to allow the transplantation of a variety of human cell sources into immunodeficient mice. The primary concept behind the SCID-hu and the hu-Peripheral Blood Lymphocyte (PBL)-SCID models was to create a small animal model useful for examining the pathogenesis and treatment of the disease caused by the human immunodeficiency virus (HIV) (McCune et al., 1988; Mosier et al., 1988). The hu-PBL-SCID (Mosier et al., 1988) model involves the intraperitoneal injection of human lymphocytes into SCID mice which leads to the selective survival of human $CD3^+$ T cells and smaller numbers of human B cells, monocytes and natural killer (NK) cells (Mosier, 1996), although the extent of engraftment in these mice is highly variable and donor-dependent (Torbett et al., 1991). Such grafts do not appear to be maintained by engraftment of a stem cell population as there are few multi-lineage progenitors or myeloid cells also present. In addition to being a model for HIV infection, the hu-PBL-SCID mouse serves as a model for the lymphoproliferative disease seen in immunocompromised transplant recipients and AIDS patients (Thomas et al., 1991), as grafts initiated from Epstein-Barr virus (EBV) seropositive donors frequently result in the development of "opportunistic lymphomas".

The second system, often described as the SCID-hu mouse, (McCune et al., 1988) uses implanted human fetal organs to provide a human microenvironment to support the further differentiation of primitive cell types. This model has been used primarily to define the phenotypic composition of human hematopoietic progenitor cells and in the evaluation of viral infectious diseases. The mostly commonly implanted tissues used to create the SCID-hu model

are human fetal thymus and liver (Thy/Liv, (Namikawa et al., 1990)) or fetal bone (Kyoizumi et al., 1992). Observations derived using these models include the following: When fetal livers and thymus are co-implanted, hematopoietic precursors from the fetal liver are able to repopulate the human thymus (Namikawa et al., 1990); Human T-cells are found in Thy/Liv implants and these show a hierachial distribution similar to those found in the normal fetal human thymus (Krowka et al., 1991). Although the SCID-hu Thy/Liv provided a model for human thymopoiesis, its utility in the evaluation of early hemapoietic progenitors was limited by the small number of cells present in the graft. To increase the utility of the model, normal fetal bone fragments were implanted under the mammary fat pads to generate the SCID-hu Bone mouse. This model has been shown to sustain human B lymphopoiesis and myelopoiesis for more than 20 weeks when unseparated cells were injected directly into the human bone fragment (Kyoizumi et al., 1992). In studies designed to examine the phenotype of the repopulating cell, grafts initiated with $CD34^{+}Thy^{+}Lin^{-}$ cells were found to have been repopulated at a high frequency and hematopoiesis was sustained for at least 8 weeks. In contrast, grafts initiated with $CD34^{+}Thy^{-}Lin^{-}$ cells were less frequently repopulated with human cells (Murray et al., 1995). In a further modification of the assay, (Fraser et al., 1995) transplanted fetal bone adjacent to a fetal thymic fragment. In these mice, donor derived B cells, myeloid cells and immature and mature T-cells were generated. In addition the SCID-hu model has been used for studies of HIV pathogenesis and is also amenable to the pre-clinical evaluation of anti HIV therapeutic modalities (McCune, 1996). Some limitations of this system include the fact that the human cells are commonly restricted to the fetal explants; few seed the BM or peripheral blood of the mice.

The third model recapitulates the steps in a standard BM transplant where human cells are injected intravenously into a sublethally irradiated host, and the cells subsequently migrate to the marrow cavities where they proliferate and differentiate (Kamel-Reid and Dick, 1988). In initial studies, recipient mice showed only low levels of engraftment with the injected human cells and this was dependent on the administration of exogenous growth factors. Subsequently it was demonstrated that if the donor cells originated in fetal tissue (cord blood, fetal liver or fetal BM) that human cytokine supplementation was not necessary for engraftment (Vormoor et al., 1994; Yurasov et al., 1997). Nolta et al, (1992) have used a similar system of transplantation but a different immunodeficient strain *bg/nu/xid* (BNX) as the recipients. They have further demonstrated that when these mice are co-transplanted with a human IL-3-producing fibroblast cell line, human myelopoiesis and T-lymphopoiesis can be sustained, albeit at low levels, for many months (Nolta et al., 1994). A major advance in the field came with the development of a more immunodeficient recipient mouse. The SCID mutation was back-crossed on the NOD/Lt strain background, resulting in the NOD/SCID mouse which has no B or T cells, reduced NK cell activity and defects in complement activity and macrophage function (Shultz et al., 1995). One of the disadvantages of this mouse strain is that the mice develop spontaneous thymic lymphomas at high frequency after 6 months. This limits the length of time that the animals can be followed in a given experiment (Shultz et al., 1995). Overall, these mice show higher levels of human hematopoietic cell engraftment with lower input cell numbers, are less dependent on exogenous cytokines, may not need preconditioning with irradiation (Lowry et al., 1996; Pflumio et al., 1996; Cashman et al., 1997) and studies using these mice have considerably advanced the field. Limiting dilution experiments (similar to those used to measure murine CRU) have now been undertaken to

quantitate the frequency and to identify the phenotype of human CRU. These experiments have been performed with both unseparated and highly purified human cells ($CD34^+CD38^-$ and $CD34^+CD38^+$) from adult BM, mobilized peripheral blood and cord blood using NOD/SCID mice as recipients (Wang et al., 1997; Bhatia et al., 1997b; Conneally et al., 1997). The results of these studies, as described in Chapter 3, have shown that the majority of NOD/SCID mice transplanted with limiting numbers of human cells from these sources will contain both lymphoid and myeloid cells of human origin indicating their common derivation from a human *in vivo* repopulating cell with lympho-myeloid potential (Conneally et al., 1997). More recently, evidence of the simultaneous production in the mice of human cells with the ability to engraft secondary mice with lymphoid and myeloid cells has also been obtained (Hogan et al., 1997; Cashman et al., 1997). Nevertheless, in spite of the readily detectable numbers of human B-lymphoid, granulopoietic, erythroid and megakaryopoietic progenitors generated in this xenogeneic transplant-model, the terminal differentiation of the human cells appears to be compromised. Moreover, this deficiency cannot be simply ascribed to a lack of species-specific cytokines as injections of such factors at doses that are clinically therapeutic in humans does not fully correct the decreased output of mature human cells in the mice (Cashman et al., 1997; Cashman et al., 1997). In addition, these animals have been used to create models of human leukemia. Such models are now being used to examine the phenotype and the frequency of the leukemia initiating cell in acute myelogenous leukemia (Lapidot et al., 1994; Bonnet and Dick, 1997).

An alternative model to immunodeficient mice is preimmune fetal sheep transplanted in utero with human hematopoietic cells (Zanjani et al., 1992; Zanjani et al., 1994; Civin et al., 1996). This system lends itself to longer term studies but is also less likely to gain widespread

use for analyses requiring human stem cell quantitation. The initial studies established that preimmune fetal sheep provide a suitable environment for the engraftment and long-term multi-lineage expression of human hematopoietic cells in a large animal model (Zanjani et al., 1992). In more recent studies this group have also examined the phenotype of the repopulating cell in these animals which was found to be enriched in the $CD34^+$ kit^{low} population. (Kawashima et al., 1996). Subsequent study of the phenotype of human marrow cells that are able to engraft primary and secondary sheep has demonstrated that both the $CD34^+CD38^-$ and $CD34^+CD38^+$ subsets are capable of repopulating primary sheep, whereas only the $CD34^+CD38^-$ cells yielded progeny that could also regenerate secondary animals (Civin et al., 1996).

1.1.3.4 Colony forming cells (CFC)

In vitro systems for supporting murine and human hematopoiesis have also been developed over the years and adapted in various ways to allow specific types of hematopoietic progenitor cells to be detected and quantified. The ability to culture hematopoietic cells *in vitro* was initially described by Pluznik and Sachs (Pluznik and Sachs, 1965) and Bradley and Metcalf (Bradley and Metcalf, 1966). These assays involved the growth of hemopoietic cells in a semi-solid matrix or agar which allows colonies of hematopoietic cells to be derived from single cells to be identified and characterized. The progenitors of such colonies were initially designated as colony-forming units-culture (CFU-C) prior to their characterization as a distinct population from CFU-S (Worton et al., 1969b). Subsequent studies showed that this methodology could be used to detect a variety of progenitor types that appear to represent biologically distinct stages of differentiation along each of the various myeloid lineages. Under

optimal assay conditions, lineage restricted CFC can be further categorized according to the size of the colonies they generate; the larger the colony, the more primitive the progenitor cell and the longer the time required until the clonal progeny complete their maturation (Eaves, 1995). Most CFC, while sometimes possessing considerable proliferative potential, are not capable of more than limited self-renewal *in vitro* (Metcalf and Moore, 1971), although during the initial phase of growth, a proportion of colonies which have the appearance of blast cells can give rise to daughter colonies on replating into secondary assays (Leary and Ogawa, 1987). Additional evidence that very few of these are likely to overlap with stem cells with long-term *in vivo* repopulating ability is provided by the finding that under current culture conditions the most primitive hematopoietic cells appear to lack the ability to proliferate *in vitro* when suspended in semi-solid medium, even when exposed to cytokines that efficiently stimulate their proliferation in liquid suspension cultures (Petzer et al., 1996a; Sitnicka et al., 1996).

1.1.3.5 Long-term culture-initiating cells (LTC-IC)

The long-term culture-initiating cell assay (Sutherland et al., 1990; Ploemacher et al., 1991; Breems et al., 1994) was developed based on the observation that granulocytes and macrophages are continuously produced for many months when hematopoietic cells are co-cultured with marrow cells in media containing horse serum and corticosteroids (Dexter et al., 1977; Gartner and Kaplan, 1980). Under such conditions, the nonhematopoietic mesenchymal precursors present at low frequency in marrow cell suspensions are stimulated to proliferate and form an adherent layer of stromal fibroblasts. The nature of the signals and the mechanisms by which stromal cells regulate stem cells remain to be defined. In LTC assays an

initial innoculum of test cells (either unseparated cells or a highly purified population) are incubated with a pre-established adherent marrow feeder or an adherent layer of stromal cells that provide the support and stimulation required by the original test cells. The endpoint in these assays is the detection of derivative clonogenic progenitors for periods exceeding the life-span of intermediate progenitors present in the initial innoculum and thus sufficient time must be allowed to elapse to allow these intermediate progenitors to have exhausted their proliferative potential. Early studies determined that this condition could be met after 4 weeks in murine studies and after 5 weeks in human assays (Sutherland et al., 1990; Ploemacher et al., 1991). To permit CFC outputs to be determined exclusively by the content of LTC-IC in input test cell suspensions, a pre-established feeder layer is used (Sutherland et al., 1990). Quantification of LTC-IC and assessment of changes in the variability or average output of CFC per LTC-IC can then be accomplished using limiting dilution analysis (Hogge et al., 1996; Sutherland et al., 1990; Udomsakdi et al., 1992).

In the standard LTC-IC assay, conditions have been optimized for detection of derivative myeloid clonogenic progeny, however the assay can be modified to detect lymphoid only progeny or to detect a lympho-myeloid cell using "switch" cultures (Lemieux et al., 1995; Lemieux and Eaves, 1996; Berardi et al., 1997). Several lines of evidence indicate that murine LTC-IC and CRU assays detect an overlapping cell population. This evidence includes the fact that both of these cell types have similarly low sensitivities to *in vitro* exposure to 4-hydroperoxycyclophosphamide by comparison to the majority of CFC (Sharkis et al., 1980; Porcellini et al., 1984; Udomsakdi et al., 1992), and that LTC-IC and CRU in both the fetal liver and adult BM of mice are phenotypically similar, exist at similar frequencies and co-purify (in contrast to all other known progenitor types detectable either *in vitro* or *in vivo*)

(Miller et al., 1997). Analogous comparisons of human LTC-IC and CRU are more limited and confounded by likely large differences in the efficiencies of the two assays. Nevertheless the experimental data presented in Chapter 3 and 4 demonstrates that in human CB these two types of functionally defined progenitors appear to be similarly distributed between the $CD38^+$ and $CD38^-$ subpopulations of the $CD34^+$ cells (Conneally et al., 1997). Also, when human CB cells were first cultured in serum-free medium containing FL, SF, IL-3, IL-6 and G-CSF, both LTC-IC and CRU numbers were subsequently found to have been modestly expanded - in contrast to the accompanying large expansion that occurs in cells detectable as CFC (Conneally et al., 1997; Bhatia et al., 1997a). Similarly, when these factors were used to enhance the infection of human cord blood cells with a neo^r-containing retrovirus, the proportion of marked LTC-IC obtained was found to correlate significantly with the proportion of marked CRU and not with the proportion of marked CFC (Conneally et al., 1998). However, differences in the factors required to elicit and sustain murine and human CRU and LTC-IC activity *in vitro* and *in vivo* have also been identified (Sutherland et al., 1993; Lemieux and Eaves, 1996; Miller et al., 1997; Gan et al., 1997). Thus, CRU and LTC-IC do not necessarily depend on the same molecules for their detection and, under certain circumstances, it may be possible for the functions that define these cells to be dissociated.

1.1.4 Regulation of hematopoiesis

Mechanisms that control the fate of hematopoietic cells and, in particular, what determines whether a stem cell undergoes a self-renewal division, or contributes to blood cell production by differentiating and producing committed progenitor cells remain outstanding issues. Various hypotheses have been proposed to explain these effects. These range from stochastic

(Till et al., 1964) to deterministic (Curry and Trentin, 1967) models. Although there are many reports that lend credence to each model, interpretation of the data is made difficult by the fact that survival of the hematopoietic cell always requires the continued presence of growth factors (GF) (Leary et al., 1992) and the target populations are often not homogeneous.

The stochastic model was first proposed by Till and colleagues (1964) to describe the heterogeneity in self renewal observed at the single cell level in the CFU-S assay. The model postulates that each cell may follow one of two pathways i.e., the cell may divide and produce two new cells with the capacity to form new colonies (birth process), or alternatively, the cell may differentiate (death process), and secondly, that these events occur randomly by a mechanism that is intrinsic to the stem cell itself. In interpreting their experimental data, Till et al (1964), found good agreement between the observed distribution of CFU-S and a computer simulation of a simplified birth-and-death process with a fixed birth and death probability and a fixed generation time. In this model, the fate of individual CFU-S is not tightly controlled but the behavior of the population as a whole is regulated by mechanisms that establish birth and death probabilities as well as the proportion of CFU-S that are cycling (Becker et al., 1963). Subsequent experimental data obtained from *in vitro* experiments, using either murine (Humphries et al., 1981; Nakahata et al., 1982) or human (Leary et al., 1984; Mayani et al., 1993a) hematopoietic cells supports the stochastic model of lineage commitment. This conclusion is also supported by data from Fairbairn et al (1993) by experiments where they infected FDCP-Mix cells (Spooncer et al., 1986)(a murine interleukin-3 (IL-3) dependent, multi-potent hematopoietic cell line) with a retrovirus encoding the *bcl*-2 gene (Hockenbery et al., 1990). In these experiments cells infected with *bcl*-2 were able to survive under serum deprived conditions in the absence of growth factor (GF) and their survival was accompanied

by multi-lineage differentiation leading to a conclusion that the differentiation process is intrinsically determined and that the role of hematopoietic GF is enabling or permissive rather than inductive.

In the alternative deterministic model the premise is that hematopoietic GF (with/without other components of the cellular microenvironment, such as cell adhesion molecules and the extracellular matrix) acts as inducers of differentiation and determine lineage choice of multipotent cells, presumably by influencing gene transcription. The model was initially proposed by Curry and Trentin (1967) (reviewed in Metcalf and Moore, 1971), to explain the predominance of erythroid colonies which developed in the spleen in contrast to a predominance of granulocytic colonies in the marrow. The theory proposed that this was indicative of different microenvironments that influenced the commitment of pleuripotent stem cells stimulated to divide in one environment versus the other. *In vitro* support for this concept came later from experiments performed by Metcalf (1980) who demonstrated that changes in GM-CSF concentrations could directly influence the differentiation program subsequently pursued by granulocyte-macrophage precursor cells. More recently it has been shown that exposure of murine lympho-myeloid progenitors to IL-1 or IL-3 containing cytokine cocktails can impair their *in vitro* self-renewal (Broccoli et al., 1996). In addition, Zandstra et al (1997a) have obtained evidence that the relative concentration of IL-3 can impact LTC-IC self-renewal, independent of effects on viability or proliferation. These examples indicate that extrinsic factors can also exert deterministic effects on primitive hematopoietic cell differentiation. However, regardless of the relative importance of external factors or poorly understood intrinsic mechanisms in regulating the self-renewal versus the

differentiation decision of a given pluripotent stem cell, increasing experimental data points to transcriptional regulators as being central to executing this process.

1.1.4.1 Transcription factors

Appropriate transcriptional control of a given gene by RNA polymerase II (Pol II) depends on contributions from a variety of factors commonly referred to as basal transcription factors (TFs). The basal TFS operate through core promoter elements (Maldonado and Reinberg, 1995). Surrounding the core promoter is the regulatory promoter. This is usually located within a few hundred base pairs of the transcription start site. Further upstream or downstream lies a second control region called an enhancer. The major contribution to precise transcriptional regulation is imparted by the binding of sequence-specific DNA binding proteins to regulatory sequences or enhancers. These proteins are grouped into families with distinct domains for DNA binding and transcriptional activation (reviewed in (Ernst and Smale, 1995)). Members of many of these families of transcription factors have been shown to play important regulatory roles in hematopoietic development. The functional roles of transcription factors known to affect hematopoiesis have been identified primarily through efforts to isolate lineage-specific genes or genes involved in leukemia-associated translocations (Nichols and Nimer, 1992). The two main experimental approaches used to determine their roles are the overexpression of the relevant gene (predominantly using retroviruses) and gene knockout studies. The development of technology for gene knockout experiments whereby mutations can be engineered into any gene in the mouse has proven to be extremely powerful in demonstrating the presence of specific factors necessary to the development of hematopoietic cells (Shivdasani and Orkin, 1996). There are, however,

limitations to the interpretation of knock-out experiments as the observed phenotype reflects the earliest requirement of the factor in development. Therefore if dysfunction of the gene is deleterious, any study of potential effects on later developmental events is compromised. More recently the *Cre* recombinase system has been used to obtain either tissue-restricted, or temporal restriction of gene inactivation to circumvent this obstacle (Gu et al., 1994; Shivdasani et al., 1997; Kolb et al., 1989). Chimera analysis and *in vitro* differentiation of ES cells can also be employed to examine effects on specific lineages that are precluded by the lethality of the knockout in the intact animal (Tsai et al., 1994).

A number of gene knockout studies have been described which are associated with embryonic lethality due to failure of the development of specific components of the hematopoietic system. Interestingly, most of these are basically different in terms of the exact phenotype observed e.g., SCL/tal-1 leads to a defect in both primitive (yolk-sac derived) and definitive hematopoiesis suggesting a function of this gene very early in hematopoietic development (Porcher et al., 1996). In contrast, animals deficient in AML-1, show normal yolk-sac derived hematopoiesis but lack fetal liver hematopoiesis (Okuda et al., 1996). Targeted disruption of the PU.1 gene results in later embryonic lethality (day 18), and a developmental block in generation of myeloid and lymphoid progenitors in yolk-sac fetal liver and thymus with normal numbers of erythroid and megakaryocytic progenitors (Scott et al., 1994). Further studies of the role of the PU.1 gene have demonstrated a differential requirement for the PU.1 gene in fetal liver vs. BM hematopoiesis (Scott et al., 1997). While PU.1$^{-/-}$ ES cells can contribute to the erythroid lineage in embryonic chimeras, they are unable to do so in adult chimeras. These studies have allowed key genes for hematopoietic stem cell development to be identified, and the concept of a hierarchy of regulatory factors is emerging.

Other genes, e.g., GATA-1 (Pevny et al., 1991), appear to play a more lineage-restricted role and have thus emerged as a candidate regulators of hematopoietic differentiation pathways. Absence of GATA-1 leads to a maturation arrest of erythroid progenitors at the proerythroblast phase.

An alternative approach using retroviral gene transfer to overexpress candidate genes of interest has also been informative. For example serial transplantation studies have revealed an enhanced ability of *HOX B4*-transduced mouse BM cells to regenerate the HSC compartment as compared to neo-infected control cells (Sauvageau et al., 1995). This suggests that the HOX family of transcriptional regulators may be involved in the self-renewal of HSC. Interestingly, studies performed with *HOX A10* resulted in the development of myeloid and lymphoid leukemias (Thorsteinsdottir et al., 1997). Forced expression of GATA-1 in retrovirally transformed myeloblasts was found to promote the differentiation of erythroblasts, thromboblasts and eosinophils (Kulessa et al., 1995). In addition, some of these effects appeared to be dose-related with the highest levels of GATA-1 expression being associated with megakaryocytic differentiation and lower levels favoring the development of eosinophils.

However, further fundamental questions about the molecular mechanisms by which such perturbations of these critical target genes interfere with cell differentiation remain largely unanswered. In addition, it is clear that multiple regulatory factors are expressed in stem or multi-potential progenitors and therefore the precise differential program of development is likely altered by a combination of external signals and an internal state of readiness for signal processing.

1.1.4.2 Growth factors

While the exact triggering mechanisms that lead to the self-renewal or the lineage commitment of HSC remain unclear, there is a large body of data showing that the continuing survival and proliferation of HSC and progenitor cells is highly dependent on their interactions with external hematopoietic GFs (Metcalf, 1993; Ogawa, 1993). GF mediate their effects by binding cognate transmembrane receptors expressed on the cell surface. This leads rapidly to the formation of ligand-receptor complexes followed by the phosphorylation of key tyrosine residues located within the cytoplasmic tail of the receptor molecules themselves, or on various adjacent kinases and other signaling intermediates. This results in the activation of various metabolic and mitogenic pathways (Kishimoto et al., 1994; O'Shea, 1997). The cloned GFs include the hematopoietic colony-stimulating factors (G-CSF, M-CSF, GM-CSF), the interleukins (IL-1 to IL-18), erythropoietin (Epo), thrombopoietin (Tpo), Steel factor (SF), flk-2/flt-3 ligand (FL) and the hematopoietic inhibitors TGF-β, TNF-α, and members of the chemokine family e.g., MIP-1α. The recent availability of biologically active recombinant GF, in combination with factor-responsive hematopoietic cell lines and purified subpopulations of hematopoietic cells has led to an explosion of knowledge about the biological and biochemical actions of these factors. Many of the cytokines initially characterized by their lineage-specific effects *in vitro* (e.g., in stimulating the formation of specific types of colonies) have since been found to have multiple effects on different types of responsive target cells both within and outside of the hematopoietic system. For example, G-CSF can stimulate the proliferation of vascular endothelial cells (Avalos, 1996), monopotent neutrophil progenitors (Ogawa, 1993), as well as activate the respiratory burst function of mature human neutrophils (Avalos, 1996).

TGF-β can inhibit primitive human hematopoietic progenitor proliferation (Ishibashi et al., 1987; Cashman et al., 1990; Keller et al., 1990) but may enhance the proliferation of later progenitor cell types (Keller et al., 1991) or accelerate their differentiation (Krystal et al., 1994). In addition, several cytokines may exert similar or overlapping functions on the same target cell, for example IL-3, G-CSF and GM-CSF can all support the proliferation and complete differentiation of granulopoietic cells into mature neutrophils (Metcalf, 1993). Finally, the most primitive hematopoietic cells appear to require simultaneous exposure to multiple cytokines to optimize their stimulation (Ogawa, 1993) the identity of which, however, may change during ontogeny (Miller et al., 1997; Rebel and Lansdorp, 1996; 1998 et al., 2001). Some of the functional overlap in activities of different GF can be explained at the molecular level as several work through common receptors or receptors that share common signaling mechanisms. For example, the binding of members of the IL-6 family of cytokines to their cognate receptors all signal through a common transducing molecule, gp130 (Kishimoto et al., 1995). Similarly the IL-2, IL-4, IL-7 and IL-15 receptors all share a common γ chain (Taniguchi and Minami, 1993).

This redundancy, pleiotropism and synergy amongst factors that can affect the behavior of primitive hematopoietic cells, makes it likely that the mechanisms regulating hematopoietic stem cell numbers *in vivo* will be found to be both complex and difficult to analyze - a prediction corroborated by both ligand and receptor gene-knockout experiments. It has long been known from studies of mice bearing naturally occurring knockout mutations of the SF gene or the SF receptor gene, c-kit, that these are embryonic lethal mutations due to failure of erythropoiesis (Huang et al., 1990; Russell, 1979; Bernstein et al., 1991). Non-lethal but specific defects have been identified in many other examples of GF knockouts (sometimes

only after the mice are challenged), e.g., knockout of the GM-CSF gene yields a mouse with no obvious deficit in granulocyte or macrophage progenitor numbers, but the mice do develop a pulmonary alveolar proteinosis-like disease secondary to a specific defect of alveolar macrophages (Dranoff et al., 1994; Stanley et al., 1994).

Until recently it was commonly believed that most primitive progenitors in the BM were permanently quiescent under-steady state conditions (Hodgson and Bradley, 1979; Lerner and Harrison, 1990). However recent evidence suggests that these cell populations are in a state of slow turnover every few weeks (Ponchio et al., 1995; Bradford et al., 1997). Nevertheless their relative quiescence in short term studies (days) has posed a major challenge to their ability to be retrovirally infected as previous studies have demonstrated that gene transfer occurs only in cells that are actively replicating at the time of infection (Miller et al., 1995). Ogawa and colleagues, amongst others, have extensively documented the different roles of various growth factors in supporting colony formation. These studies (Ogawa, 1993) have led them to group GFs into three categories: late acting lineage-specific factors such as Epo, M-CSF, IL-5, intermediate-acting lineage-nonspecific factors, such as IL-3, IL-4, GM-CSF, and factors with effects on more primitive progenitors for example IL-6, IL-11, SF, FL and Tpo. These early acting cytokines increase the recruitment into the cell cycle of HSC from both human and murine sources (Ikebuchi et al., 1987; Musashi et al., 1991; Ponchio et al., 1995; Jordan et al., 1996; Nordon et al., 1997).

The ability to recruit primitive cells into cycle combined with rapid changes in cell numbers has led to substantial interest in the possibility of *ex vivo* expansion of primitive HSC and progenitor cells. For the purposes of HSC transplantation, the use of *ex vivo* expanded cells may offer several potential advantages over unmanipulated material. These include the

possibility of decreased toxicity due to faster hematological recovery, gene therapy applications, tumor purging by $CD34^+$ selection, the potential to increase dose intensity by the provision of hematological support for multiple cycles of intensive chemotherapy and development of novel applications e.g., for *in vivo* immune therapy. In addition, the ability to expand primitive progenitor cells from small initial collections, for example CB collections, may be facilitated by the ability to expand the relevant cells, *ex vivo*. The challenge in all of these situations is to ensure that HSC functions are maintained under conditions that stimulate their proliferation. Several murine studies using either purified or unseparated marrow and a variety of cytokine combinations have clearly demonstrated a dramatic decrease of *in vivo* repopulating potential as a result of cytokine-driven *in vitro* proliferation (Knobel et al., 1994; Traycoff et al., 1996; Peters et al., 1996). Interestingly a number of these combinations contained IL-3 or IL-1 which have recently been reported to abrogate the reconstituting ability of murine HSC (Yonemura et al., 1996). However combinations that allow maintenance of long-term repopulating ability have also been described (Bodine et al., 1989; Luskey et al., 1992; Neben et al., 1994; Rebel et al., 1994; Muench et al., 1993; Holyoake et al., 1996; Yonemura et al., 1997; Miller and Eaves, 1997). Interpretation of the results of *ex vivo* expansion is additionally compounded by the finding that cell surface antigen expression is not necessarily a reliable indicator of HSC function (Rebel et al., 1994; Spangrude et al., 1995).

In parallel studies, requirements for the amplification of human stem and progenitor cells have been similarly investigated. In early studies Haylock et al (1992) demonstrated that significant expansion of CFU-GM could be obtained when $CD34^+$ cells from mobilized peripheral blood were cultured in IL-1, IL-3, IL-6, G-CSF and SF. Using relatively similar

protocols these results have been reproduced by several groups. (Brugger et al., 1993; Srour et al., 1993; Sato et al., 1993; Shapiro et al., 1994). LTC-IC have been shown to be maintained by Henschler et al (1994)using IL-1, IL-3, IL-6, Epo and SF. These cytokines have subsequently been used in a clinical trial in which expanded PBPC were used to support reconstitution of hematopoiesis after high-dose chemotherapy (Brugger et al., 1995). In this study, expanded cells reinfused after a 12 day culture period gave an identical reconstitution pattern compared to historical controls treated with unmanipulated $CD34^+$ cells.

Over the last few years, attention has focused on two more recently cloned cytokines: Flk-2/flt-3 ligand (FL) and Thrombopoietin (Tpo) as both of these have been demonstrated to have effects on primitive HSC. Flk-2/flt-3 [a member of the platelet-derived growth factor (PDGF) receptor tyrosine kinase family] was initially cloned from a population of murine fetal liver cells that were enriched for HSC (Matthews et al., 1991). Initial studies demonstrated restricted expression of Flk-2/flt-3 in both murine and human hematopoietic progenitors which suggested that the ligand might play an important role in regulating the growth and development of early hematopoietic cells (Matthews et al., 1991; Rosnet et al., 1993). FL has since been shown to induce the proliferation of highly purified HPC in synergy with a number of other growth factors (Lyman et al., 1993; Hannum et al., 1994; Muench et al., 1995). In human studies it has been further demonstrated that the population of hematopoietic cells that respond to FL includes more primitive elements as defined by their $CD34^+CD45RA^-/CD71^-$ phenotype (Nordon et al., 1997). This population has also been functionally defined to contain all of the LTC-IC (Lansdorp and Dragowska, 1992). FL also stimulates $CD34^+CD38^-$ cells which are additionally rhodamine 123 low and resistant to 4-HC (Shah et al., 1996; Haylock et al., 1997). FL enhances the rate of recruitment and increases the number of cells stimulated

to divide within these primitive cell populations (Nordon et al., 1997; Shah et al., 1996; Haylock et al., 1997) as well as promoting their self-renewal (Petzer et al., 1996a).

Tpo is a primary regulator of megakaryocyte and platelet production (de Sauvage et al., 1994; Lok et al., 1994; Kaushansky et al., 1994; Wendling et al., 1994). Recent observations have suggested that Tpo may also play a role in the proliferation of some early hematopoietic cells (Kobayashi et al., 1996). Tpo has now been demonstrated to promote the growth of $CD34^+CD38^-$ cells and appears to be more potent than IL-3, SF or FL in supporting the viability of $CD34^+CD38^-$ cells (Ramsfjell et al., 1997; Borge et al., 1997). The finding that both Tpo receptor (c-mpl) and Tpo knockout mice have reduced levels of progenitors of multiple lineages, suggests that Tpo may be a non-redundant cytokine playing a key role in regulating HSC *in vivo* (Alexander et al., 1996). The use of systematic studies (e.g., factorial analysis) (Box et al., 1978) to define the role of individual cytokines has allowed additional HSC-specific activities of FL and Tpo to be identified, as only these 2 factors (from a large number tested) were individually able to increase the number of cells detectable as LTC-IC in cultures of $CD34^+CD38^-$ cells in a stroma-free, serum-free system, (Petzer et al., 1996b). The addition of SF and IL-3 to this combination further enhanced this increase. These studies suggest that these factors would be likely to be important in *ex vivo* expansion of HSC, in addition to improving retroviral gene transfer to HSC.

1.2 Genetic manipulation of hematopoietic cells using recombinant retroviruses

The concept of gene therapy was first proposed in the early 1970s (Friedmann and Roblin, 1972) and the field of clinical gene transfer has progressed from speculation to reality in a relatively short period of time (Anderson, 1992; Miller, 1992). In addition to clinical

applications of gene therapy, the use of recombinant retroviruses as genetic tags represents a powerful approach to track the proliferative and differentiative behavior of individual stem cell clones (Keller and Snodgrass, 1990; Jordan and Lemischka, 1990; Pawliuk et al., 1996). In addition they can be used to analyze the activity of genes regulating hematopoiesis both in normal and disease states (for example, as described above to study the role of HOX genes in normal hematopoiesis (Sauvageau et al., 1995) or in the development of murine models of chronic myelogenous leukemia (Daley et al., 1990; Elefanty et al., 1990)). Studies using retroviral marking of HSC have demonstrated the ability of murine HSC to undergo self-renewal both *in vitro* and *in vivo* through the detection of identical proviral banding patterns in both lymphoid and myeloid cells of more than one primary recipient, or in additional secondary recipients (Fraser et al., 1992; Keller and Snodgrass, 1990; Jordan and Lemischka, 1990). In addition these studies have demonstrated that individual HSC can contribute to hematopoiesis for many months to years (Keller and Snodgrass, 1990; Capel et al., 1990), however the clonal contribution of individual transplanted HSC to mature hematopoietic tissues can change over time, particularly during the first 4 to 6 months post-transplant (Lemischka et al., 1986; Snodgrass and Keller, 1987; Capel et al., 1990). These fluctuations have been interpreted as a reflection of clonal succession (Kay, 1965). However, when the clonal contribution to the peripheral blood has been followed for longer periods of time in animals transplanted with marked cells, hematopoiesis became more stable and was subsequently dominated by a small number of totipotent stem cells clones (Jordan and Lemischka, 1990). In an elegant study, Jordan et al proposed that the initial clonal instability observed post-transplant occurred as a result of the expanding pool of HSC undergoing lineage-restricted differentiation versus self-renewal (Jordan and Lemischka, 1990). Thus

some clones in the early transplant period differentiated rapidly and contributed progeny only at early time points. Others were postulated to undergo some self-renewal and some differentiation with the result that they would contribute to both early and late blood cell production. Finally a third pool was postulated to undergo only self-renewal divisions at early time points resulting in no contribution to blood cell output in the initial post-transplant period. However, much of this model was based on the assumption that the number of marked hemopoietic HSC engrafting each mouse was non-limiting (at all time points), an assumption which was not tested and may well be suspect given what we now appreciate in terms of the lack of understanding of factors that can sustain fetal as opposed to adult murine HSC in vitro (Rebel and Lansdorp, 1996; Miller and Eaves, 1997).

Human gene therapy has been heralded for its potential to revolutionize modern medicine through the use of recombinant DNA technology to treat inherited and acquired diseases. It is interesting to note that, although initially conceived for the treatment of monogenic genetic diseases, at the end of 1996, only 10% of the clinical trials approved by the Recombinant DNA Advisory Committee were for classical genetic disorders (Marcel and Grausz, 1997). Moreover, although considerable progress has been made in the field and high levels of gene transfer to murine repopulating cells has been achieved, studies of gene transfer to human or primate repopulating cells have, overall, yielded disappointing results. Nevertheless, despite the low levels of gene transfer obtained in the early clinical studies, important observations derived from these include the demonstration that gene transfer into primary human hematopoietic stem cells is achievable, (Brenner et al., 1993b; Brenner et al., 1993a; Deisseroth et al., 1994; Dunbar et al., 1995) and that the initial safety concerns have not proven to be warranted. In addition, biological activity resulting from transferred genes

relevant to the targeted disease has been detected in patients (Kohn et al., 1995). Gene marking studies have perhaps yielded the most useful information, demonstrating that malignant cells in hematopoietic grafts used in patients undergoing autologous transplants can contribute to relapse and thereby, establishing the need for effective purging strategies in autotransplant protocols (Brenner et al., 1993b; Brenner et al., 1993a; Deisseroth et al., 1994). Through systematic improvement of infection protocols, very recent reports have indicated the possibility of gene transfer to human hematopoietic cells capable of engrafting immune-deficient mice (Nolta et al., 1996; Larochelle et al., 1996; Yurasov et al., 1997; Conneally et al., 1998) and non-human primates (Dunbar et al., 1996).

The most commonly used vehicles for gene transfer studies are replication-incompetent retroviral vectors. Enthusiasm for this method of gene transfer is based in part on the ability of retroviruses to infect a wide range of cell types as well as their capacity to integrate stably into the DNA of the infected cell. However, retroviruses have many limitations, including a relatively low efficiency of gene transfer to human hematopoietic stem cells (despite the fact that high levels of gene transfer can be achieved into murine repopulating cells), the ability to integrate only into cells in cycle, lack of stable expression of the transduced gene, and difficulties in producing large amounts of high-titer, clinical grade material. Much of the recent effort of the field has therefore been focused on the development of strategies to independently overcome these various limitations, some of which are described briefly below.

1.2.1 Recombinant retroviruses as vectors for gene transfer

No group of infectious agents have received as much attention in recent years as retroviruses. The retroviral family display a variety of interesting biological features which include marked variability in the interaction between host and virus ranging from completely benign infections by endogenous viruses through to the generally fatal consequences of viruses such as HIV. Retroviruses can rapidly alter their genomes by mutation and thus change in response to altered environmental conditions, a feature which has contributed to the difficulty encountered in treating HIV infected individuals. Studies of the ability of retroviruses to acquire and alter the structure and function of host-derived sequences to create "oncogenes", have provided fundamental insights into carcinogenesis. Finally, and of greatest relevance to this thesis is the fact that retroviruses can serve as vectors for foreign genes, allowing their controlled transmission to and expression in a wide variety of cells and organisms.

Despite the variety of host species that retroviruses infect and the outcome of such interactions, all retroviral isolates are basically similar in virion structure, genomic organization and mode of replication (Coffin, 1996). The virion is about 100 nm in diameter and is enveloped by a lipid bilayer containing the *env* glycoprotein. This is a heterodimeric complex of both transmembrane (TM) and surface (SU) domains. The larger SU domain is responsible for recognition of the cell surface receptor. The TM domain anchors the complex to the virion envelope and contains domains responsible for fusion of the viral and cellular membranes. Retroviruses have 2 identical molecules of a single-stranded RNA genome complexed with viral-encoded proteins derived from the *gag* and *pol* genes. The order of the genes encoding structural proteins is *gag-pro-pol-env* (Figure 1.1). Retroviral *gag* genes encode polyproteins that are cleaved into at least 3 proteins designated matrix (MA), capsid (CA) and nucleocapsid (NC). The *pro* region encodes a protease (PR) responsible for the

cleavage of the *gag* and *pol* polyproteins. The *pol* gene encodes 2 proteins, reverse transcriptase, (RT) an RNA-dependent DNA polymerase and the integrase protein necessary for integration of viral DNA into cellular DNA. Retroviral genomes are arranged so that all non-coding sequences that contain the recognition sequences for DNA and RNA synthesis and processing are located in the terminal regions or long terminal repeats (LTRs), with the internal regions being given over to protein coding functions.

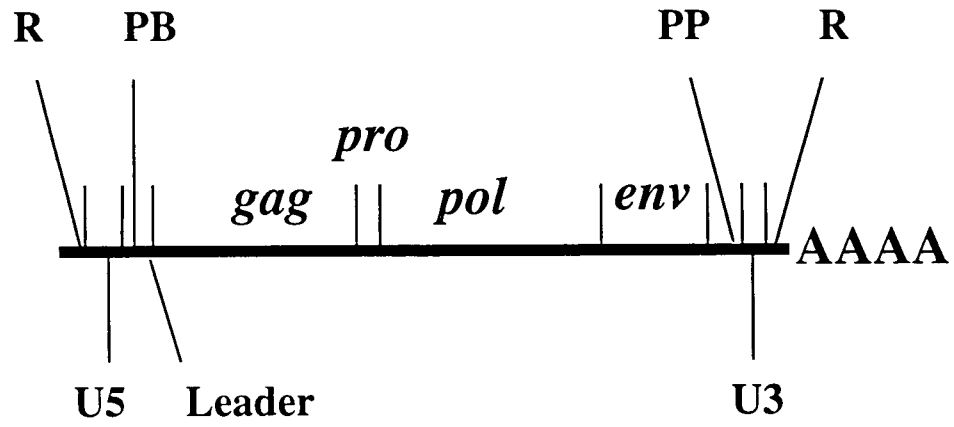


Figure 1.1 Sequence features of the retroviral genome. R- the sequence at the terminal ends of the retrovirus plays a role during reverse transcription in permitting the transfer of the nascent DNA from one end of the genome to the other. U_5 is the first region copied into DNA during reverse transcription and becomes the 3' region of the LTR. PB- the nucleotides of the primer binding (PB) site are complementary to the tRNA primer to which it binds. The leader sequence contains the donor site for the generation of spliced, subgenomic mRNAs. This area also contains the packaging signal (ψ) which specifies incorporation of the genome RNA into virions. In MuLV this signal extends into the *gag* region. In the majority of retroviruses the sequence from the beginning of *gag* to the end of *env* is translated in its entirety. Preceding U_3 is the polypurine tract which contains the initiation site for the synthesis of the plus strand of viral DNA. U_3 contains a number of cis acting sequences necessary for viral replication. It forms the 5' region of the LTR. At the end of the 3' genome is the other copy of R which may contain the poly (A) addition signal. Adapted from Retroviridae: the viruses and their replication (Coffin, 1996).

1.2.2 Lifecycle of the retrovirus

The retroviral replication cycle can be divided basically into two phases (see Figure 1.2). The first portion includes binding of the virus particle to a specific receptor on the cell surface, entry of the virion core into the cytoplasm, synthesis of the double-stranded DNA using the virion RNA as a template, transfer of the core structure to the nucleus and integration of the DNA into the host genome forming the provirus. These steps are mediated by proteins found within the virion and proceed in the absence of viral gene expression. The second phase consists of the synthesis and processing of the viral genome, mRNAs and proteins, assembly of the virion and finally release of competent mature virions.

Several features of the retroviral lifecycle make retroviruses suitable as vectors for gene transfer. Integration of the DNA copy of the viral genome is efficient and, like the control of transcription, is directed by sequences within the LTRs. Therefore viral sequences whose functions can be supplied in trans can be deleted. The integrated viral genome subsequently behaves as a cellular gene and is transferred to all progeny of the infected cell. Also budding of the virus is non-lytic. This allows the generation of permanent cell lines that can continuously produce recombinant retroviruses (Williams and Orkin, 1986).

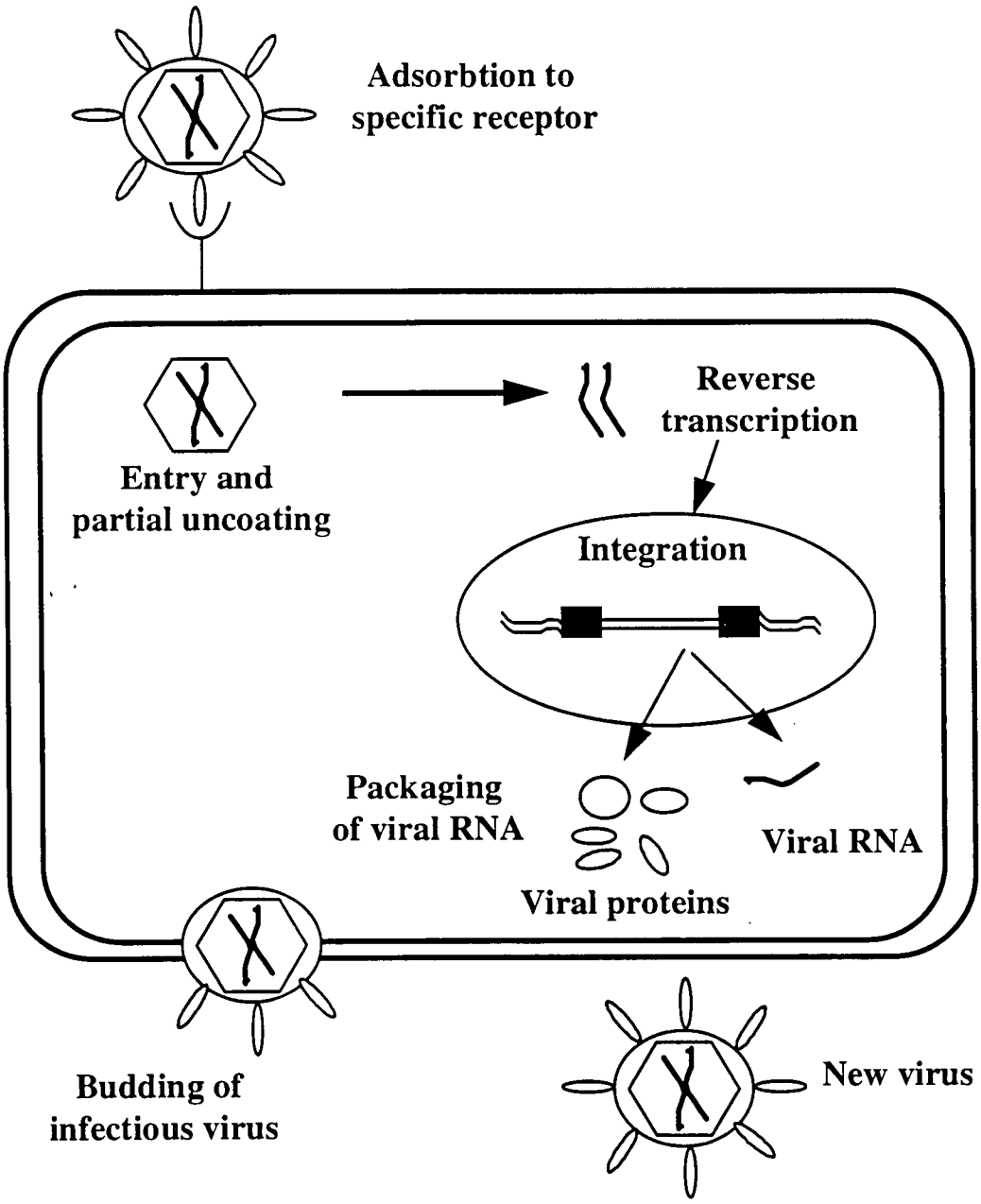


Figure 1.2 Overview of the retroviral lifecycle. The early phase of the replication cycle involves attachment by the virion to a cell surface receptor, entry and uncoating of the virus, viral DNA synthesis by reverse transcription in the cytoplasm, transport of the viral DNA to the nucleus and integration into the host cell DNA to produce the provirus. The late phase starts with the synthesis of viral transcripts from the provirus and continues to the release of the progeny virions.

1.2.3 Production of helper-free recombinant retroviruses

To generate a recombinant replication-incompetent retrovirus, the *gag, pol* and *env* genes are deleted from the wild-type retrovirus and replaced by a marker gene or the gene of interest. The development of cell lines (designated packaging cell lines) that can provide the functional viral proteins were essential to the development of retroviral vectors for human gene therapy applications. These packaging cell lines are fibroblast-derived lines that have been engineered to produce retroviral proteins through the introduction of *gag*, *pol* and *env* genes, but are unable to package the viral RNA, itself. A retroviral vector can be then introduced into the packaging cell line using standard transfection techniques (e.g., electroporation, $CaPO_4$ precipitation, or lipofectin). The genomic length RNA is produced from the recombinant retroviral vector and combines with the retroviral proteins produced by the packaging cell line resulting in recombinant viral particles capable of one round of infection but unable to sustain further replication due to the absence of the required viral genes (Figure 1.3). The concept of a helper free retroviral packaging cell line was introduced in 1983 by Mann et al who developed the ψ-2 packaging cell line. This involved deleting a sequence adjacent to the 5' LTR which is necessary for the packaging of genomic RNA into virions (Mann et al., 1983). However, in the earliest packaging cell lines, a low frequency of encapsidation of packaging cell line RNA with the deleted ψ region allowed recombination with the vector RNA, resulting in the generation of replication-competent virus having an intact ψ region (Miller and Buttimore, 1986). Newer generations of packaging cell lines have, therefore, been developed to minimize the risk of helper virus production. These modifications involved removing sequences in the packaging cell line homologous to the vector DNA to decrease further the risk of recombination, as well as introducing mutations in the LTRs (e.g., PA317) (Miller and

Buttimore, 1986). Today third generation packaging cell lines (GP+ E86) (Markowitz et al., 1988a; Markowitz et al., 1988b) and ψCRE (Danos and Mulligan, 1988) are in use. In these later lines, the *gag-pol* and *env* are separated on different plasmids, so that at least three recombination events are needed before replication-competent retroviruses (RCR) can be produced. However recently, a report of RCR even using the GP+E86 cell line has been reported (Chong and Vile, 1996) stressing the need for vigilance and constant screening of producer cell lines for helper virus.

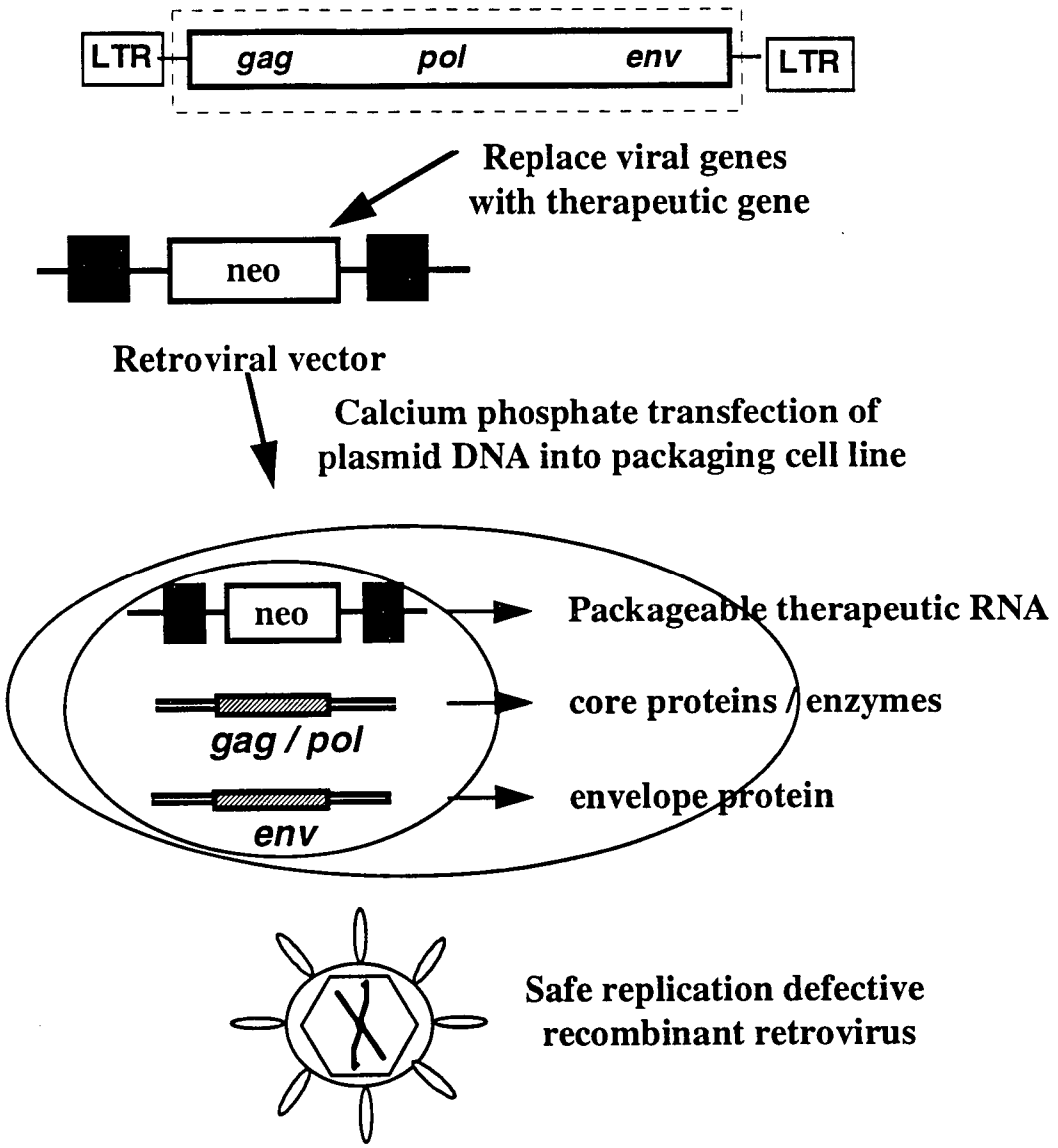


Figure 1.3 To generate a retroviral vector the wildtype structural genes are replaced with the gene of interest, but the viral packaging signal is maintained. This plasmid is then transfected into a packaging cell line which can produce the viral proteins. However, the wildtype viral RNA is unable to be packaged due to deletion of the ψ signal. The subsequently released infectious retrovirus is able to undergo only one round of infection prior to its integration into the genome of the host target cell.

1.2.4 Optimization of retroviral gene transfer to primitive hematopoietic cells

The efficiency of retroviral infection of HSC is dependent on a large number of factors and can be considered to involve a series of steps. These include targeting the virus to the cell, specific binding and internalization of the retrovirus using the retroviral receptor on the cell surface, integration of the retrovirus into the target cell genome (which is dependent on cell division) and, finally, expression of the transferred gene. The entire process can require several days depending on the target cell of interest. Therefore, the challenge for retroviral infection of HSC includes the use of conditions that allow its stem cell attributes to be maintained while achieving their efficient infection. Researchers involved in gene transfer to HSC have attempted to understand and optimize each of the above steps and some of the approaches used will be discussed in the final section of this Chapter.

Palsson et al have recently examined the physical characteristics of retroviruses and based on their size and density have determined that retroviral movement is governed by random Brownian motion (Chuck and Palsson, 1996; Palsson and Andreadis, 1997). Retroviruses have a short half-life (approx 5-8 hours). Accordingly, the distance that they can move by Brownian motion is limited and has been estimated to be only 480-610 μm in the 5-8 hour $t_{1/2}$ period (Chuck et al., 1996). Therefore the probability that a retrovirus will encounter a cell depends on the initial distance between them. This prediction is borne out by the observation that co-cultivation of target cells and producer cells, which maximizes the proximity of the virus and the target cells, is associated with the highest infection efficiencies (Bodine et al., 1991). This limitation can be overcome by increasing the motion of the retrovirus toward the target cell using either centrifugation (Kotani et al., 1994) or "flow through" transduction (Chuck and Palsson, 1996). Using a "flow through" system with 3T3

cells as a target, Chuck et al (1996) demonstrated that this system can result in high transfection efficiencies independent of the viral titre, and does not require the use of cations (e.g., polybrene) which have previously been demonstrated to be toxic to hematopoietic cells

As noted above, cocultivation of the hematopoietic target cells and viral producer cell lines results in the highest efficiencies of gene transfer. However, it has also been noted that infection efficiencies obtained by exposing cells to viral supernatants protocols could also be augmented simply by having stromal cells present (Moore et al., 1992; Bodine et al., 1993; Nolta et al., 1995), although the mechanism for this has remained unclear. The role of defined components of the extracellular matrix was further examined by Moritz et al who determined that gene transfer was increased in the presence of fibronectin. This improvement in gene transfer efficiency was subsequently localized to a 30/35 kD fragment of the fibronectin molecule (Moritz et al., 1994). More recent studies have indicated that retroviruses bind specifically to sequences within the fibronectin molecule (Hanenberg et al., 1996) It had previously been demonstrated that hematopoietic cells bind to either the VLA-4 (Verfaillie et al., 1991) or VLA-5 (Patel and Lodish, 1984) binding region of fibronectin. Thus incubation on fibronectin or the 30/35 kD fragment would be expected to serve as a means to co-localize retroviral particles and hematopoietic cells (see Figure 1.4 for a schematic model).

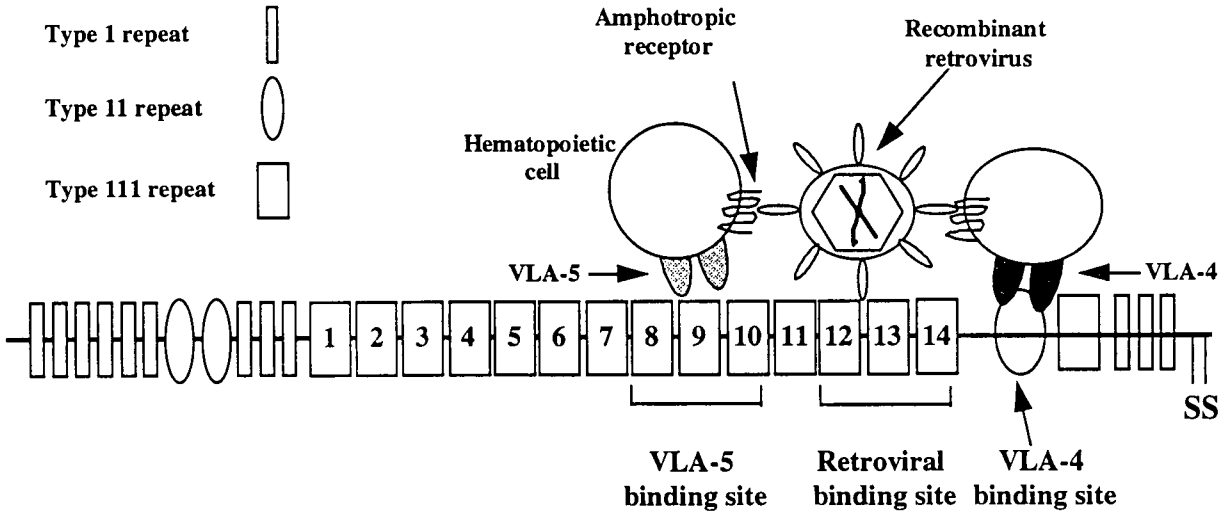


Figure 1.4 Schematic representation of the mechanism by which fibronectin potentiates gene transfer HSC. The fibronectin type 1, 11, 111 repeats are indicated. The type 111 repeats are numbered from 1-14. The VLA-5 binding region is in repeat $111_{(8\text{-}10)}$, the putative retroviral binding region is in repeats $111_{(12\text{-}14)}$ and the VLA-4 binding region is in the CS-1 region. (Adapted from information derived from Hanenberg et al., 1996)

The retroviral receptor is the primary determinant of the range of cells that can be transduced by a virus. Retroviruses utilize a variety of unrelated cell surface receptors to initiate infection, although in general individual retroviruses only recognize a single receptor (Miller, 1996). In 1984, CD4 was identified as a receptor for HIV-1 and became the first known retroviral receptor (Dalgleish et al., 1984; Klatzmann et al., 1984). Since then, several additional retrovirus receptors have been identified and their cDNAs cloned (reviewed in (Miller, 1996)). Many of the receptors have multiple transmembrane-spanning domains and function as transporter molecules. For example, the ecotropic receptor for the murine leukemia virus is a cationic amino acid transporter (Kim et al., 1991; Wang et al., 1991), and the gibbon ape leukemia virus receptor (Glvr-1) and Ram-1, the receptor for the amphotropic murine leukemia virus are both phosphate transporters (Kavanaugh et al., 1994; Miller et al., 1994; Van Zeijl et al., 1994). Studies of the distribution of the various receptors show that Glvr-1 is expressed at a higher level than Ram-1 on hematopoietic cells (Kavanaugh et al., 1994) and that, overall, the expression of Ram-1 is low. Indeed this has been proposed as one of the reasons for the low infection efficiency of human HSC by recombinant retroviruses (Orlic et al., 1996). Furthermore levels of Ram-1 expression in the $CD34^+CD38^+$ and $CD34^+CD38^-$ subpopulations of human marrow cells was found to correlate with their infection efficiencies (Orlic et al., 1996). Potential solutions to up-regulate the expression of Ram-1 include culturing the cells in phosphate-free medium as this has been demonstrated to increase amphotropic receptor expression (5-fold) (Kavanaugh et al., 1994). In addition, Crooks et al have demonstrated that amphotropic virus binding can be increased by culturing the target cells in SF, IL-3 and IL-6 (Crooks and Kohn, 1993). Another approach has been the development of packaging cell lines that use the Glvr-1 env protein (Lynch and Miller, 1991),

or are pseudotyped with the vesicular stomatitis virus (VSV) (Yee et al., 1994). Both of these modifications have been reported to result in the production of modified retrovirus that give increased gene transfer efficiencies to human hematopoietic cells (von Kalle et al., 1994; Akkina et al., 1996). An additional advantage of the VSV system is the increased stability of the virus which allows it to be concentrated without loss of biological activity (Burns et al., 1993). To target the virus to certain cell types, additional modifications of the envelope protein have been carried out. For example, a portion of the erythropoietin molecule was incorporated into the envelope protein to allow the specific infection of cells displaying the erythropoietin receptor (Kasahara et al., 1994). Similar approaches have targeted the low density lipoprotein receptor (Somia et al., 1995), epidermal growth factor receptor (Cosset et al., 1995), as well as major histocompatibility class I and class II antigen receptors (Roux et al., 1989). The attraction of such approaches is that the cells with the defective gene of interest can be specifically targeted, although there are concerns that fusion of the viral *env* gene with a foreign molecule may result in a noninfectious viral particle and that the receptors chosen for targeting may need to be expressed at a sufficient level on the cells for the approach to work.

Retroviral entry into the cell cytoplasm takes place by two mechanisms: fusion of the viral membrane with the cell plasma membrane, or endocytosis of the viral particle. The Mouse Mammary Tumor Virus and the ecotropic strain of Moloney Murine leukemia virus (MMuLV) utilize the endocytic pathway (Coffin, 1996), whereas amphotropic MMuLV is thought to enter by direct fusion (Ragheb and Anderson, 1994).

The final phase in the process of retroviral-mediated gene delivery is integration. This requires active replication of the cells at the time of infection (Miller et al., 1995). As

discussed above, many primitive hemopoietic cells are members of, at best, slowly proliferating populations (Reems and Torok-Storb, 1995; Ponchio et al., 1995; Hao et al., 1996; Zakian, 1996; Bradford et al., 1997). Efforts to improve gene transfer by increasing the number of cycling cells have included either *in vivo* manipulations (e.g., by the administration of 5-fluorouracil or cytokines (Bodine et al., 1991; Bodine et al., 1994)), or *in vitro* manipulations (e.g., by exposure of the cells to various cocktails of hematopoietic GFs (Bodine et al., 1989; Luskey et al., 1992; Nolta et al., 1992)). Recently Dunbar et al using $CD34^+$ cells derived from *in vivo* SF and G-CSF primed peripheral blood or BM from Rhesus monkeys demonstrated rapid reconstitution of blood counts post-transplant and readily detectable gene transfer for up to 5 months (Dunbar et al., 1996). The use of *in vitro* manipulations with cytokines has raised concerns, however, similar to those raised by expansion studies, of the ability of the infected HSC to initiate and sustain durable blood cell production. In studies performed by Nolta et al, human $CD34^+$ cells cultured for 72 hours in IL-3, IL-6 and SF lost the ability to sustain engraftment in *bnx* mice (Nolta et al., 1995). This problem, however, appeared to be reversed when the cells were transduced in the presence of stromal cells (Dao et al., 1997).

1.2.5 Post-infection selection of infected cells

As an alternative approach to obtaining populations of cells with high levels of gene transfer and/or to provide a rapid and sensitive method for monitoring and tracking infected cells, many of the vectors in use were designed to contain sequences that encode a selectable marker. Most of these confer resistance to a toxic compound e.g., neomycin, hygromycin, or methotrexate (Dick et al., 1985; Palmer et al., 1987; Miller et al., 1985; Flasshove et al., 1995)

which requires the continued proliferation of the infected cells for their subsequent identification or selection (Hughes et al., 1989; Apperley et al., 1991). An alternative approach has been to use the bacterial β–galactosidase gene or the human alkaline phosphatase gene as reporters (Nolan et al., 1988; Fields-Berry et al., 1992). Both of these latter genes enable the sensitive and specific detection of individual transduced cells but are not readily adapted to the isolation of viable populations that are enriched in their content of transduced cells. Recently a number of groups have focused on reporter genes that encode cell surface molecules detectable by staining with monoclonal antibodies (Pawliuk et al., 1994; Valtieri et al., 1994; Conneally et al., 1996; Pawliuk et al., 1996; Planelles et al., 1995; Tumas et al., 1996), although at the time that this thesis was initiated the use of genes encoding cell surface markers for the selection of a retrovirally-marked human cells had not yet been reported. A further development of selectable markers has been the incorporation of the human multi-drug resistance gene (MDR-1) as the selectable marker (Ward et al., 1994; Richardson and Bank, 1995). The MDR-1 gene product, p-glycoprotein, is a transmembrane efflux pump that shunts a variety of commonly used chemotherapeutic drugs (e.g., anthracyclins, taxol, vinca alkaloids) out of cells (Geissler et al., 1981). Normal BM cells express low levels of this protein and are therefore susceptible to the toxic effects of these drugs. Expression of the MDR-1 gene product can thus function as both a fluorescence-activated cell sorting (FACS) selectable marker and a therapeutic gene. Murine studies have shown that *in vivo* taxol selection can increase the number of cells expressing MDR protein (Sorrentino et al., 1992; Podda et al., 1992). The potential applications of such an approach would include the possibility of dose escalation of chemotherapeutic agents after re-infusing transduced hematopoietic cells, or its use as a dominant selectable marker to promote

expansion of a transduced clone of cells carrying an additional gene of interest (Aran et al., 1994) thus increasing the applicability of this approach to many disorders.

1.3 Thesis objectives

The overall goal of the work described in this thesis was to develop methodologies that would allow primitive human hematopoietic cells to be genetically modified at frequencies that could be exploited for experimental and clinical applications of this technology. At the time this work was initiated, efficient gene transfer to murine cells had been demonstrated and retroviruses were being used to examine the proliferative capacity of individual murine HSC. In addition, gene transfer to murine cells was being used for the first time to assess the role of various genes in the regulation of hematopoiesis e.g., GM-CSF (Johnson et al., 1989). Retroviral-mediated gene transfer to hematopoietic cells from larger animals including humans had also been demonstrated but data from the initial gene therapy trials also showed that conditions for obtaining clinically relevant levels of gene transfer into human hematopoietic cells with retention of their *in vivo* reconstituting abilities had not been identified.

The hypothesis on which this thesis was developed was that conditions for achieving improved gene transfer to HSC with *in vivo* reconstituting ability required the identification of culture conditions that would reproducibly ensure their division, and that to achieve this, conditions that stimulate a net expansion of LTC-IC numbers should be useful. The latter was based on the assumption that human LTC-IC and *in vivo* reconstituting cells would prove to be closely related or highly overlapping cells as was indicated by studies of these cells in the murine system. However, this was still an assumption that could not be examined due to the fact that no quantitative procedure that could be used to characterize human HSC with *in vivo*

reconstituting potential was available. Several groups had identified conditions that allow human hematopoietic cells to be transplanted into immuno-deficient mice and hence the development of a small animal model system using human progenitor cells capable of proliferation and differentiation in an *in vivo* setting appeared an important opportunity to meet this need. The first step therefore focused on the adaptation of this xenotransplant model to develop a quantitative assay of human HSC with *in vivo* lympho-myeloid regenerating activity. Because of the superior engrafting potential already associated with human cord blood cells (Lapidot et al., 1992; Vormoor et al., 1994), I focused on this source of HSC for this phase of the work.

Previous studies had also shown that cells targeted for gene transfer must be actively cycling in order for any retrovirally-introduced genetic material to be stably integrated into the DNA of the infected cell.. Available data suggested that most human HSC, even in CB, would at any given time be quiescent and thus difficult targets for retroviral-mediated gene transfer unless adequately stimulated. However experience with both human and murine adult BM cells indicated that this would likely require a period of exposure of the HSC's to cytokines *in vitro* for at least 4 or 5 days (Hao et al., 1996; Jordan et al., 1996). It was therefore necessary to determine if human HSC could be stimulated to divide *in vitro* without loss of their *in vivo* repopulating potential as shown by rigorous quantitative evidence that their numbers could be expanded. The results of these studies are described in Chapter 3.

The successful outcome of these experiments laid the groundwork for testing the hypothesis that the same conditions might allow improved gene transfer to human HSC with *in vivo* (NOD/SCID) repopulating activity. A series of experiments were performed to optimize gene transfer efficiencies to these cells incorporating these conditions as well as

several other features that had been reported to enhance gene transfer efficiencies using a supernatant infection protocol. The results obtained from these studies, in which a high and correlated level of retroviral mediated gene transfer to the clonogenic progeny of NOD/SCID repopulating cells and LTC-IC from $CD34^+$ populations are described in Chapter 4.

In addition to manipulating the infection conditions, I also considered the possibility of using a post-infection selection strategy to allow a highly enriched population of genetically modified HSC to be selected. For these experiments, a retroviral vector encoding a selectable marker detectable by FACS analysis to enable rapid, efficient and non-toxic identification and selection of retrovirally transduced BM cells was developed. The marker chosen was the murine Heat Stable Antigen (HSA) gene product. The successful development and validation of this system for analyzing and/or selecting rare subpopulations of primitive human hematopoietic cells is described in Chapter 5.

Chapter 2 Materials and Methods

2.1. Human Cytokines

Highly purified recombinant IL-3 and granulocyte-macrophage CSF (GM-CSF) were gifts from Novartis (formerly Sandoz, Basel, Switzerland). IL-6 and SF were purified from media conditioned by COS cells that had been transiently transfected in the Terry Fox Laboratory with the corresponding human cDNAs. FL was a gift from Immunex Corp (Seattle, WA) and purified human erythropoietin (Ep) and G-CSF were kindly provided by StemCell Technologies Inc, (StemCell) (Vancouver, B.C).

2.2 Cell Lines

The amphotropic packaging cell line GP-*env* AM12 (Markowitz et al., 1988b) and the ecotropic packaging cell line GP-E86 (Markowitz et al., 1988a) were used for the generation of helper-free recombinant retroviruses. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; StemCell.) supplemented with 10% heat-inactivated newborn calf serum (Bio-Whittaker, Walkersville, Maryland), 15 μg/mL hypoxanthine (Sigma Chemicals, St. Louis, MS), xanthine 250 μg/mL (Sigma) and 25 μg/mL mycophenolic acid (Gibco-BRL, Burlington, Ont.) (abbreviated as HXM). For the maintenance of the GP-*env* AM12 cell line, 200 μg/mL hygromycin B (Calbiochem, San Diego, CA) was also added to this medium. Viral packaging cells were maintained in HXM supplemented with 1 mg/mL of the neomycin analog, G418 (Gibco-BRL). NIH-3T3 fibroblasts (American Type Culture Collection [ATCC], Rockville, MD) were maintained in DMEM with 10% bovine calf serum (Gibco BRL). HL60 cells were maintained in RPMI supplemented with 10% fetal calf serum (FCS,

Stem Cell). Mo7e cells (Avanzi et al., 1988) were maintained in DMEM supplemented with 10% FCS, 10% 5637 cell (ATCC) conditioned medium, 5ng/mL IL-3, (Novartis) and $5x10^{-5}$ M β-mercaptoethanol (Sigma). All cells were kept at 37° C in a humidified atmosphere of 5% CO_2 in air.

2.3 Hematopoietic cells

Surplus human BM cells were obtained with informed consent from normal adult donors of allogeneic BM transplants or were cadaveric samples obtained from the Northwest Tissue Centre (Seattle, WA). To generate marrow fibroblasts for the infection experiments, fresh BM cells were first cultured for at least 5 weeks in Iscove's medium with 20% FCS (StemCell) and then subcultured repeatedly until a pure fibroblast monolayer was obtained. Mobilized peripheral blood cells were obtained with informed consent from breast cancer patients pretreated with 10 μg /kg G-CSF for 7 days. Leukopheresis was performed on days 5, 6 and 7 of the mobilization regimen. CB cells from normal, full-term infants delivered by cesarean section were collected in tubes containing heparin according to protocols approved by the University of British Columbia Clinical Screening Committee for Research Involving Human Subjects. This included obtaining informed consent from the mother prior to delivery.

In most experiments, low density (<1.077 g/cm^3) cells were obtained by centrifugation of the initial cell sample on Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden). In 3 experiments using CB cells, RBC were removed either by lysis at 4°C in 0.83% ammonium chloride with 0.1% sodium bicarbonate at a pH of 7.0 or by hydroxyethyl starch (Dupont)-assisted sedimentation. The enriched white blood cell fraction was then used either without further manipulation, or after being enriched for $CD34^+$ cells using a high gradient magnetic

cell separation procedure in which cells expressing markers of mature human hematopoietic cells were removed on a *StemSep*™ column (StemCell) according to the manufacturer's directions. Aliquots of cells were stained before and after this separation with a fluorescein-isothiocyanate (FITC)-conjugated anti-CD34 antibody (8G12) (kindly provided by Dr. P. Lansdorp, Terry Fox Laboratory) to calculate the recovery, enrichment and purity of the $CD34^+$ cells isolated. This magnetic separation procedure (Thomas et al., 1995) enriched the $CD34^+$ cell content of mobilized peripheral blood cells from 1.4% to 49% with 77% recovery of the $CD34^+$ cells. With previously frozen BM, the final $CD34^+$ cell content of the resultant lin^- cells was increased approximately 8-fold (from 10% to 83%) with a recovery of $CD34^+CD38^-$ cells of approximately 50%. The average $CD34^+$ cell content of the starting CB cell suspensions were 0.4% and after depletion of the lineage-$marker^+$ cells, this increased to 43%. The corresponding $CD34^+$ cell recovery and enrichment values for these lin^- CB preparations were 120 ± 20% and 160 ± 30-fold, respectively. In some experiments, highly purified (>99.9%) $CD34^+$ or $CD34^+CD38^{lo}$ cells were isolated from these lin^- cells by FACS. In the remainder, the enriched $CD34^+$ cells were used without further purification.

2.4 Retroviral Vectors

The MSCV-NEO virus (Hawley et al., 1994) constructed using the MSCV 2.1 vector (kindly provided by Dr. R. Hawley, University of Toronto, Toronto, ONT) was used to establish a GP-env AM12 MSCV-NEO producer cell line. The titer of these producer cells was 10^7 colony-forming units/mL as assessed by the transfer of G418 resistance to NIH-3T3 cells (Cone and Mulligan, 1984). The MSCV-HSA.NEO construct was made by insertion of a 271

base pair Hinf I fragment of a cDNA encompassing the entire HSA coding region into the MSCV-2.1 vector upstream of the pgk-neo cassette by blunt-end ligation using standard procedures. The Hinf I fragment was isolated from the pSL87c4 M1/69 cDNA (Kay et al., 1990). High-titer retroviral producer cells were obtained using a serial infection strategy in which GP-E86 cells were first transfected with a calcium-phosphate preparation of MSCV-HSA.NEO and the supernatant harvested 48 hours later. This was then used to infect GP-*env* AM12 cells. Infected GP-*env*AM12 cells were selected in 1 mg/mL G418 (active concentration 688μg/mg)to obtain a polyclonal population of GP-*env* AM12 MSCV-HSA.NEO producers. The resultant titer of the producer cell line thus obtained was 10^6 colony forming units/mL (CFU/mL) as assessed by transfer of G418 resistance to NIH-3T3 cells (Cone and Mulligan, 1984). Subsequent FACS selection of biotin-labeled M1/69 (anti-HSA (Springer et al., 1978; Milstein et al., 1979)) antibody-stained producers enabled a 10-fold increase in viral titer to be obtained (see Results).

Both producer cells were shown to be free of helper virus, as indicated by the inability to recover infectious virus from MSCV-NEO or MSCV-HSA.NEO-infected NIH-3T3 cells (capable of transferring G418 resistance to a culture of naive NIH-3T3 cells). To generate viral conditioned medium, viral supernatants were collected from confluent cultures of MSCV-NEO virus-producing cells after incubation of these overnight with fresh Iscove's medium containing 20% FCS or bovine serum albumin, insulin and transferrin (BIT, StemCell) as indicated. The medium was then harvested, filtered through 0.4 μm filters and stored frozen at -196°C, or was used directly.

2.4.1 Analysis of RNA Transcripts

Total cellular RNA was purified from the producer cell lines using TRIzol (Gibco/BRL) and separated using formaldehyde/agarose gel electrophoresis. After transfer to nylon membranes (ZetaProbe, BioRad, Richmond, CA) mRNAs were detected by hybridization as described (Dougherty et al., 1989), using DNA probes labeled with ^{32}P by random priming (Hodgson and Fisk, 1987). The HSA probe was the 270-bp HinfI fragment of pSL87c4. The neo probe was the XhoI/SalI fragment of pMC 1neo (Thomas and Capecchi, 1987).

2.4.2 Polymerase chain reaction (PCR) Analysis

Colonies generated in CFC assays that did not contain G418 were plucked and analyzed individually using the PCR and Southern blotting with a neo^r probe to amplify and identify incorporated neo-specific sequences as previously described (Hughes et al., 1989).

2.4.3 Retroviral Infection Protocol

In the experiments performed with the MSCV.NEO retrovirus a significant portion of the work concerned the development and validation of a supernatant infection protocol. Therefore the methods involved are described with these experiments in Chapter 4. In the experiments performed with the HSA retrovirus (described in Chapter 5) cells from the mononuclear fraction (post-ficoll) were first incubated at a density of 5 x 10^5/mL in petri dishes for a period of 48 hours in Alpha medium supplemented with 20% FCS, 20 ng/mL human IL-3, 10 ng/mL human IL-6, and 50 ng/mL of human SF. In two experiments designed to assess gene transfer to LTC-IC, lin^- cells (obtained using the *StemSep*™ column) were prestimulated in 20 ng/mL each of IL-3 and IL-6, and 100 ng/mL each SF and FL for 48 hr. The cytokine-activated

hematopoietic cells were then harvested, washed and placed directly in tissue culture dishes containing irradiated (1,500 cGy) producer cells and fresh medium of the same composition (with the same cytokines) plus 4 μg/mL polybrene (Sigma). 48 hours later, loosely adherent and non-adherent cells were recovered by gentle agitation and washing of the dishes. The cells were then pelleted, resuspended in fresh cytokine-supplemented culture medium and incubated for a further 24 hours at 37° C to allow expression of the transferred HSA and neo^r genes. The cells were finally analyzed by FACS and/or plated directly in methylcellulose and LTC-IC assays to measure total and G418-resistant clonogenic progenitor and LTC-IC numbers. HL60 and Mo7e cells were infected without prestimulation by co-cultivation with the retroviral producers in their normal growth medium supplemented with 20% FCS and 4 μg/mL polybrene and thereafter handled in the same way as the infected BM cells.

2.5 FACS Analysis

To isolate the $CD34^+CD38^-$ and $CD34^+CD38^+$ subpopulations from these lin^- cells, they were first suspended in Hank's balanced salt solution with 2% fetal calf serum (FCS) and 0.02% sodium azide (HFN) supplemented with 5% human serum, then incubated on ice for 10 minutes, followed by staining with anti-CD34-FITC and anti-CD38-phycoerythrin (PE) (Becton Dickinson, San Jose, CA). This was followed by 2 washes in HFN, in the last case, in the presence of 2 μg/mL propidium iodide (PI; Sigma) to allow exclusion of nonviable (PI^+) cells. Throughout the staining procedure, the cells were maintained at 4°C. Additional aliquots of cells were stained with irrelevant isotype-matched control antibodies labeled with FITC and PE to establish gates for identifying positively stained cells (fluorescence greater than that exhibited by 99.9% of cells in the corresponding controls).

For analysis of cells post retroviral infection with the HSA containing retrovirus as described in Chapter 5, mock infected, neo^r control and HSA-infected cell populations were first suspended in HFN supplemented with 5% human serum and then incubated on ice for 10 minutes. Aliquots of cells were then stained with a directly conjugated anti- CD34-FITC antibody (8G12) (Lansdorp et al., 1989), and either a directly conjugated M1/69-cyanine-5-succinimidyl (CY5) antibody or a biotinylated M1/69 antibody (Springer et al., 1978; Milstein et al., 1979). In the case of the biotinylated M1/69 antibody, the cells were washed twice in HFN and then incubated with PE - labeled streptavidin for an additional 30 minutes. Appropriate isotype-matched controls were performed as above for each analysis. Following antibody labeling, two further washes in HFN were performed, the last in the presence of 2 μg/mL PI (Sigma) to exclude nonviable cells. In some experiments, specific subpopulations of infected $CD34^+$ cells were analyzed after staining with an anti- CD38-PE antibody. All cells were analyzed and sorted on a FACStar$^+$ (Becton Dickinson) equipped with a 5W argon laser and a 30 mW helium neon laser.

2.6 Progenitor Assays

2.6.1 CFC Assays

Methylcellulose assays (all reagents from StemCell) were performed essentially as previously described (Hogge et al., 1996). Cell suspensions to be assayed for CFC were plated at suitable concentrations (to give < 100 colonies per 1 mL culture) in Iscoves's medium containing 0.9% methylcellulose, 30% FCS, 1% bovine serum albumin (BSA), 10^{-4} M 2-ME (Methocult H4430). In CFC assays initiated with $CD34^+CD38^-$ cells, the methylcellulose was

supplemented with 3U/mL highly purified erythropoietin, 50 ng/mL of SF and 20 ng/mL each of IL-3, IL-6, G-CSF, and GM-CSF. In experiments initiated with less purified cells, the methylcellulose was supplemented with 50 ng/mL of SF and 10 ng/mL each of IL-3 and GM-CSF. Methylcellulose cultures were incubated at 37°C for 2-3 weeks. At the end of this period of time colonies were distinguished by direct visualization in situ using well established criteria (Eaves, 1991).

Following retroviral infection, cells were plated in methylcellulose both with and without G418 (Gibco BRL). The dose of G418 necessary to ensure no colony growth from uninfected cells was determined by preliminary dose response studies of cells from each source used. For HL60 and Mo7e final G418 concentrations of 1.3 and 1.4 mg/mL (dry weight), respectively were used. In experiments where mobilized PBPC were the target cells the final G418 concentration was 1.5mg/ml and in experiments using cord blood a final concentration of 1.6 mg/mL was used. Throughout all the experiments the same batch of G418 was used. In all experiments, control cells were subjected to the same manipulations and then plated with and without G418 at the same time as the infected cells. Gene transfer efficiencies, assessed by G418 resistance, were calculated by dividing the number of colonies observed in cultures containing G418 by the number of clonogenic cells plated (as determined by plating aliquots of the same cells in the same medium but without G418). The surviving fraction of untransduced cells plated in G418 was always less than 5%.

2.6.2 LTC-IC Assays

LTC-IC assays were performed essentially as described (Hogge et al., 1996). Briefly the test cells were seeded into 35 mm tissue culture dishes on 3 x 10^5 irradiated (8000 cGy) murine

fibroblast feeder layers consisting of an equal mixture of M2-10B4 and Sl/Sl cells engineered by retroviral-mediated gene transfer to produce human G-CSF, IL-3 and SF (Sauvageau et al., 1994). Thereafter, the cultures were maintained at 37°C with weekly half-medium changes with fresh hydrocortisone-supplemented Myelocult medium. At the end of the 6 week incubation period, the non-adherent and adherent fractions of the cultures were harvested, pooled and plated in methylcellulose media with and without G418 as indicated. The total CFC content thus obtained provides a relative measure of the in-put LTC-IC since these two parameters are linearly related under the assay conditions used (Sutherland et al., 1990). The CFC output per LTC-IC does not change even when the LTC-IC are purified or cultured (Sutherland et al., 1990; Petzer et al., 1996a).

2.6.3 Serum-Free Suspension Cultures

$CD34^+CD38^-$ cells were incubated at ~ 10^3 to 10^4 cells/mL (0.1 to 10 mL per culture) in Iscove's medium supplemented with 10 mg/mL bovine serum albumin, 10 µg/mL human insulin, and 200 µg/mL human transferrin (BIT, StemCell), 10^{-4} M 2-mercaptoethanol (Sigma), plus 40 µg/mL low density lipoproteins (Sigma) and the following recombinant human GF: 20 ng/mL each of IL-3, IL-6 and G-CSF, 100 ng/mL each of SF, and FL. These suspension cultures were incubated unperturbed, usually for 5 or 6 days (five experiments) and in one experiment for 8 days at 37°C. At the end of this time, all cells were harvested, counted and aliquots assayed *in vitro* for CFC, LTC-IC and in NOD/SCID mice for CRU. In two additional experiments $CD34^+CD38^-$ CB cells were cultured as single cells (deposited using the FACS cloning attachment) in the individual wells of 96 well plates, each of which had been preloaded with 100 µl of the same medium. At the end of 6 days, 70 of the clones

produced were harvested individually and injected into 70 sublethally irradiated NOD/SCID mice (one clone per recipient).

2.7 Mice

The colony of NOD/LtSZ-*scid/scid* (NOD/SCID) mice was established in the animal facility of the British Columbia Cancer Research Center from breeders originally provided by Dr. L. Schultz (Jackson Laboratory, Bar Harbor, ME). All NOD/SCID mice were kept under sterile conditions in microisolator cages and were provided exclusively with autoclaved food and water. Just before, and for 2 months after, total-body irradiation the mice received acidified H_20 (pH=3).

2.7.1 Competitive Repopulation Assay

Mice were given 350 cGy of total body ^{137}Cs γ-irradiation (~ 1 cGy/min) and were then injected intravenously with varying numbers of test cells (as indicated). For grafts of $\leq 10^6$ cells per mouse, 10^6 irradiated (1500 cGy) carrier normal human BM cells were co-injected. Mice were killed 6 to 8 weeks post-transplant (or as indicated) for assessment of the number and types of human cells detectable. Human CRU frequencies were determined by the method of maximum likelihood from the proportions of negative recipients measured in groups of mice injected with different numbers of test cells as described (Szilvassy et al., 1990). In the present study, negative recipients were defined as mice that did not contain detectable numbers of *both* human B-lymphoid cells; i.e., ≤ 5 positive $CD34^-CD19^+$ cells per 5000 cells analyzed *and* human $CD34^+$ myeloid progenitors (CFU-GM and/or BFU-E and/or CFU-

GEMM). In experiments performed to assess the frequency of gene transfer to human CRU, the mice were injected with cells obtained at the end of the infection protocol being investigated.

2.7.2 Analysis of Human Cells in NOD/SCID Mice

Femurs and tibias were flushed with HFN and cell counts performed. Human Fc receptors were blocked by a first incubation of the cells in 5 % normal human serum and murine Fc receptors by a second incubation of the cells in 2.4G2 [an anti-mouse Fc receptor monoclonal antibody (Unkeless, 1979)]. To quantitate the total number of human cells present, a small aliquot was stained with anti-CD45-FITC (HLel, Becton Dickinson) and anti-CD71-FITC (OKT9). This aliquot was also stained with anti-CD19-PE (Leu-12, Becton Dickinson) and CY5-labeled anti-CD34 (8G12) to allow the exclusive detection of human pre-B ($CD34^-$ $CD19^+$) cells (Tjonnfjord et al., 1996). The majority of the cells were stained with anti-CD34-PE and anti-gpIIb/IIIa CD41-FITC (3H2) (Hogge et al., 1997) antibodies to allow quantitation of cells with these markers and isolation of human $CD34^+$ cells by FACS for subsequent plating in CFC and LTC-IC assays. All cells were also stained with PI (see above) in the final wash to allow exclusion of dead (PI^+) cells from these analyses and collections. Aliquots of the same original cell suspension were stained with both FITC-conjugated and PE-conjugated mouse Ig as negative controls. Positive cells were defined as those demonstrating greater fluorescence than that exhibited by $\geq$ 99.9% of these negative control cells. Incubation of normal NOD/SCID marrow cells with all anti-human antibodies used showed $\leq$ 0.1% of murine myeloid cells to be stained non-specifically. In the animals reconstituted with infected cells which contained both human lymphoid ($CD19^+$) and human $CD34^+$ populations, the

human $CD34^+$ cells were sorted and plated in CFC assays with and without G418 as described above.

2.8 Statistics

Differences between test populations were determined by two-tailed Student t test. Values derived from replicate measurements are reported as the mean ± SEM. CRU frequencies were calculated by the method of maximum likelihood from the proportion of animals that were negative, when these mice were assessed 6-8 weeks post transplant, using a generalized linear model with binomial errors and a complementary log-link function.

Chapter 3 Expansion *in vitro* of Transplantable Human Cord Blood Stem Cells Demonstrated Using a Quantitative Assay of their Lympho-Myeloid Repopulating Activity in Nonobese Diabetic-Scid/Scid mice*

*The contents of this Chapter are essentially as published in Conneally al., 1997, Proc Natl Acad Sci USA, 94, 9836-9841. J. Cashman made a significant contribution in the FACS analysis and the assays of the human cells in the mice. A. Petzer also made a significant contribution in the development of the purified cell expansion protocol and in the isolation of some of the purified cells.

3.1 Introduction

Analysis of various endpoints of hematopoietic reconstitution from defined transplants has provided extensive information about HSC in several species including man. In mice, this approach has been further developed to allow both adult and fetal hematopoietic stem cells to be specifically identified and quantitated using an assay that measures the frequency of cells in a given test cell suspension that at limiting dilutions can be seen to individually, competitively and durably repopulate both the lymphoid and myeloid systems of histocompatible but genetically distinguishable recipients (Szilvassy et al., 1990; Fraser et al., 1992; Rebel et al., 1994; Harrison et al., 1993). As described in Chapter 1, to maximize the efficiency of detection of these so-called competitive repopulating units (CRU), the recipient mice are pretreated with a myeloablative conditioning regimen and then transplanted with sufficient additional cells to allow their survival independent of the stem cell content of the test transplant. Alternatively, the recipients may be given a sublethal dose of radiation (Trevisan et al., 1996). Clinical studies with purified subpopulations of human cells (Berenson et al., 1991; Shpall et al., 1994; Korbling et al., 1995; Dunbar et al., 1995) have suggested some of the properties of transplantable human hematopoietic cells, but an experimental method for their enumeration has not been available. In fact, until recently, attention has focused primarily on the identification of properties of hematopoietic cells that might prove useful as surrogate endpoints of transplantable totipotent HSC, for example, those that allow cells to be identified as LTC-IC (Sutherland et al., 1990). The fact that LTC-IC and CRU in both the fetal liver and adult BM of mice are phenotypically similar, exist at similar frequencies and copurify (Ploemacher et al., 1991; Lemieux et al., 1995; Miller et al., 1997) has suggested that LTC-IC

and CRU may be the same cells, i.e., that the functions required for cells to be detectable in these two assays are coordinately regulated during normal hematopoietic cell development. This concept is further supported by the demonstration that at least some CRU proliferate and undergo self-renewal divisions under the same culture conditions as are used to stimulate LTC-IC proliferation and differentiation into CFC (Fraser et al., 1992). However, differences in the factors required to elicit and sustain murine CRU and LTC-IC activity *in vitro* and *in vivo* have also been identified (Lemieux and Eaves, 1996; Miller et al., 1997).

Recently, a variety of immunodeficient xenogeneic recipients have been found to support the growth of transplanted human hematopoietic cells (Zanjani et al., 1994; Dick, 1996; Nolta et al., 1996) with higher overall levels of human hematopoiesis obtained in sublethally irradiated NOD/SCID mice than in any other strain thus far tested (Pflumio et al., 1996; Cashman et al., 1997). The amplification, multi-lineage composition, durability, continuing proliferation and retransplantability of the human hematopoietic cell populations regenerated in these mice all suggest their origin from a transplantable human cell type with extensive proliferative and differentiation potential (Cashman et al., 1997; Cashman et al., 1997). In this Chapter, I describe a simple method for quantitating human cord blood (CB) cells that produce both lymphoid and myeloid progeny in sublethally irradiated NOD/SCID mice injected intravenously with limiting dilutions of test cells (without exogenously administered cytokines). The assay can be applied to highly purified populations and has been used to demonstrate human CRU expansion in 5 to 8 day cultures of $CD34^+CD38^-$ CB cells in serum-free cultures containing FL, SF, IL-3, IL-6 and G-CSF.

3.2 Results

3.2.1 Both Human Lymphoid and Human Myeloid cells are found in NOD/SCID Mice Transplanted with limiting numbers of human CB cells.

Groups of sublethally irradiated NOD/SCID mice were injected with decreasing doses of light density CB cells or highly enriched (> 99.9% pure) $CD34^+CD38^-$ or $CD34^+CD38^+$ CB cells. Six to 8 weeks later their marrows were assessed for the presence of various types of human hematopoietic cells. In a total of 115 mice in which human cells were detected, 91% contained both lymphoid ($CD34^-CD19^+$) and myeloid ($CD34^+$ CFC) elements (Figure 3.1). Analysis of the mice injected with limiting numbers of transplantable human CB cells (i.e., cell doses that, on average, should have resulted in < 15% of the mice being engrafted with human cells) showed that examples of mice containing human pre-B cells in the absence of human myeloid cells, or vice versa, were rare regardless of whether the mice had been transplanted with unseparated light-density CB cells, or highly purified $CD34^+CD38^-$ or $CD34^+CD38^+$ cells (Table 3.1). Thus, no evidence of either lymphoid- or myeloid-restricted repopulating cells in human CB was obtained using a 6 to 8 week readout in NOD/SCID mice. In fact, essentially all of the regenerating activity detected using this endpoint could be attributed to transplantable CB cells with lympho-myeloid potential. In addition, 40% of these clonally repopulated mice showed evidence of human erythroid as well as granulopoietic cell differentiation (i.e., both BFU-E and CFU-GM were detected).

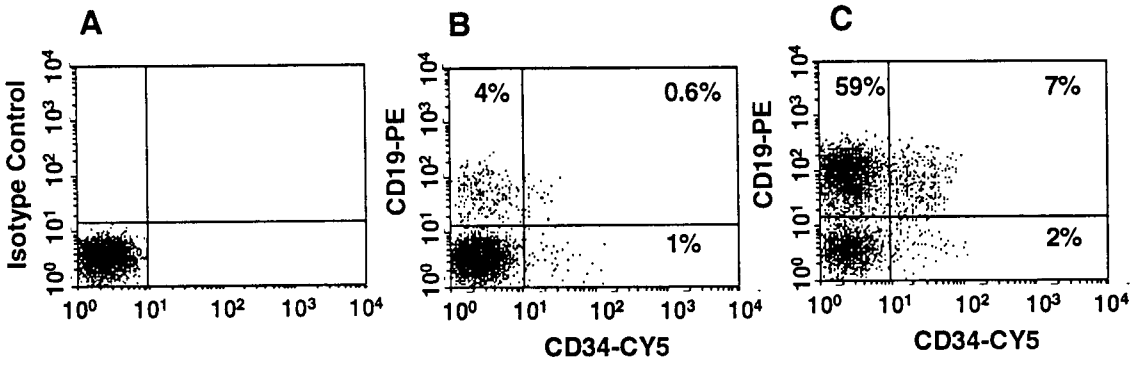


Figure 3.1 Comparison of FACS profiles of marrow cells from NOD/SCID mice transplanted 6 and 8 weeks previously with 300 $CD34^+CD38^-$ cells (A and B). (i.e., at limiting dilution) and 3000 $CD34^+CD38^-$ cells (C) from the same original CB sample. In A the cells were stained with an irrelevant isotype mouse IgG. In B and C, the cells were stained with anti-human CD34-CY5 and anti-human CD19-PE. Evidence of human cells of all 3 phenotypes examined in these analyses is seen in both mice (B and C).

Table 3.1 NOD/SCID mice transplanted with limiting numbers of human CB repopulating cells usually contain both lymphoid and myeloid cells of human origin.*

Progeny Combinations	Proportion of Positive Mice Transplanted With:					
	Light Density Cells (< 4×10^5 / mouse)		$CD34^+CD38^-$ Cells (< 800 / mouse)		$CD34^+CD38^+$ Cells (< 10^4 / mouse)	
Myeloid and/or Lymphoid (M^+ &/or L^+)	67%	(12/18)	36%	(20/55)	43%	(7/16)
M^+ only	6%	(1/18)	2%	(1/55)	6%	(1/16)
L^+ only	0%	(0/18)	4%	(2/55)	6%	(1/16)
Expected L^+ M^+ (due to coincidence)	0%		0.1%		0.4%	
Observed L^+ M^+	61%	(11/18)	31%	(17/55)	31%	(5/16)

* Only mice injected with doses of cells expected to contain < 1 repopulating cell of any kind (based on limiting dilution analysis of the entire data set in that group) were considered in this analysis, i.e., < 15% of these mice would have received > 1 repopulating human cell (same experiments as those shown in Table 3.2, but for the analysis shown here, mice were considered negative only if they contained neither). Note also that these cell doses represent the calculated numbers expected to contain < 1 such repopulating cell but, as can be seen, did not always give 37% negative mice.

3.2.2 Frequency and Characterization of CRU in Human CB

Limiting dilution analysis was then used to determine the frequency of these transplantable lympho-myeloid cells in various subpopulations of human CB (Table 3.2). Individual mice were scored as positive only when the number of human lymphoid ($CD34^-CD19^+$) cells present 6 to 8 weeks post-transplant constituted $\geq 0.1\%$ of the total marrow population *and* human ($CD34^+$) granulopoietic progenitors, with or without erythroid clonogenic progenitors, were also demonstrable in the cells removed from two femurs and two tibias. Since these 4 bones contain ~ 25% of the total marrow volume of a mouse (Boggs, 1984), the minimal output required for assignment of human CRU activity was thus ~ 2×10^5 pre-B cells and ~ 200 CFC at 6 to 8 weeks post-transplant. Any recipient who did not fulfill both of these criteria was scored as negative. The average frequency of CRU in the light density fraction of CB cells calculated from the results of these experiments was ~ 1 per 6×10^5 cells (range defined by $\pm$ SE = 1 per 5×10^5 to 1 per 8×10^5 cells, Table 3.3), or 4.5 (3.4 to 5.4) CRU per mL of CB (n=3).

Table 3.2 Frequency of "negative" NOD/SCID mice 6 to 8 weeks after transplantation with varying numbers of freshly isolated or cultured CB cells. (Negative mice were defined as those that did not contain detectable levels of *both* human $CD34^-CD19^+$ (B-lymphoid) *and* $CD34^+$ CFC (myeloid) cells).

Type of Cell	No. of Exp'ts	Total No. of CB Assessed	Cells/Mouse	Proportion of Negative Mice
Light Density	3	7	3×10^6	2/10
			1×10^6	2/8
			8×10^5	0/6
			3×10^5	3/8
			2×10^5	1/6
			5×10^4	3/4
$CD34^+CD38^+$	2	4	5×10^4	0/5
			1.5×10^4	5/6
			7500	2/6
			3000	5/5
			600	4/5
$CD34^+CD38^-$	7	16	4400	1/2
			3000	2/11
			2000	5/5
			1000	5/17
			500	5/12
			300	1/4
			200	10/14
			50	21/25
Expanded $CD34^+CD38^-$	6	14	4400	0/3
			1000	2/11
			500	3/5
			200	9/12
			100	3/3
			50	5/6
			40	3/7

Pooled data from the number of experiments shown (2 to 3 CB samples pooled per experiment).

Table 3.3 Comparison of the frequencies of different types of progenitors in the light-density, purified and cultured human CB populations studied.

Progenitor	Light Density (per 10^6 cells)	$CD34^+CD38^+$ (per 10^3 cells)	$CD34^+CD38^-$ (per 10^3 cells)	Cultured $CD34^+CD38^-$ (per initial 10^3 cells)
CFC	4,000 ± 2,300	500	310 ± 130	30,000 ± 12,000
LTC-IC	52 ± 46	52	560 ± 230	2,100 ± 900
CRU	1.7	0.06	1.1	2
	(1.3 - 2)	(0.04 - 0.08)	(0.9 - 1.3)	(1.6 - 2.5)
No. of expts	3	2	7	6

Values shown for CFC and LTC-IC are the mean ± SEM and for CRU are the mean with the range defined by ± SE shown in brackets. The total cell expansion in the cultures of $CD34^+CD38^-$ cells was 78 ± 33-fold.

Several lines of evidence, including the finding that clinical transplants of $CD34^+$ cell-enriched BM transplants give timely hematopoietic reconstitution of patients treated with myeloablative conditioning therapy (Berenson et al., 1991; Shpall et al., 1994; Korbling et al., 1995) suggest that human cells with repopulating ability are $CD34^+$. However, human $CD34^+$ hematopoietic cells are now known to be heterogeneous both functionally and in terms of other markers they may express (Krause et al., 1996; Tjonnfjord et al., 1996). One such marker, whose absence on a minor subset of human hematopoietic $CD34^+$ cells (including human CB cells) has proven useful for isolating a population that is highly enriched in cells with properties of primitive progenitors is CD38 (Terstappen et al., 1991; Rusten et al., 1994; Murray et al., 1995; Issaad et al., 1993; Hao et al., 1995; Hao et al., 1996; Rawlings et al., 1997; Civin et al., 1996). To investigate the phenotype of human CB cells capable of regenerating lympho-myelopoiesis in NOD/SCID mice, the $CD34^+$ light density CB cells were subdivided into a $CD38^-$ or $CD38^{lo}$ and a $CD38^+$ fraction and then limiting dilution CRU assays were performed on these subsets. The average frequency of CRU in the $CD34^+CD38^{lo}$ population determined from the pooled results of 7 experiments was found to be 1 per 900 cells (range defined by ± SE = 1 per 750 to 1 per 1,100 cells, (Table 3.3). This represents a 600-fold enrichment of CRU relative to their frequency in the light density fraction of CB cells. In two experiments, CRU assays were performed on the $CD38^+$ and $CD38^-$ fractions of the same CB samples. From these assays, values of 1 CRU per 18,000 $CD34^+CD38^+$ CB cells and 1 CRU per 400 $CD34^+CD38^-$ CB cells were obtained. Thus some CRU could be detected in the $CD34^+CD38^+$ fraction and at > 33-fold higher frequencies than in the original light density fraction of CB cells, but at a 45-fold lower frequency than in the corresponding $CD34^+CD38^-$

fraction. However, because the majority of the $CD34^+$ cells are also $CD38^+$, absolute numbers of $CD38^+$ CRU are much higher than predicted by a comparison of their frequencies. The ratio of total $CD34^+CD38^-$ CRU to $CD34^+CD38^+$ CRU in CB was calculated to be 4:1. LTC-IC and CFC assays of these same fractions showed that both of these assays detect cells at much higher frequencies than CRU (several hundred-fold) and in fact account for the majority of all the $CD34^+$ cells (Table 3.3). However, the distribution of LTC-IC between the $CD38^-$ subset and $CD38^+$ subsets (64% and 36%, respectively) proved to be similar to that calculated for CRU whereas the corresponding values for CFC were 20% and 80%.

3.2.3 Quantitation of CRU and other Progenitors in Short Term Cultures of $CD34^+CD38^-$ Human CB cells

On the basis of previous studies indicating FL, SF and IL-3 to be important stimulators of BM LTC-IC amplification (Petzer et al., 1996b), and SF and IL-6 to be effective stimulators of primitive cells in both marrow and CB (Lansdorp et al., 1993; Sui et al., 1995; Traycoff et al., 1995; DiGiusto et al., 1996), we set up experiments to determine whether CRU activity would be amplified, maintained, or lost when $CD34^+CD38^{lo}$ CB cells were incubated in serum-free media supplemented with FL and SF (both at 100 ng/mL) and IL-3, IL-6, and G-CSF (all at 20 ng/mL). The results of the CRU assays performed on aliquots of the cells used to initiate these cultures as well as on aliquots of the cells harvested from them 5 to 8 days later (6 experiments) are shown in Tables 3.2 and 3.3. The frequency of CRU in the cultured cells was 1 per 500 input $CD34^+CD38^-$ CB cells (range defined by $\pm$ SE = 1 per 400 to 1 per 600). This represents a small (2 - fold) but significant ($p < 0.02$, Student's 2-tailed t-test) increase over the input values of these experiments.

In two additional experiments, 120 $CD34^+CD38^-$ CB cells were cultured as single cells under the same conditions. At the end of 5 days, 33 of the original 120 wells (i.e., 28%) did not appear to contain any viable cells. In each of the remaining 87 wells, between 2 and 270 viable (refractile) cells were seen (with no wells containing only one viable cell). The following day (day 6), the first 70 clones were injected individually into 70 sublethally irradiated NOD/SCID mice (1 clone per mouse). Six to 8 weeks later, 68 of these recipients showed no evidence of engraftment with human cells. In the other 2 mice, a small proportion (0.2%) of all the cells present in the marrow appeared to be positive human $CD34^+$ cells, but no $CD19^+$ cells were detected and when the $CD34^+$ cells were isolated (972 and 115 cells, respectively) and assayed, none of these were found to be CFC.

In addition to evaluating CRU, we also measured the frequency, and hence total number of CFC and LTC-IC present in the starting light density population, the $CD34^+CD38^+$ cells, the $CD34^+CD38^{lo}$ cells, and in the cell populations generated from $CD34^+CD38^{lo}$ cells in the 5 to 8 day cultures. As previously demonstrated (Hao et al., 1995), LTC-IC were detectable in both the $CD34^+CD38^{lo}$ and $CD34^+CD38^+$ fractions of CB (Table 3.3). In the cultured population, the total number of cells increased 78 ± 33-fold, the number of CFC 98-fold and the number of LTC-IC 4-fold (Table 3.3).

3.2.4 Comparison of the Cellular Output of Different Sources of Human CRU

Table 3.4 compares the average output of different types of human hematopoietic cells generated in NOD/SCID mice, per injected CRU, for each type of CB population transplanted, i.e., light-density cells, $CD34^+CD38^+$ cells, $CD34^+CD38^{lo}$ cells and the cells produced in 5 to 8 day-old cultures of $CD34^+CD38^{lo}$ cells. In all cases, the predominant types

of progeny present 6 to 8 weeks post-transplant were human pre-B ($CD34^-$ $CD19^+$) cells (~ 10^6 to 10^7 per mouse per injected CRU). However, the output of very primitive human myeloid cells, i.e., LTC-IC and CFU-GEMM, was substantial (~ 50 to 1300 per mouse per injected CRU), with intermediate numbers of progeny indicative of differentiation along the erythroid, megakaryocyte and granulopoietic lineages (10^3 to 5 x 10^5 per mouse per injected CRU). These values were similar for the $CD34^+CD38^+$ and $CD34^+CD38^-$ CRU in fresh CB but were lower for the culture-derived CRU.

Table 3.4 Comparison of the numbers and types of human progeny present after 6 to 8 weeks in NOD/SCID recipients of various subsets of fresh or cultured human CB cells expressed per injected CRU.*

Endpoint	Light-Density	$CD34^+CD38^+$	$CD34^+CD38^-$	Cultured Cells
$CD34^-CD19^+$	0.9×10^7	0.8×10^7	1.0×10^7	0.1×10^7
$CD34^+$	2.2×10^6	2.5×10^6	2.2×10^6	0.4×10^6
CD41+	4.0×10^5	2.0×10^5	5.0×10^5	2.0×10^5
BFU-E	2,800	7,000	3,500	1,300
CFU-GM	27,000	28,000	28,000	9,000
CFU-GEMM	150	130	520	180
LTC-IC	200	1,300	240	50

*Values shown are average estimates calculated by dividing the average total number of each type of human progeny measured in recipients in the limiting dilution experiments shown in Table 3.2 by the total number of CRU that were assayed (based on the CRU frequency values shown in Table 3.3).

3.3 Discussion

In this Chapter I describe a quantitative *in vivo* assay for transplantable normal human cells with lympho-myeloid differentiation potential. Quantitation of these cells was achieved by limiting dilution analysis of the frequency of cells that were individually able to regenerate detectable numbers of both lymphoid ($CD34^-CD19^+$) and myeloid ($CD34^+$ erythroid and/or granulopoietic progenitors) within the marrow of immunodeficient (NOD/SCID) mice transplanted 6 to 8 weeks previously. The mice were pretreated with a close to lethal dose of radiation, sufficient to provide a potent stimulus for intravenously transplanted human HSC to proliferate and differentiate, but insufficient to kill more than a small proportion of the mice even in the absence of any protection provided by the injected cells (Cashman et al., 1997). Strong evidence that this assay detects single human cells with lymphoid and multilineage myeloid potential was provided by the demonstration that both human pre-B cells and human CFU-GM were usually seen in individual mice (and in almost half of these human BFU-E were also demonstrable), even when unseparated human CB cells were injected at doses that were insufficient to engraft more than two thirds of the recipients. This assay thus successfully incorporates the same principles as the murine CRU assay (Szilvassy et al., 1990) and therefore the same term (CRU) is proposed for the human cells it detects.

The average frequency of human CB CRU measured using this assay is 1 per 6 x 10^5 light density cells or 5 CRU per mL of CB. These values are much lower than the numbers of cells detectable as LTC-IC; however, both were found predominantly, although not exclusively, in the $CD34^+$ $CD38^-$ subpopulation. The presence of $CD34^+CD38^+$ as well as $CD34^+CD38^-$ cells in adult human BM that can engraft fetal sheep has recently been reported

(Civin et al., 1996), as has the ability of NOD/SCID mice to be engrafted with $CD34^+CD38^-$ human CB cells (Larochelle et al., 1996). Notable differences between $CD38^+$ and $CD38^-$ subpopulations of $CD34^+$ cells have been demonstrated even for functionally similar cells (Issaad et al., 1993; Sauvageau et al., 1994; Hao et al., 1995; Prosper et al., 1996), including those with *in vivo* repopulating activity (Civin et al., 1996). In the present study, no obvious difference was seen in the types or numbers of 6 to 8 week progeny generated in NOD/SCID recipients of human $CD34^+CD38^+$ and $CD34^+CD38^-$ CB CRU. The culture-derived CB CRU which produced the same spectrum of progeny types but at somewhat reduced levels. Recent studies have indicated that NOD/SCID mice engrafted with light density human CB cells can also regenerate progeny CRU (Cashman et al., 1997). It will, therefore, be of interest in future work to determine whether a greater self-renewal capacity in the NOD/SCID system is associated with a lack of CD38 expression by the original $CD34^+$ hematopoietic cells transplanted, as suggested by studies in the sheep model (Civin et al., 1996). In addition, the NOD/SCID recipient could offer other opportunities to investigate potential molecular determinants of human totipotent stem cell self-renewal (Sauvageau et al., 1995; Yonemura et al., 1996).

Human CB has recently attracted attention as a source of hematopoietic stem cells both for transplantation and gene therapy applications. However, concern that a single CB collection may not be sufficient to guarantee engraftment of adult allogeneic recipients has also stimulated considerable interest in developing methods for expanding CB stem cell numbers *in vitro*. Similarly, gene transfer using retroviral vectors requires that the target stem cells be proliferating under conditions where stem cell functions are retained. In related studies of the responses of $CD34^+CD38^-$ cells isolated from human marrow to various cytokine

combinations, Zandstra et al have found that LTC-IC function can be maintained or lost according to the relative or absolute concentrations of FL, SF and IL-3 to which the cells are exposed, without significant effects on their viability or mitotic activation (Zandstra et al., 1997a). As a first application of the CRU assay, I therefore asked whether conditions that expand the LTC-IC population from the $CD34^+CD38^-$ subset of cells in adult human marrow 20 to 30-fold (Petzer et al., 1996a; Petzer et al., 1996b) would similarly expand the CRU population in cultures of $CD34^+CD38^-$ CB cells. The results show that, in spite of the large increases in total cells and CFC (80 and 100-fold, respectively) anticipated from earlier studies (Lansdorp et al., 1993), LTC-IC and CRU numbers were increased only 4-fold and 2-fold respectively. Similar results for CB LTC-IC and CRU expansion under these or similar conditions have recently been reported by others (Kogler et al., 1996; Bhatia et al., 1997a). It thus appears that $CD34^+CD38^-$ CB and adult BM cells may differ in their cytokine requirements for promoting self-renewal divisions. Subsequent multi-factorial design experiments have confirmed this (Zandstra et al., 1997b) (see also Chapter 6). Nevertheless, the fact that a net increase in CRU numbers can be obtained under conditions that yield only 4-fold expansions of LTC-IC is encouraging and supports the concept that the modified LTC-IC assay used in the present studies (Hogge et al., 1996) is highly predictive of changes that may occur in CRU numbers. Thus, even though the cells identified by these two assays do not appear to represent identical cell populations, it can be anticipated that conditions able to more effectively stimulate CB LTC-IC expansion may also stimulate greater increases in CB CRU.

Chapter 4 Efficient Retro-Viral Mediated Gene Transfer to Human Cord Blood Stem Cells with *In vivo* Repopulating Activity

The contents of this chapter are as described in Conneally et al, 1998, Blood, *91* (9).

4.1 Introduction

Transduction of pluripotent HSC using recombinant retroviruses forms the basis of most current strategies for the correction of single gene defects. Efficient transfer of genes into murine hematopoietic stem cells with long-term *in vivo* repopulating ability can now be routinely achieved using this approach (Williams et al., 1984; Szilvassy et al., 1989a; Sorrentino et al., 1992; Einerhand et al., 1993). Encouraging results have also been obtained with human progenitors detectable *in vitro* as colony-forming cells (CFC) and their more primitive precursors identified as long-term culture-initiating cells (LTC-IC) (Moore et al., 1992; Hughes et al., 1992; Nolta et al., 1992; Moritz et al., 1993; Lu et al., 1993a). More recent findings indicate the possibility of gene transfer to human hematopoietic cells capable of engrafting immune deficient mice (Nolta et al., 1996; Larochelle et al., 1996; Yurasov et al., 1997) and improved gene transfer to primate repopulating cells (Dunbar et al., 1996). However, as described in Chapter 1, the application of this technology to clinical transplants has, overall, yielded disappointing results with a few notable exceptions.

In this Chapter, I describe studies in which I focused on the identification of factors that might rapidly stimulate the proliferation of human cell populations that include transplantable progenitors without loss of their original functional potential. As described in Chapter 3, LTC-IC (defined using a 6 week CFC output endpoint (Hogge et al., 1996)) and cells able to regenerate human lympho-myelopoiesis in sublethally irradiated NOD/SCID mice (CRU) are similarly amplified in short term cultures of $CD34^+CD38^{lo}$ human CB cells stimulated by high concentrations of FL, SF, IL-3, IL-6 and G-CSF (Conneally et al., 1997). In addition, LTC-IC and CRU in freshly isolated CB cells are similarly distributed between the

$CD38^+$ and $CD38^-$ subsets of the $CD34^+$ CB population. These findings suggested a close relationship between the cells identified by these two assays and encouraged the continued use the LTC-IC assay as a means to identify conditions for optimizing retroviral-mediated gene transfer to CRU. This strategy allowed the development of a supernatant infection protocol that gives reproducibly high levels of retroviral-mediated gene transfer to human CB CRU (~30%) which is significantly correlated with the levels of gene transfer obtained for co-infected 6 week LTC-IC but not for co-infected CFC.

4.2 Results

4.2.1 Validation of the Supernatant Infection Protocol

In an initial series of experiments, the efficiency of infecting human CB CFC and LTC-IC when the target cells were incubated with MSCV-NEO virus-containing supernatants under various culture conditions was compared to the levels of gene transfer obtained by co-cultivation with MSCV-NEO viral-producer cells. The conditions chosen were based on previously reported findings that coincubation of the target cells on fibronectin (Moritz et al., 1994; Hanenberg et al., 1996) or fibroblasts (Moore et al., 1992; Nolta et al., 1995) could improve the efficiency of gene transfer to primitive human hematopoietic cells. Either light density (10^6/mL) or lin^- (36 ± 6 % $CD34^+$, 10^5/mL) CB cells were first incubated in the absence of virus for 48 hours in Iscove's medium with 20% FCS and 20 ng/mL IL-3, 10 ng/mL IL-6 and 50 ng/mL SF. Aliquots of these "prestimulated" cells were then incubated in the same culture volume for an additional 48 hours either in cell-free virus-containing medium supplemented with the same cytokines in petri dishes, or in dishes that had been pre-coated

with human full length fibronectin (Sigma) at a concentration of 5 μg/cm^2, or on top of a monolayer of irradiated (1500 cGy) allogeneic human BM-derived fibroblasts, or in fresh medium containing the same cytokines on top of a monolayer of irradiated (150 cGy) producer cells, as indicated. Polybrene was added to all media to give a final concentration of 4 μg/mL. The cytokine-supplemented viral supernatants (and control media) were replaced halfway through the 48 hour infection period at the end of which all nonadherent cells were harvested, washed and assessed for G418-resistant CFC and LTC-IC. The results of these experiments are summarized in Table 4.1. Supernatant infection on fibronectin-coated plates gave similarly high levels of gene transfer to LTC-IC as were obtained by cocultivation (44% vs. 39%) and both conditions also gave a high level of gene transfer to CFC. Supernatant infection in the absence of either fibronectin or human BM fibroblasts produced very low levels of gene transfer to any type of progenitor. The presence of human fibroblasts improved gene transfer efficiencies to CFC but the gene transfer efficiencies and recoveries of LTC-IC were reduced to levels that precluded their assessment.

Table 4.1 Comparison of transduction efficiencies (% G418-resistant progenitors) obtained using supernatant infection alone, supernatant with fibronectin, supernatant with stromal support or cocultivation.*

	No of Expts.	BFU-E	CFU-GM	CFU-GEMM	LTC-IC
Sup. on Fibronectin	7	54 ± 10	52 ± 5	40 ± 7	44 ± 14
Sup. on Fibroblast	3	23 ± 6	28 ± 3	15 ± 4	--‡
Sup. alone	2	0	7 ± 0.5	3 ± 3	6 ± 1
Cocultivation	3	90 ± 20	56 ± 11	43 ± 9	39 ± 12

The values represent the mean ± SEM.

* All cells were incubated in cytokines for 2 days prior to 2 days of infection in the same virus-containing medium. For details, see text. Sup. = supernatant

‡Low LTC-IC recoveries precluded measurements of gene transfer efficiency.

4.2.2 Retention of CRU Activity During Infection

A series of 6 experiments was then undertaken to determine how the maintenance of CRU activity might be influenced by incubation of the cells with a retroviral supernatant generated in medium containing 20% FCS or medium supplemented with a defined serum substitute (BIT, StemCell). At the time of starting these experiments, we had just determined that FL, in addition to SF, IL-3 and IL-6 (or G-CSF), is important for achieving optimal expansion of LTC-IC and CFC in short term cultures of normal adult human BM (Petzer et al., 1996b), and that this combination of cytokines would also support some expansion of CB LTC-IC and CRU (4-fold and 2-fold, respectively), in 5 to 8-day cultures (Conneally et al., 1997). Therefore, the cytokines selected for use in this next set of gene transfer experiments were changed from the previous combination to FL and SF (100 ng/mL each) plus IL-3, IL-6 and G-CSF (20 ng/mL each). To avoid the toxicity that polybrene had been found to have on primitive cells (Flasshove et al., 1995), the polybrene was replaced with 5 μg/mL of protamine sulphate. In addition, the period of prestimulation was extended from 48 to 72 hours. This latter change was based on our observations of single $CD34^+CD38^-$ CB cells which showed that, under the conditions used, all viable cells would divide within 5 days, but not before (Conneally et al., 1997). Four of the experiments were set up with lin^- CB cells (at 10^5 cells/mL), one with FACS-purified $CD34^+$ CB cells (at 10^5 cells/mL), and one with FACS-purified $CD34^+CD38^{lo}$ CB cells (at 10^4 cells/mL). The rest of the protocol was the same as had been found to be optimal in the previous experiments, i.e., the cells were prestimulated in cytokine-supplemented, serum-free medium followed by 48 hours of infection on fibronectin-coated petri dishes with replacement of the cytokine-supplemented viral supernatants (prepared either in medium plus 20% FCS or serum-free plus BIT) after the first 24 hours. At the end of the second 24 hours of infection, the cells were harvested and assayed for CFC, LTC-IC and for their ability to generate lymphoid and

myeloid progeny following their transplantation into sublethally irradiated NOD/SCID mice. The input cell type and numbers and the number of resulting positive mice for human CFC, and $CD19^+$ cells is shown in Table 4.2. Figure 4.1 shows a representative dot plot of the relative numbers of total human cells and human $CD34^+$ cells present in the marrow of one of these mice.

Table 4.2 Type and number of cells initially cultured and number of resulting positive mice

Cell Type	No. of Expts.	Input Cell Number Used to Generate the Cells Injected into Each Mouse (x 10^4)	Number of Positive Mice (%)**
Lin- cells*	3	32 ± .3	11/13 (85%)
$CD34^+$	1	3.6	4/4 (100%)
$CD34^+CD38^{lo}$	1	7	5/6 (83%)

* The mean ± SEM starting $CD34^+$ cell content of the lin- CB cells was 43 ± 2%.

** Positive mice refers to mice in which human $CD19^+$ lymphoid cells and human myeloid CFC were detected after 6 weeks

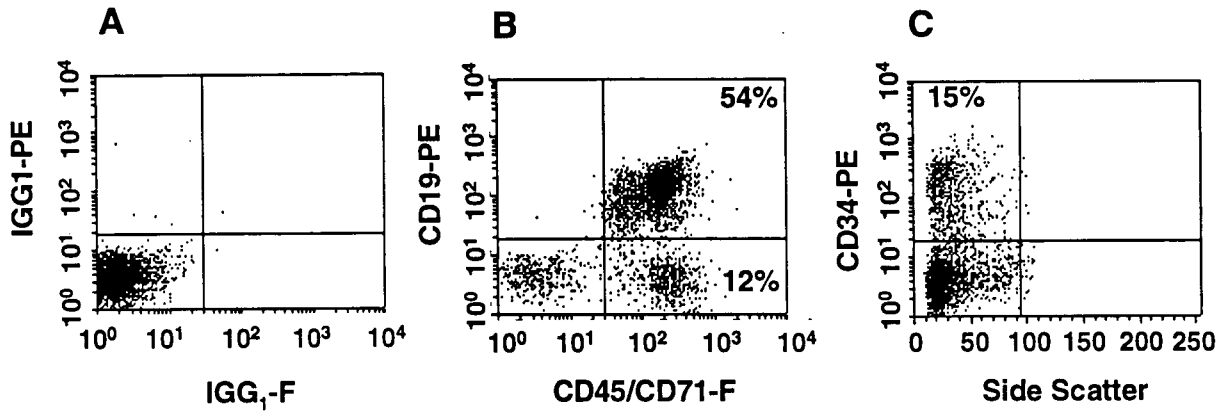


Figure 4.1 Phenotypic analysis of BM cells derived from a NOD/SCID mouse transplanted 20 weeks previously with the infected progeny of 3.5 x 10^4 FACS-purified $CD34^+$ human CB cells. In panel A, the cells were stained with irrelevant isotype-matched mouse IgG labeled with FITC and PE and the gates shown set to exclude 99.9% of these cells. In panel B the cells were stained with a combination of anti-CD45/71-FITC and anti-CD19-PE. In panel C, the cells were stained with anti-CD34-PE.

As shown in Table 4.3, the presence or absence of serum in the cultures from which the cells transplanted were obtained made no consistent difference to any of the endpoints of human engraftment assessed in mice up to 15 weeks post-transplant. In addition there was also no difference in the total numbers of cells, CFC or LTC-IC recovered from the two types of infection culture (i.e., viral supernatants prepared in serum-free or serum-replete medium, data not shown). The results from both procedures were therefore pooled to derive mean (± SEM) yields of each progenitor cell type at the end of the 5 day infection culture period (per 10^5 input $CD34^+$ cells) as follows: 3.4 ± 1.1 x 10^6 total cells, 1.4 ± 0.4 x 10^6 CFC and 790 ± 290 LTC-IC. (The results from the experiment that was performed with $CD34^+CD38^{lo}$ cells was excluded from this analysis).

Table 4.3 Output of human myeloid CFC and CD19$^+$ cells from NOD/SCID mice engrafted with cells harvested from cultures of cord blood cells which contained serum-free medium (SFM) for the first 3 days and SFM or FCS replete medium for the final 2 days.

			No. of Human Cells Regenerated in Each Mouse Tested	
Expt. No.	Culture Condition	Time of Assessment of Engrafted Mice	CFC (x 10^3)	CD19$^+$ Cells (x 10^6)
1	FCS	6 wks	32, 25	2.3, 4.9
	SFM	6 wks	3, 8	2.1, 1.9
2	FCS	8 wks	2	0.8
	SFM	8 wks	2	2.0
3	FCS	15 wks	83, 7	37, 34
	SFM	15 wks	81, 150, 89	16, 59, 26

Values shown are the results obtained from individual mice calculated in each case assuming 2 femurs and 2 tibias contain 25% of the total marrow population. Each of the 3 experiments was initiated with lin$^-$ CB cells and the output values shown have been adjusted to correspond to the yield of progeny expected from 10^5 input CD34$^+$ CB cells originally placed in culture.

4.2.3 Gene Transfer to Human CB Progenitors

To assess gene transfer efficiencies in these latter experiments, the proportion of G418-resistant CFC, or progeny CFC derived from LTC-IC, or (*in vivo*) from the CRU injected into the NOD/SCID mice, was determined. The results, shown in Figure 4.2, reveal average gene transfer efficiencies that range from a maximum of 68% (BFU-E exposed to FCS-containing supernatants) to a low of 8% (CRU exposed to BIT-containing supernatants). However, for each progenitor type there was a ~ 2 to 3-fold higher proportion of G418-resistant cells when these were infected with FCS-containing supernatants, in spite of the fact that the total number of progenitors present had not been affected. Assessment of the viral titer of the supernatants prepared with FCS and BIT showed a 3-fold difference (6×10^6 in FCS vs. 2×10^6 in BIT, n = 2). Thus, the most likely cause of the reduced gene transfer obtained with the BIT-containing supernatants was simply their reduced content of virus.

The results shown in Figure 4.2 also indicate a similar efficiency of gene transfer to human CB CRU and LTC-IC under the best conditions ($17 \pm 3\%$ and $17 \pm 8\%$, respectively). When gene transfer efficiencies to CRU, LTC-IC and CFU-GM in individual experiments were compared, the results for LTC-IC and CRU were significantly correlated ($r^2 = 0.85$, $p < 0.01$), whereas there was no correlation between the corresponding gene transfer efficiencies to LTC-IC or CRU and CFU-GM ($r^2 = 0.16$ and 0.17, respectively, $p > 0.05$) (Figure 4.3). Results for BFU-E and CFU-GEMM were similar to those shown for CFU-GM, although the numbers of these were lower and hence the data less reliable (data not shown).

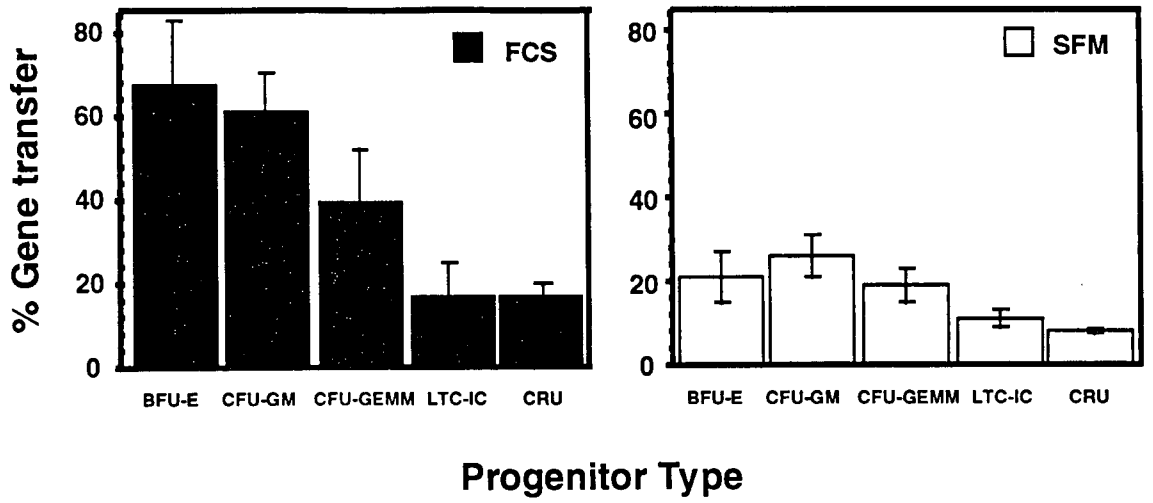
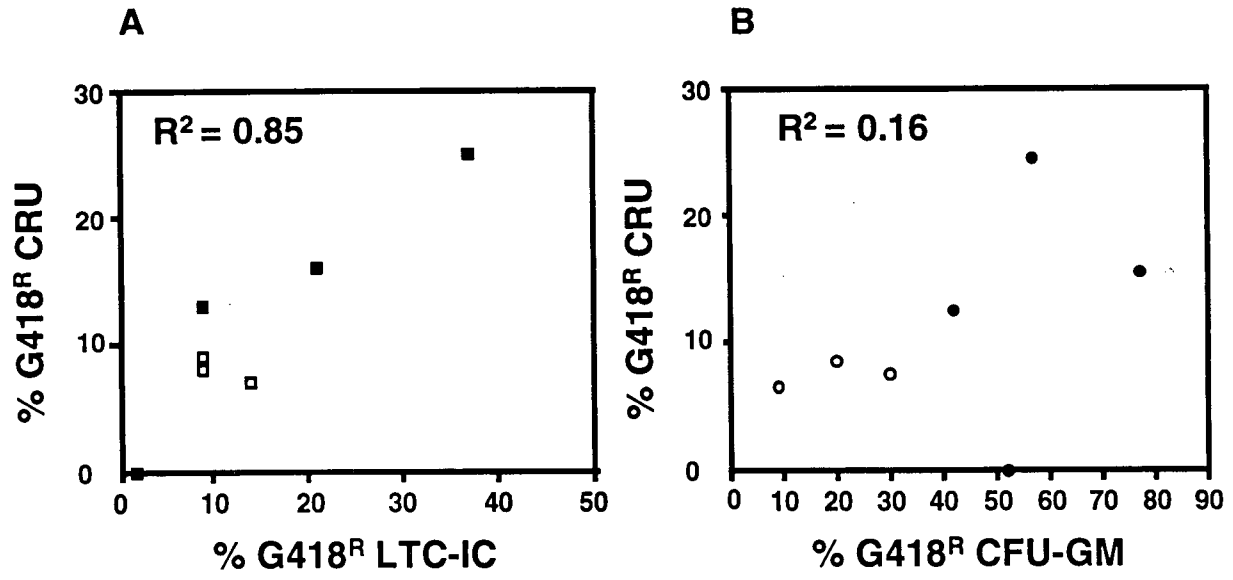


Figure 4.2 Comparison of gene transfer efficiencies to different types of human CB progenitors infected under serum-free vs. serum-replete conditions and assessed by measurement of G418-resistance. Values represent mean ± SEM



4.3 Correlation analysis of gene transfer efficiency to CRU and LTC-IC (panel A) and to CRU and CFU-GM (Panel B). Results shown include data from protocols in which either FCS (solid symbols) or BIT-containing (open symbols) viral supernatants were used. The average number of colonies counted to calculate the efficiency of gene transfer to CFU-GM ranged from 77 to 404 (mean = 154) and from 41 to 350 (mean = 123) in the presence and absence of G418, respectively. For assessment of LTC-IC, the numbers of colonies counted ranged from 12 to 115 (mean = 57) and from 1 to 85 (mean = 20) in the presence and absence of G418, respectively

Although assessment of G418-resistant colony formation provides a convenient method of quantitating gene transfer efficiency using neo-containing retroviruses, such measurements typically underestimate the frequency of infected cells due to a variety of mechanisms that may block or reduce expression of the integrated retroviral cDNA. In addition, some cell types cannot be monitored this way. Therefore, to further characterize the progeny of the infected CB cells that engrafted the NOD/SCID mice, some of the human colonies generated *in vitro* from the human $CD34^+$ cells isolated from their marrow's (6 - 20 weeks post-transplant) were plucked and assessed individually for the presence of the neo gene by PCR. In addition, in one experiment, highly purified human $CD19^+$ (B-lineage) cells sorted from 3 mice were similarly analyzed. The human lymphoid and human myeloid cells from all mice analyzed showed integration of the neo gene. Results from a representative experiment are shown in Figure 4.4. Table 4.4 shows a detailed comparison of the estimates of gene transfer to the CRU obtained from the infected CB cultures based on the G418 resistance of, vs. the presence of neo sequences in human CFC obtained from mice 6 to 20 weeks after they had been injected with the infected human cells. Values determined by PCR analysis were consistently ~ 2 to 3-fold higher, suggesting that the actual efficiency of gene transfer to the transplanted CRU was correspondingly higher (i.e., $32 \pm 12\%$).

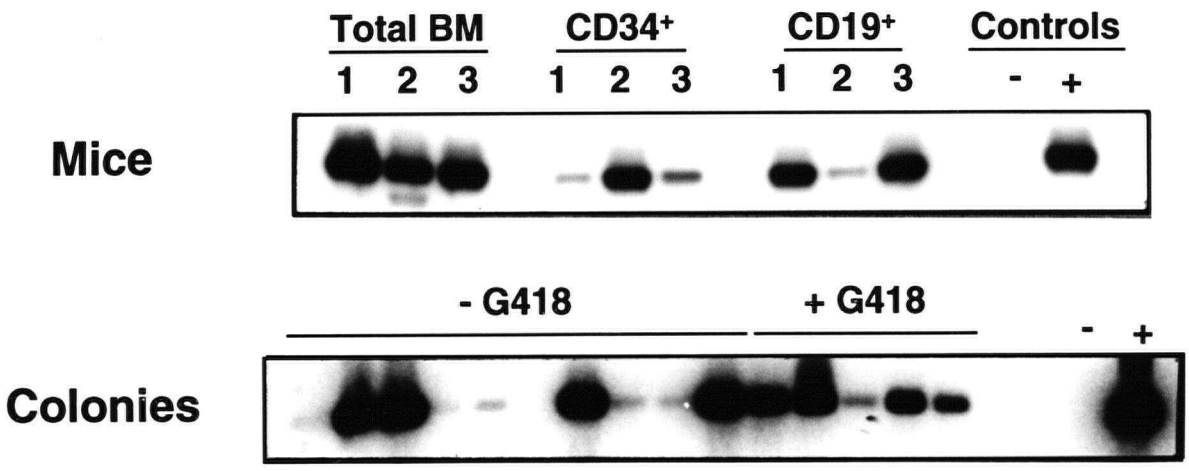


Figure 4.4 PCR detection of neo sequences in cells obtained from NOD/SCID recipients engrafted with infected human CB cells (Exp. 3 in Table 4.4). The upper panel shows results for total BM and isolated human CD34+ and CD19+ populations. The lower panel shows results for individual colonies derived from the sorted human CD34+ cells plated with and without G418. Densitometric analysis (from 2 films exposed for different lenghts was performed) of the amount of DNA in each lane relative to PCR of the actin gene done on the same colonies were as follows: Lane 1 to 16: 0.8, 0.7, 0.7, 0.4, 0.3, 0.5, 1, 0.4, 0.4, 0.9, 0.7, 0.6, 0.4, 0.4, 0.7, 0.7. As the NEO signal in lane 1 is weaker than expected relative to the amount of actin present we have not called this colony positive.

Table 4.4 Comparisons of gene transfer efficiencies measured by assessing G418-resistance and PCR detection of the neo gene in individual colonies for human CFC obtained from NOD/SCID mice.

Method	% Marked CFC			
	Exp 1	Exp 2	Exp 3	Mean ± SEM
$G418^r$	10	11	7	9 ± 2
	(13,18,5,3)	(10,11)	(14,7,2,4)	
PCR	43	11	44	32 ± 12
	(30,48,50,44)	(0,22)	(80,30,13,N/D)	

Values shown for the individual experiments are means ± SEM of the values shown in brackets which represent the proportion of marked CFC determined in individual mice.
N/D = not done.

4.3 Discussion

The studies described in this report identify conditions that allow human *in vivo* repopulating cells to be reproducibly infected by recombinant retroviruses at high efficiency (~ 30%) using a protocol that should be readily adaptable to clinical applications. This infection procedure builds on a series of previous important observations including the identification of a cytokine cocktail that stimulates the expansion *in vitro* of human CB *in vivo* repopulating cells (Conneally et al., 1997) and recognition of the ability of fibronectin-coated dishes to enhance gene transfer efficiencies using cell-free viral supernatants (Moritz et al., 1994; Hanenberg et al., 1996). The toxicity that polybrene has for primitive human hematopoietic cells (Flasshove et al., 1995) was circumvented by using protamine sulphate as a substitute. We also chose to focus on human CB as a source of the HSC to be infected. This was based on previous work suggesting that these might be more susceptible to retroviral infection (Moritz et al., 1993; Lu et al., 1993a), and that they also regenerate larger numbers of lymphoid and myeloid progeny in NOD/SCID mice and for more prolonged periods by comparison to the repopulating cells present in normal adult human BM (Cashman et al., 1997). The adoption of a 5 day infection protocol (3 days of prestimulation plus 2 days of infection) was based on studies indicating that even under conditions of optimized cytokine stimulation, some human $CD34^+CD38^{lo}$ cells require this duration of cytokine exposure before they will divide (Petzer et al., 1996a; Jordan et al., 1996; Conneally et al., 1997).

To document gene transfer to human CB cells with *in vivo* repopulating activity, sublethally irradiated NOD/SCID mice were injected with the 5 day progeny of relatively large numbers of input $CD34^+$ cells, sufficient to obtain > 1% engraftment of human cells in > 80%

of the recipients (20 of 23). Evidence of the expression and/or presence of the neo gene in the human CFC and $CD19^+$ (B-lineage) cells isolated from the BM of the engrafted mice 6 to 20 weeks post-transplant was used to infer the presence of infected human CRU in the cells originally injected (i.e., *in vivo* repopulating human stem cells with lympho-myeloid differentiation potential). In the data presented in Chapter 3, I demonstrated that > 90% of NOD/SCID mice transplanted with limiting numbers of human CB cells produce both lymphoid and myeloid progeny, indicative of the origin of both of these populations from a common stem cell. Thus, although direct evidence of clonal populations containing both retrovirally marked lymphoid and myeloid elements was not obtained in the present experiments, the previous findings would suggest that the genetically marked CFC and pre-B cells detected were generated from infected human CB CRU. It should be noted that I made a conscious effort to transplant non-limiting numbers of infected CRU into the NOD/SCID mice in order to minimize variability between recipients in the proportion of regenerated human cells that would be genetically marked. This was then tested by comparing the proportions of G418-resistant and neo sequence-positive human CFC demonstrable in individual mice injected with aliquots of the same infected CB cell population. The level of gene transfer achieved was sufficient to mark a readily detectable proportion of the human CFC present in the 80% of mice where CFC were regenerated, and the proportion of marked CFC was highly consistent between all mice in a given set, regardless of the method used to identify the marked cells. If the mice had been injected with only 1 or 2 CRU, a larger proportion of mice in each experiment would have been expected to not contain any human cells, and all of the human cells in the engrafted mice would have been either marked or not, a situation which, interestingly, fits the findings reported by Larochelle et al. (1996).

Efficient gene transfer to human *in vivo* repopulating HSC present in adult BM (Nolta et al., 1996; Dao et al., 1997) or fetal liver (Yurasov et al., 1997) has also been reported recently by other groups. In two of these studies, beige-nude-xid (bnx) mice were used as recipients. These latter mice allow human myeloid and T cell progeny to be generated, but not B-lineage cells, and overall appear to support much lower levels of human hematopoiesis than NOD/SCID mice. Nevertheless, high level gene transfer to human BM cells able to engraft bnx mice was reported for cells infected in the presence of stroma and FL could partially overcome this, which our present studies confirm. In the studies of Yurasov et al, who used human fetal liver cell targets (Yurasov et al., 1997), greater infectivity would be expected from their likely increased proliferative activity (Fleming et al., 1993). Our findings thus extend those recently reported by others highlighting the importance of using an infection protocol that optimizes stem cell recovery as well as infection efficiency. Moreover, the present studies show that these requirements can be met under conditions that are suitable for clinical application. In the future, the possibility of adding other strategies to selectively isolate retrovirally infected human HSC (Conneally et al., 1996) as has been achieved with murine HSC (Richardson and Bank, 1995; Pawliuk et al., 1997), or other types of human cells (Mavilio et al., 1994) should, with the gene transfer efficiencies now achievable, allow useful numbers of viable 100% gene-modified human HSC populations to be obtained.

Many groups have shown that LTC-IC from different sources can be subdivided into biologically distinct subtypes according to the longevity of their CFC-producing ability (Ploemacher et al., 1989; Prosper et al., 1996; Hao et al., 1996). In fact, this principle was first used to discriminate LTC-IC as a population distinct from CFC (Ploemacher et al., 1989; Sutherland et al., 1989). It has also allowed murine cells with short versus long-term *in vivo*

repopulating abilities to be distinguished (Magli et al., 1982; Ploemacher and Brons, 1989; Jordan and Lemischka, 1990; Miller et al., 1997). Thus some functional heterogeneity amongst human cells detectable either as LTC-IC or as CRU likely exists, consistent with their heterogeneity in CD38 expression (Conneally et al., 1997). However, because human CB CRU are identified at a frequency that is several hundred-fold lower than the frequency of CB LTC-IC, independent of their phenotype (Conneally et al., 1997), it is difficult to establish the precise relationship of CRU and LTC-IC, particularly in the absence of any independent information concerning the relative efficiencies of the procedures used to detect them. Nevertheless, the highly significant correlation demonstrated here between the efficiency of gene transfer to LTC-IC and CRU in human CB (both assessed using a ≥6 week endpoint) provides further evidence of a close relationship between these two populations and also serves to emphasize the predictive value of measuring gene transfer to such LTC-IC as a prelude to more ambitious and labor-intensive *in vivo* experiments.

Chapter 5 Rapid and Efficient Selection of Human Hematopoietic Cells Expressing Murine Heat Stable Antigen as Indicator of Retroviral-Mediated Gene Transfer

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The contents of this Chapter are essentially as published in Conneally et al, 1996, Blood, 87, 456-464. P. Bardy made a significant contribution to the establishment of the retroviral producer cell line and in the initial analysis of the hemopoietic cell lines.

5.1 Introduction

To facilitate the identification of transduced hematopoietic cells, genes encoding a variety of selectable markers have been incorporated into retroviral vectors. As described in Chapter 1, these have typically included genes that confer cellular resistance to drugs such as neomycin, hygromycin or methotrexate (Dick et al., 1985; Palmer et al., 1987; Miller et al., 1985). Subsequent exposure of the cells to concentrations of these drugs that are lethal for normal cells has provided a useful strategy for detecting and quantitating gene transfer to different hematopoietic cell targets; however, this has proven to be of limited value for obtaining enriched populations of transduced cells either pre- or post- transplant. There are several reasons for this, including a usually poor differential in the selective survival advantage conferred by the transferred gene, metabolic co-operation between transduced and non-transduced cells resulting in a further decrease in the specificity of the selection procedure (Bayever, 1990), and a requirement for the infected cells to complete several cell divisions in order for the differential survival of the transduced progenitors to be manifested in a fashion that can be quantitated.

A number of groups have explored several approaches to the development of an alternative selection strategy in which a gene encoding a molecule that is normally expressed on the cell surface (e.g., CD24, the nerve growth factor receptor, or the multi-drug resistance gene or more recently the green fluorescent protein (GFP) (Pawliuk et al., 1994; Valtieri et al., 1994; Ward et al., 1994; Persons et al., 1997)) is incorporated into the vector and the transduced cells are then identified by staining with a specific antibody. This type of selection procedure offers a number of potential advantages including the application of multiparameter

flow cytometry for the immediate and quantitative analysis and selection of phenotypically defined subpopulations of transduced cells (Pawliuk et al., 1994; Richardson and Bank, 1995). Such a method might thus allow a variety of parameters that affect gene transfer efficiency to a specific and rare cell type to be systematically and rapidly evaluated; for example, those cells likely to possess long-term *in vivo* reconstituting ability. Pawliuk et al (1994) have previously demonstrated the potential of this approach in a murine model using a retroviral vector encoding CD24. These studies included the isolation within 48 hours of infection, of transduced murine CRU as well CFC and day 12 CFU-S. In this Chapter, I describe the application of a similar strategy to primary human hematopoietic cell targets using an amphotropic vector encoding HSA, the murine homologue of CD24, as a selectable marker.

HSA and its human homologue CD24 are small (27 amino acid) glycosyl phosphatidylinositol-linked glycoproteins (Kay et al., 1990) that are expressed on the surface of a large number of hematopoietic cell types (Hough et al., 1994). Neither the function of CD24 nor of HSA is well understood, although HSA has been shown to be involved in murine T-cell development and to play a co-stimulatory role on murine antigen-presenting cells and other events where cell adhesion is important (Kadmon et al., 1992; Liu et al., 1992; Hahne et al., 1994; Sammar et al., 1994). The mature HSA and CD24 proteins share only limited sequence homology (57%) and antibodies to HSA and CD24 are non-cross reactive. Nevertheless, both are encoded by relatively small DNA fragments [consisting of 228 bp in the case of HSA, (Kay et al., 1990)] and have the potential for being expressed on the cell surface. In addition, the availability of a non-cross reactive murine monoclonal antibody against HSA makes HSA-containing retroviruses ideal for use in studies of human cells.

5.2 Results

5.2.1 The HSA Viral Vector

To explore the utility of HSA as a selectable marker, a MSCV-based HSA vector was constructed as described in Chapter 2 (see also Figure 5.1A). This vector, in addition to encoding HSA, included the neo^r gene to provide an independent means of assessing the efficiency of gene transfer. As shown in Figure 5.1B, Northern blot analysis of RNA obtained from the MSCV-HSA.NEO producer cells demonstrated a single HSA transcript of 2.7 kb (lane 1). Hybridization of the same blot with a neo^r-specific probe revealed both the 2.7 kb LTR-driven transcript and a reduced level of the expected 1.3 kb transcript driven by the internal pgk promoter.

The initial HSA viral producer had a titer of 10^6 CFU/mL. As a first test of the potential of the transduced HSA gene to allow the selection of infected cells, the producer cells were stained with M1/69 and those expressing the highest levels of cell surface HSA (Figure 5.2) were then isolated. Subsequent titration of supernatants conditioned by these selected producers, as assessed by transfer of G418 resistance to 3T3 cells, showed that the viral titer had been increased approximately 10-fold.

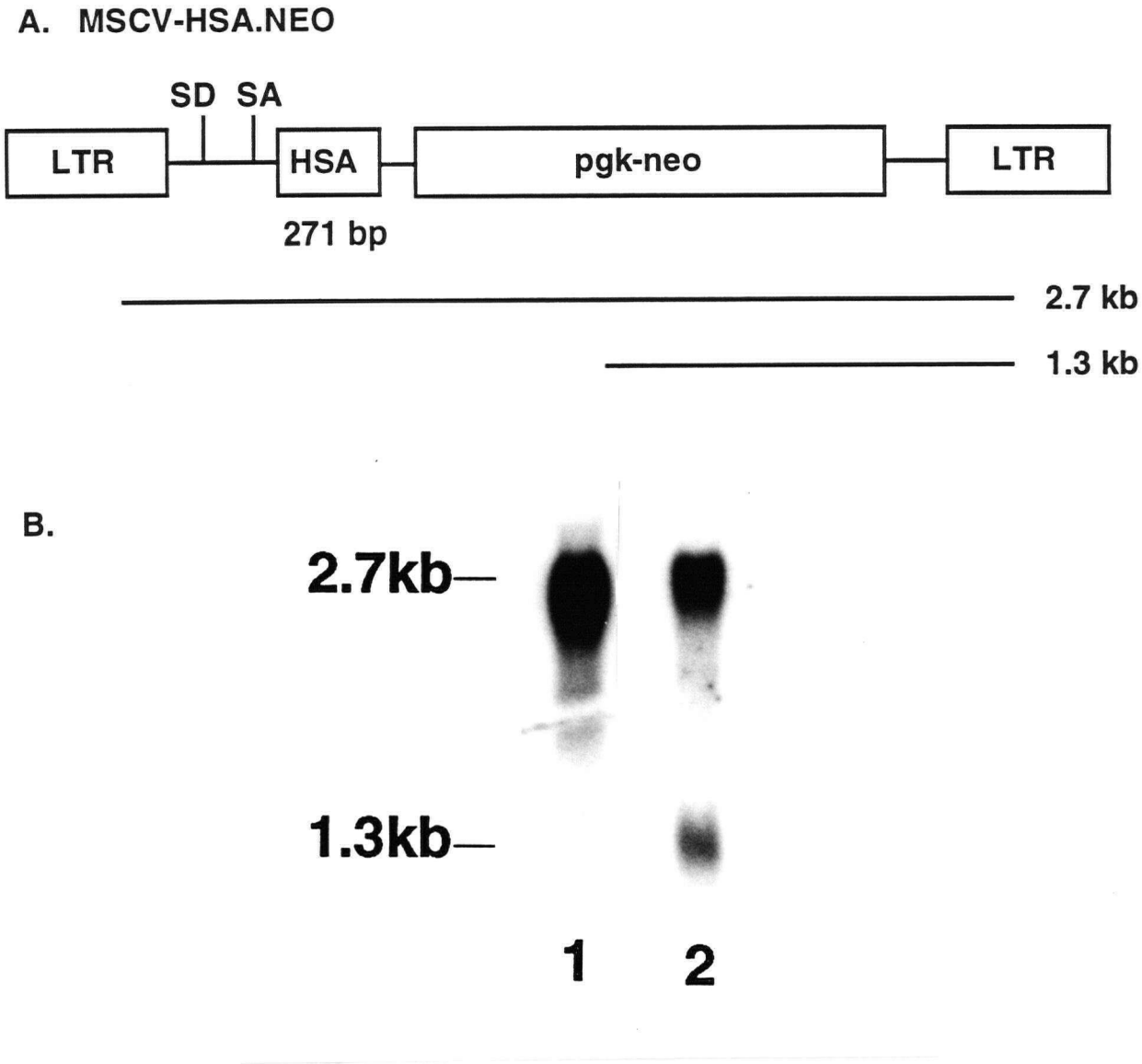

FIgure 5.1 Structure and expression of the MSCV-HSA.NEO retrovirus used in this study. (A) Diagrammatic representation of the MSCV-HSA.NEO provirus. Expected full length transcripts and those initiated from the internal pgk promoter are shown. SD and SA denote the splice donor and splice acceptor sites. (B) Northern blot analysis of the MSCV-HSA.NEO viral producer cell line. The membrane was sequentially hybridized to a probe specific for HSA (lane 1) and neo^r (lane 2).

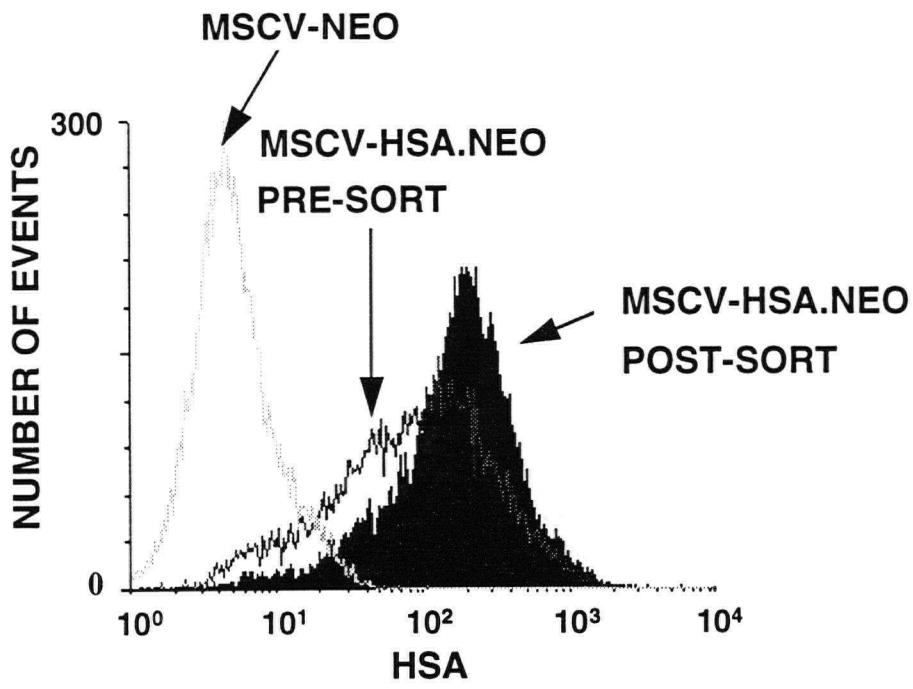

Figure 5.2 FACS analysis of HSA expression by GP-*env* AM 12 MSCV-HSA.NEO and GP-*env* AM 12 MSCV-NEO producer cells after labeling with biotinylated M1/69 followed by streptavidin-PE as described in the Materials and Methods. GP-*env* AM 12 MSCV-HSA.NEO cells were sorted to isolate those expressing the highest levels of cell surface HSA.

5.2.2 Infection of Human Hematopoietic Cell Lines

A series of experiments were then undertaken to evaluate the level of HSA expression obtainable 24 hours after infection of human hematopoietic cells with the MSCV-HSA.NEO retrovirus. In the first of these, two established human leukemic cell lines (HL60 and Mo7e) were used as model targets. These were infected by co-cultivation with the producer cells for 48 hours and the non-adherent cells then stained and analyzed for HSA expression. The results of representative experiments are shown in Figures 5.3 and 5.4, respectively. For both lines, an HSA^+ fraction (7% of the infected HL60 cells and 40% of the Mo7e cells) could be readily resolved. These values correlate well with the gene transfer efficiencies of 10% and 46%, respectively, obtained from measurements of the proportion of infected, but unselected HL60 and Mo7e cells that formed colonies in methylcellulose medium containing selective concentrations of G418. In these experiments, sorting of the infected HL60 cells into an HSA^+ and an HSA^- fraction (24 hours after infection) prior to assessment of their resistance to G418, revealed a > 95-fold difference in the proportion of cells that were G418-resistant in the HSA^+ as compared to the HSA^- fractions (95% vs. < 1%). In the experiment with Mo7e cells, a > 10-fold difference in the G418 resistance of the HSA^+ and HSA^- cells (90% vs. 6%) was demonstrated.

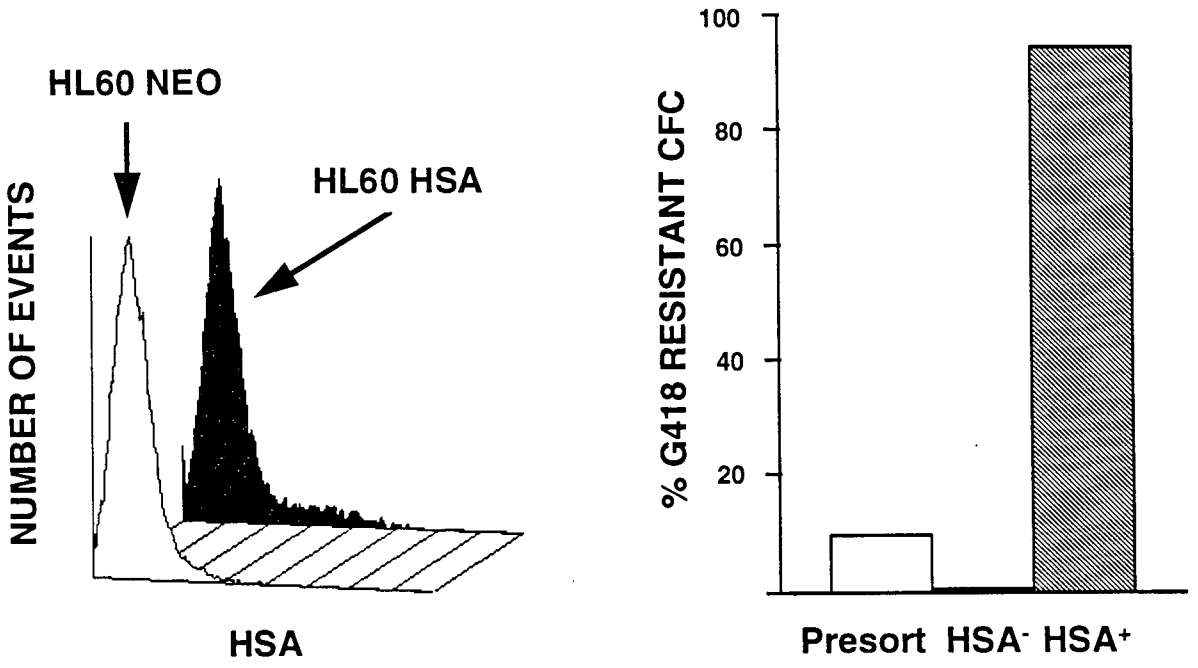

Figure 5.3 FACS analysis of HSA expression on HL60 cells infected by co-cultivation for 48 hours with MSCV-HSA.NEO or MSCV-NEO producers and stained with M1/69-Cy5 24 hours later. HSA^+ and HSA^- cells were then sorted and plated with and without G418 to estimate the relative enrichment and depletion of infected cells in each fraction. HSA^+ cells were defined by gates set to exclude 99% of cells from control cultures exposed to MSCV NEO producers and then stained at the same time with the same procedure as the MSCV-HSA.NEO-infected cells.

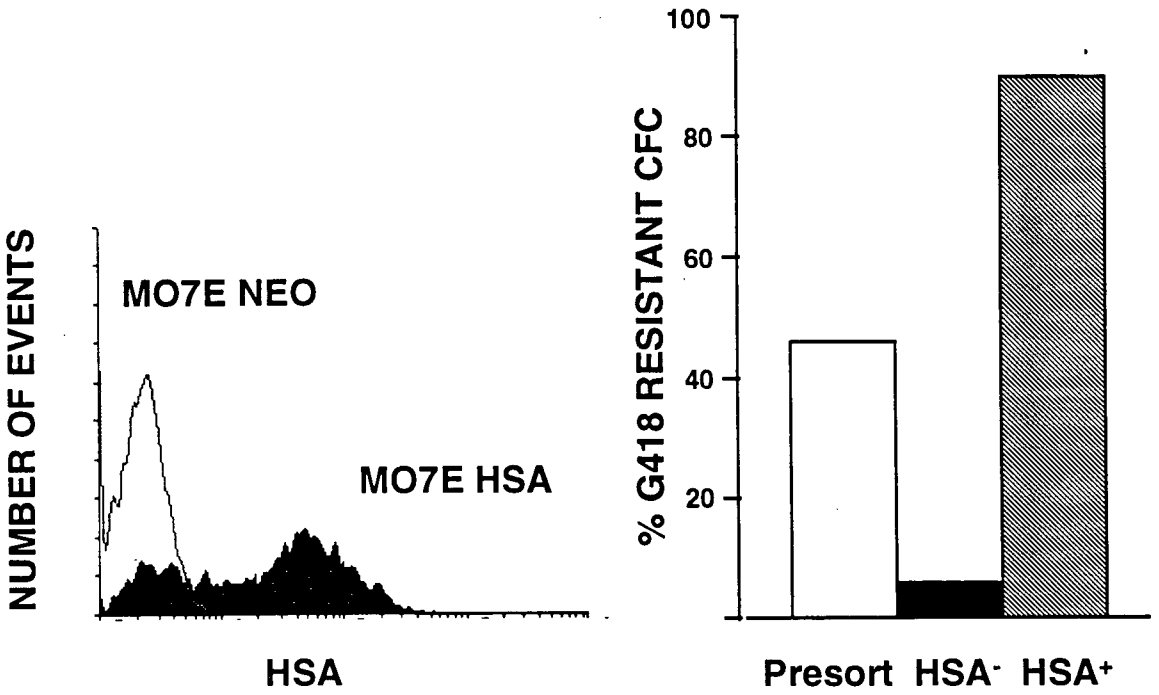

Figure 5.4 FACS analysis of HSA expression on Mo7e cells, infected, stained and analyzed for G418 resistance using the same procedures as described for HL60 cells in the caption to Figure 5.3.

5.2.3 Infection of Primary Human Hematopoietic Cells

Experiments were then undertaken using normal human BM or mobilized PBPC as a source of target cells. These were also infected by co-cultivation with MSCV-HSA.NEO or MSCV-NEO producer cells for 48 hours (after an initial 48 hours of prestimulation with IL-3, IL-6 and SF as described in Chapter 2) and then assessed for HSA expression and G418 resistance 24 hours later. An initial 6 such experiments were performed (5 with normal BM and 1 with mobilized PBPC) and Table 5.1 shows the proportions of G418-resistant BFU-E and CFU-GM in the infected cells prior to selection of HSA^+ and HSA^- fractions. Overall the results for both of these progenitor types were similar at 11% and 12%, respectively. In 2 of these 6 experiments, LTC-IC assays were also performed on the infected (but unselected) cells and the extent of gene transfer to these cells then assessed 6 weeks later by plating their clonogenic progeny in the presence and absence of G418. The results of these measurements demonstrated gene transfer efficiencies to LTC-IC of <1% and 11%.

In each of these 6 experiments, the remaining cells were then analyzed by FACS for their levels of CD34 and HSA expression. As shown in Table 5.2, on average, approximately 26% of all the cells were still $CD34^+$ after a total of 5 days in culture and approximately 27% of these also showed positive staining for HSA. Figure 5.5 shows representative FACS profiles of the HSA staining obtained with cells infected with the MSCV-HSA.NEO virus by comparison to the level of background fluorescence exhibited by cells infected with the control MSCV-NEO virus.

Table 5.1 % Gene Transfer to CFC Assessed by G418 Resistance

Experiment	Unsorted		$CD34^+HSA^+$		$CD34^+HSA^-$	
BFU-E						
1	n/d[¶]		225	(5/2)*	6	(2/32)
2	0	(0/0)	0	(0/1)	0	(0/4)
3	0	(0/2)	74	(32/43)	1	(6/98)
4	4	(1/26)	166	(5/3)	4	(2/48)
5	36	(4/11)	61	(77/127)	2	(2/102)
6	17	(23/136)	73	(23/31)	0	(0/68)
Mean ± SEM	11 ± 6		100 ± 31		2 ± 0.9	
CFU-GM						
1	n/d		63	(42/67)	7	(14/200)
2	11	(15/137)	90	(51/57)	5	(9/196)
3	12	(9/77)	60	(58/96)	3	(8/302)
4	6	(8/129)	91	(93/102)	1	(3/234)
5	11	(22/209)	62	(109/176)	3	(11/394)
6	21	(80/384)	93	(54/58)	10	(15/146)
Mean ± SEM	12 ± 2		77 ± 6		5 ± 1	

[¶]n/d = not done. * Values represent % gene transfer derived from the number of colonies scored in assays (+ G418/-G418).

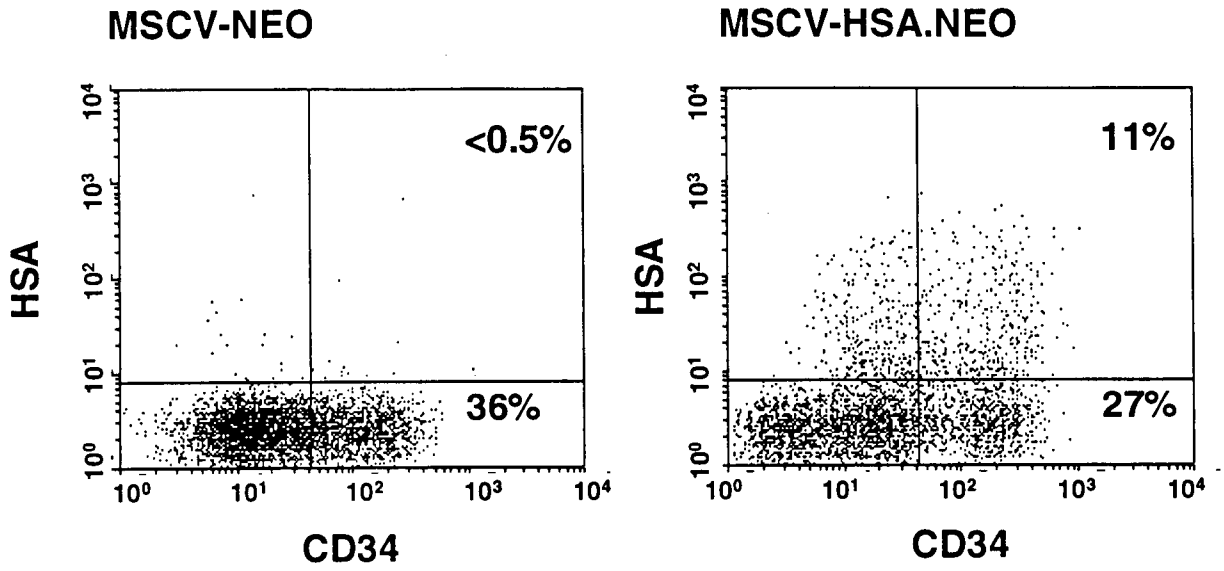

Figure 5.5 FACS analysis of HSA expression on CD34$^+$ cells isolated from normal human BM infected by co-cultivation with MSCV-HSA.NEO or MSCV-NEO and then stained with M1/69-Cy5. The CD34$^+$HSA$^+$ and CD34$^+$HSA$^-$ cells were sorted and plated separately in G418 to compare these two endpoints of gene transfer efficiency (G418 resistance and HSA expression). Numbers shown indicate the percent of the total population that were contained within the gates shown.

Table 5.2 Gene Transfer Assessed by FACS Analysis

Experiment	HSA$^+$ Cells (% of Total)	CD34$^+$ Cells (% of Total)	CD34$^+$HSA$^+$ Cells (% of CD34$^+$)
1	15	34	21
2	8	3	26
3	21	35	41
4	7	14	21
5	14	35	22
6	12	36	31
Mean ± SEM	13 ± 2	26 ± 6	27 ± 3

The $CD34^+HSA^+$ and $CD34^+HSA^-$ cells were then also sorted and aliquots from each of these fractions finally plated in methylcellulose assays with and without G418. Subsequent colony counts from these assays showed that isolation of the HSA^+ fraction allowed a population to be obtained in which most, if not all, of the clonogenic progenitors were G418-resistant, with a corresponding decrease (to 5%) of G418-resistant clonogenic progenitors in the isolated HSA^- fraction (Table 5.1). In two of these experiments, colonies generated (in the absence of G418) from both the $CD34^+HSA^+$ and $CD34^+HSA^-$ fractions, as well as from the original unfractionated population, were plucked and analyzed individually for the presence of the neo^r gene. Figure 5.6 shows a Southern blot of PCR products from the $CD34^+HSA^+$ and $CD34^+HSA^-$ colonies produced in one of these experiments. Overall 46% (7/15) of the colonies derived from the unseparated cells were positive for neo^r, whereas 96% (26/27) of the colonies derived from the $CD34^+HSA^+$ cells were neo^r-positive. In contrast, only 7% (2/27) of the colonies derived from the $CD34^+HSA^-$ cells were neo^r-positive.

Having determined that HSA is readily detected on $CD34^+$ cells which can then be specifically isolated by FACS selection, we then investigated whether enriched populations of transduced LTC-IC can also be obtained using this approach. In 2 experiments lin^- cells were isolated from normal human marrow and prestimulated in IL-3, IL-6, SF and FLT-3 ligand for 48 hr and infected by co-cultivation. 24hr later the cells were analyzed by FACS. Unsorted and FACS-sorted $CD34^+HSA^+$ and $CD34^+HSA^-$ populations were plated directly in methylcellulose with and without G418 and in LTC-IC assays.

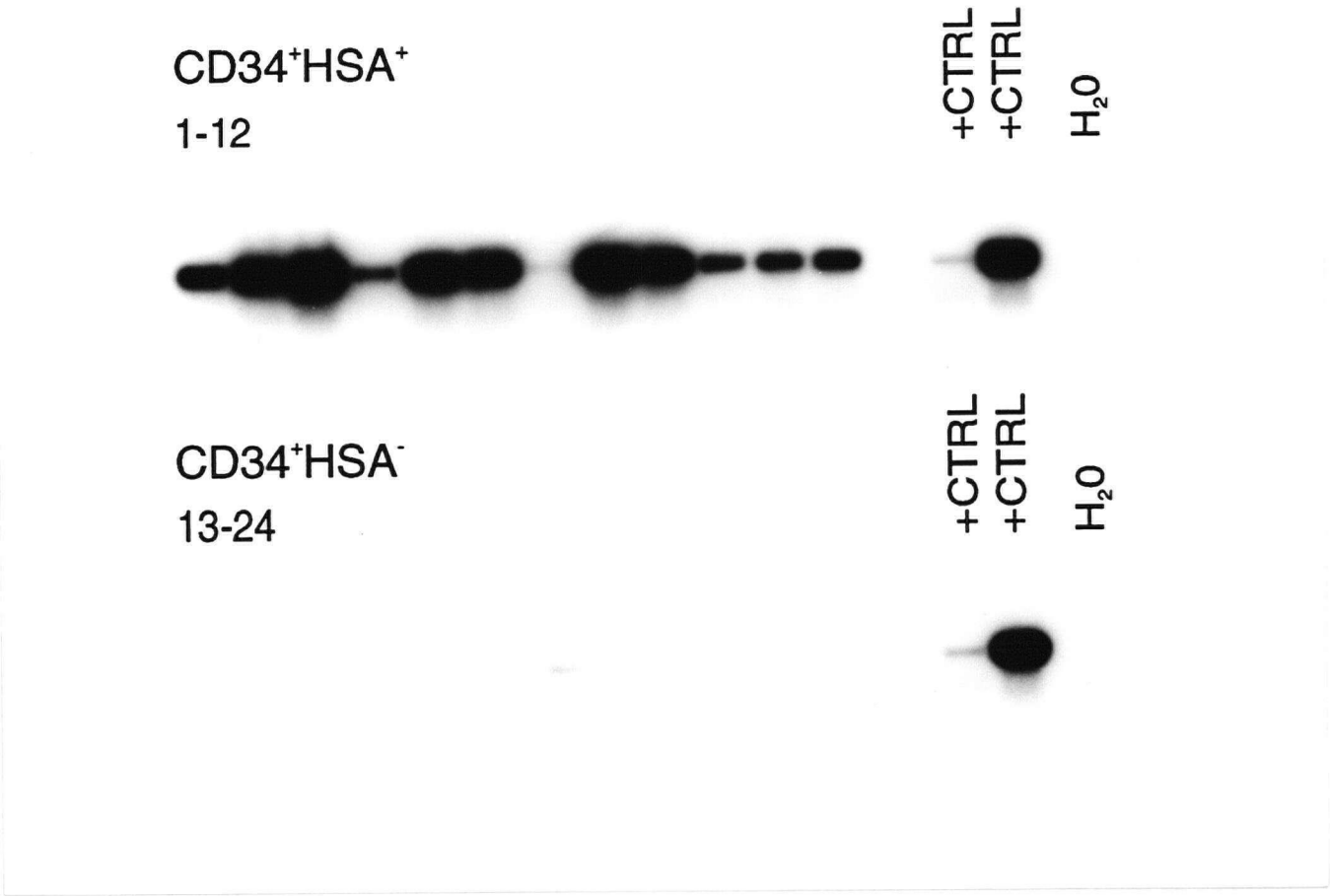

Figure 5.6 Demonstration of transduced clonogenic progenitors by PCR analysis of colonies plucked from dishes containing FACS-selected $CD34^+HSA^+$ cells (lanes 1-12) or $CD34^+HSA^-$ cells (lanes 13-24) plated in methylcellulose medium without G418. The positive controls were dilutions of a neo^r transduced K562 cell line. The negative control consists of all the PCR reagents plus H_2O.

As shown in Table 5.3, the frequency of total G418-resistant CFC (BFU-E plus CFU-GM plus CFU-GEMM) in the pre-sort population averaged 12%. G418-resistant CFC were enriched in the $CD34^+HSA^+$ fraction as found in previous experiments (Table 5.1), and were correspondingly depleted in the $CD34^+HSA^-$ fraction. Similarly, the frequency of LTC-IC-derived G418-resistant CFC was increased from 9% in the pre-sort population to 86% in the $CD34^+HSA^+$ fraction and was decreased to 3% in the $CD34^+HSA^-$ fraction. The decreased frequency of both CFC and LTC-IC in the $CD34^+HSA^+$ fraction in experiment 1 is likely due to a less stringent setting of the sort gate thus allowing inclusion of some non-transduced cells.

In addition to analyzing the efficiency of HSA gene transfer to the total $CD34^+$ cell population, the extent of HSA gene transfer to a phenotypically-defined primitive subset of these cells ($CD34^+CD38^{low}$) was also assessed 24 hours after infection in some experiments. In these a lin^- (~50% $CD34^+$) starting population was used for the infections. FACS plots of the HSA^+ cells seen in a representative experiment with infected lin^- normal BM cells are shown in Figure 5.7. The proportion of $CD34^+CD38^{low}$ cells that were also HSA^+ in the these experiments was $22\pm4\%$ (n=6). This value is similar to the value of 27% obtained for the efficiency of HSA gene transfer measured for the total $CD34^+$ population (Table 5.2).

Table 5. 3 % Gene transfer to CFC and LTC-IC-derived CFC Assessed by G418 Resistance

Experiment	Unsorted	$CD34^+HSA^+$	$CD34^+HSA^-$
CFC*			
1	12	59	3
2	11	96	2
Mean ± SEM	12 ± 0.5	78 ± 19	3 ± 0.5
LTC-IC-derived CFC			
1	7	53	2
2	10	118	3
Mean ± SEM	9 ± 2	86 ± 33	3 ± 0.5

*CFC= BFU-E plus CFU-GM plus CFU-GEMM

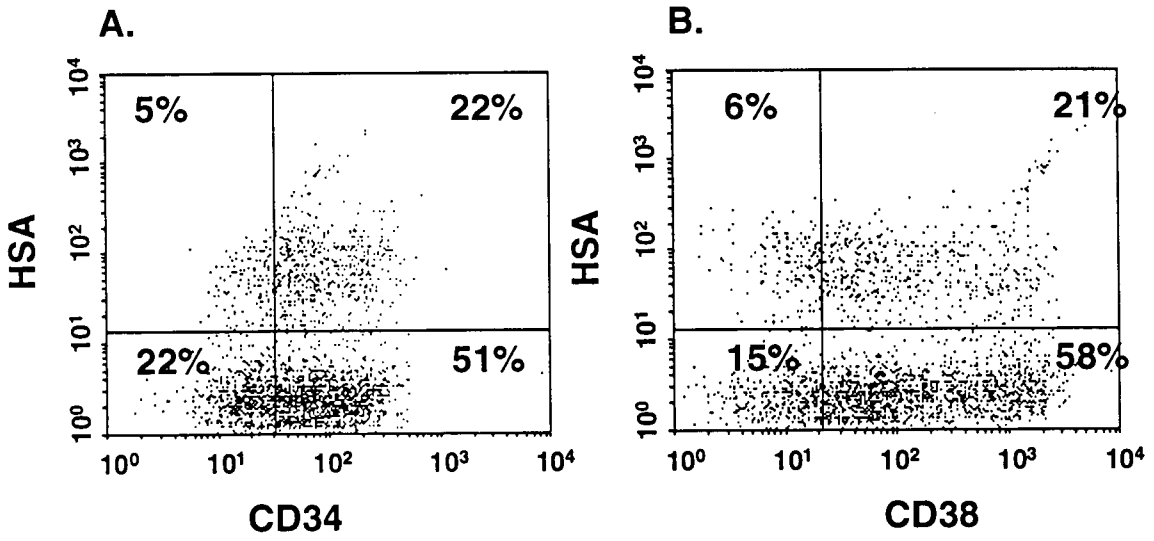

Figure 5.7 Expression of HSA versus CD34 (Panel A) and of HSA versus CD38 amongst the $CD34^+$ cells (Panel B) 24 hours after infection of a population of lin^- normal marrow cells with MSCV-HSA.NEO. The cells were stained with M1/69-Cy5 (HSA), 8G12-FITC (CD34) and anti-CD38-PE as described in Chapter 2. The gate used to define $CD34^+$ cells is shown in Panel A. The gate for HSA expression was set to exclude ≥ 99% of cells from control cultures exposed to MSCV-NEO producers and then stained at the same time with the same procedure as the MSCV-HSA.NEO-infected cells.

5.3 Discussion

Pawliuk et al (1994) have previously described the use of CD24 as a selectable marker of retroviral-mediated gene transfer to murine hematopoietic stem cells, as defined by their capacity for the long-term production of myeloid and lymphoid cells after transplantation into myeloablated recipient mice. In addition, CD24 gene transfer to more mature murine progenitor cell types was shown. In the present study, I tested a retrovirus encoding HSA, a related murine cell surface antigen, for its utility in allowing the identification and selection immediately post-infection of primitive transduced hematopoietic cells of human origin. FACS analysis showed that the human leukemic cell lines, HL60 and Mo7e, as well as CFC and $CD34^+CD38^{low}$ cells from adult BM expressed readily detectable levels of HSA within 24 hours of termination of a 48 hour infection procedure. Furthermore, effectively transduced cells from both the infected cell lines and primary hematopoietic cells (including LTC-IC) could then be specifically isolated in a viable state on the basis of their ability to express the transferred HSA gene using standard antibody staining techniques and sorting by FACS.

The proportion of HSA^+ cells in the $CD34^+$ fraction of MSCV-HSA.NEO-infected cultures of primary human BM or mobilized PBPC was consistently higher than the proportion of CFC (all of which have previously been shown to be $CD34^+$) that were classified as G418-resistant under the conditions used to detect cells with these two acquired phenotypes (overall, 25% versus 12%). In these studies, expression of the HSA gene was driven off the LTR which, as shown in Figure 5 1B, acts as a stronger promoter than the internal pgk promoter placed 5' of the neo^r gene. In addition, it should be noted that both gene transfer and expression are known to vary in different cell types and that only approximately 20% of the harvested $CD34^+$ cells were detectable as CFC. Within the CFC

population, there was, in fact, a close concordance between the efficiency of gene transfer assessed by detection of retroviral DNA in individual colonies and HSA expression on the progenitors, with a slightly lower proportion of the latter showing G418 resistance.

These studies highlight the potential of using retroviral constructs encoding cell surface markers not normally expressed on the target cells of interest to facilitate the selection immediately post-infection of those to which gene transfer has been achieved. For many gene therapy applications, the capacity to control the number or proportion of transduced HSC could be of considerable significance, particularly in those settings where replacement of most of the endogenous hematopoietic cells with genetically modified cells would be required.

A number of groups are also currently evaluating the use of cell surface markers for the selection and tracking of marked cells. For example, the use of a vector encoding the low affinity nerve growth factor receptor (NGFR) to allow the selection *in vitro* of both infected human peripheral blood lymphocytes and hematopoietic progenitor cells has been described (Valtieri et al., 1994). The use of a retroviral construct encoding the human multi-drug resistance (MDR-1) gene for a similar purpose has also been reported (Ward et al., 1994). However, to date neither of these approaches has been shown to result in expression of the transferred gene immediately post-infection in human cells at a level sufficient to allow the discrimination and physical isolation of pure populations of infected cells as has been described here using HSA. The use of the MDR-1 approach has, however, a potential additional advantage since it encodes a therapeutic gene that can increase cellular resistance to a variety of chemotherapeutic drugs including anthracyclines, taxol, and the vinca alkaloids (Ward et al., 1994). As a result, transduction of HSC with this gene might endow these cells and/or their progeny with properties that would allow them to survive and hence be selected

in vivo following the administration of higher doses of drugs than are tolerated by normal hematopoietic or drug-sensitive malignant cells. However, whether it will be possible in practice to exploit these principles to achieve a therapeutic benefit has yet to be established.

One of the major advantages of the HSA/CD24 family of vectors is the ability to assess gene transfer to specific subpopulations of cells immediately post-infection. Of particular interest to hematopoietic stem cell transplants are cells with a $CD34^{+}CD38^{low}$ phenotype (Terstappen et al., 1991). In normal marrow these rare cells have been shown to be highly enriched in their content of LTC-IC (Sauvageau et al., 1994) and to contain few cells detectable as CFC even when assayed in the presence of multiple cytokines (Rusten et al., 1994). In this study, I have shown that it is possible to infect normal BM or mobilized PBPC with a retroviral vector that encodes a gene of interest (HSA) and then, within 24 hours, demonstrate the subsequent expression of the transferred gene at a high level in cells that are still $CD34^{+}CD38^{low}$. It is known that insertion into undifferentiated cells like embryonal carcinoma cells of DNA sequences contained in Mo-MuLV constructs is typically followed by a rapid silencing of the promoter function of the retroviral LTR (Petersen et al., 1991). The MSCV vectors used in this study have a number of point mutations in the LTR which overcome the transcriptional block seen when embryonal carcinoma and embryonal stem cells are transduced with Mo-MuLV-containing constructs (Hawley et al., 1994). The use of the MSCV LTR may, therefore, also be important in allowing expression of the gene of interest in infected HSC. The fact that it is now possible to demonstrate and quantitate the expression immediately post-infection of a transduced gene in human $CD34^{+}CD38^{-}$ cells should open the way to a more rapid and critical evaluation of a variety of parameters that may limit the efficiency of current procedures for gene transfer to HSC.

The data presented in this Chapter have shown the feasibility of using HSA both as a selectable marker and as a reporter gene in primary hematopoietic cells of human origin including those with a $CD34^{+}CD38^{low}$ phenotype that is exhibited by functionally defined types of very primitive cells. The small size of the HSA (and CD24) molecule in combination with the ease of selection of transduced cells expressing these genes should make them ideal candidates for inclusion in retroviral vectors encoding almost any therapeutic gene of interest. Such applications for vectors encoding glucocerebrosidase and globin genes are currently under investigation (Medin et al., 1994; Migita et al., 1994; LeBoulch et al., 1995).

Chapter 6 Summary and Discussion

The development of *in vivo* reconstitution assays in the murine system has yielded extensive information regarding the phenotype, cycling characteristics and expansion potential of murine hematopoietic cell with long-term *in vivo* repopulating activity (Spangrude et al., 1991; Morrison and Weissman, 1994; Miller and Eaves, 1997; Rebel et al., 1994; Spangrude and Johnson, 1990; Bradford et al., 1997). Until recently, *in vivo* assays have not been available to address similar questions about the properties and regulation of analogous human cells. In Chapter 3 of this thesis I described the development of a quantitative *in vivo* assay that detects a cell in human CB that has lympho-myeloid repopulating activity in NOD/SCID mice. This assay was used to quantitate the number of these "Competitive Repopulating Units" (CRU) in the light density fraction, in highly purified subpopulations of CB cells and in cells generated *in vitro* from input populations of $CD34^+CD38^-$ CB precursors. The findings presented in Chapter 3 demonstrate that CRU are detectable within both the $CD34^+CD38^-$ and $CD34^+CD38^+$ subpopulations of human CB cells. In addition, as a first test of this assay it was shown that cells detectable as CRU and LTC-IC could both be modestly expanded *in vitro* cultures containing in FL, SF, IL-3, IL-6 and G-CSF.

In a previous study (Petzer et al., 1996b) the same cytokine cocktail was found to be necessary and sufficient to stimulate greater than 50 fold increases in LTC-IC and maximal CFC expansion from highly purified $CD34^+CD38^-$ cells isolated from normal adult human BM. It was, therefore, somewhat surprising, to find that when $CD34^+CD38^-$ CB cells were cultured under similar conditions, the LTC-IC population was only slightly expanded. This marked difference in the extent of expansion of LTC-IC from similarly stimulated CB and BM

cells prompted additional studies to reexamine and compare the specific cytokines required to stimulate the amplification and differentiation of hematopoietic cells from these two sources (1998 et al., 2001). Interestingly the results from these studies demonstrated that the kinetics of CB and BM LTC-IC expansion under the conditions originally used are different, with CB LTC-IC showing less expansion (maximum 4 -fold over input) in the first two weeks. In contrast BM LTC-IC numbers expanded rapidly and continuously over the first 3 weeks to achieve a maximal increase of 91±42 fold. This suggests that primitive cells in CB and adult BM may respond differently to specific growth factors or growth factor combinations. Subsequent factorial design analysis studies have demonstrated that FL and IL-6 plus the soluble IL-6 receptor (sIL-6) are the most important factors for stimulating LTC-IC amplification in 10 day serum-free cytokine supplemented cultures initiated with human CB cells. The marked difference in the expansion of LTC-IC from CB and BM under similar conditions bring into focus another emerging theme in HSC biology - i.e., that ontogeny adds another dimension to the patterns of change that differentiating hematopoietic cells may undergo and, in particular, the ways in which these changes may be regulated. It will be critical to determine whether these results will prove predictive for the expansion of human CB CRU and, indeed, such studies have already been initiated. The human CRU assay should also be an important tool for examining factors that allow HSC from BM and mobilized peripheral blood to retain their defining attributes after their proliferation *in vitro*.

While this thesis was being completed a similar *in vivo* assay was developed independently (Wang et al., 1997; Bhatia et al., 1997b). Although the endpoints of the two assays are slightly different (detection of lympho-myeloid progeny in this thesis and the detection of human cells by Southern analysis in the assay described by Wang and Bhatia), the

actual frequencies of repopulating cells detected by both assays are very similar. In this thesis the frequency of CRU in the light density fraction of CB cells was found to be 1 per 6 x 10^5 cells (Conneally et al., 1997) vs. 1 per 9.3 x 10^5 cells as described by (Wang et al., 1997). In the $CD34^+CD38^-$ population of CB, the frequency was 1 per 900 (Conneally et al., 1997) and 1 per 600 (Bhatia et al., 1997b). On the other hand, Dick and colleagues have suggested that the repopulating cell is found exclusively within the $CD34^+CD38^-$ population (Larochelle et al., 1996; Bhatia et al., 1997b). In contrast, results derived from the engraftment of immunodeficient sheep with human cells have shown that $CD34^+CD38^-$ and $CD34^+CD38^+$ cells are capable of regenerating multi-lineage hematopoiesis that is sustained in this model for many months. Interestingly, however, retransplantable cells were found only in the sheep that had originally been transplanted with $CD34^+CD38^-$ cells.

Differences in surface antigen phenotype, failure to detect expansion of CRU (Bhatia et al., 1997a) under conditions that support extensive expansion of adult BM LTC-IC (Petzer et al., 1996b) combined with differences in their apparent frequencies have led to the suggestion that some LTC-IC and CRU may be different cells (Civin et al., 1996). The LTC-IC assay as performed in this thesis utilizes a 6 week period of coculture of the test cells on growth factor producing fibroblast feeders. These conditions have been found to detect a population of LTC-IC many of which are functionally silent in the original 5 week LTC-IC assay (Hogge et al., 1996). It is, therefore, possible that the LTC-IC detected in the 6 week assay used here may be closer to the type of LTC-IC that would be detected using an 8 week period of incubation under the conditions used in the original assay. We have also found that the relative frequencies of LTC-IC and CRU vary considerably, depending on the CB population studied: a 30 fold difference in their apparent frequencies was, in fact, encountered

in the light density fraction, whereas, this difference became >1,000 fold for cultured CD34 $CD38^-$ cells. This raises the possibility that small numbers of highly purified populations of human cells may be less efficient at homing to the BM. Alternatively, there may be a need for an accessory population of cells to facilitate their engraftment (e.g., by preventing their non-specific clearance or as a source of species-specific cytokines etc.). In the current studies, irradiated normal BM cells were co-injected with all grafts of $<10^6$ cells in an attempt to overcome any non-specific cell losses. We are currently investigating whether additional carrier cells or the administration of cytokines can also improve the detection of human CRU in NOD/SCID mice.

Previous studies have demonstrated that LTC-IC are a heterogeneous population of cells with respect to their phenotype, the length of time that they can sustain hematopoiesis (Prosper et al., 1996; Hao et al., 1996), and their ability to be expanded *in vitro* (Prosper et al., 1997). The results presented in this thesis suggest that human LTC-IC and CRU, although closely related, will be similarly heterogeneous.

The use of recombinant retroviruses to transfer genes into murine HSC has provided important insight into the organization and regulation of the murine hematopoietic system and this technology is now also likely to play a major role in the further development of gene therapy protocols. Despite initial enthusiasm and early signs of safety and biological feasibility, the lack of therapeutic benefit, and questions regarding what gene delivery systems will prove effective and which diseases are appropriate targets for gene therapy prompted a 1995 National Institutes of Health sponsored review of the field. This review panel concluded that although somatic gene therapy is a logical progression of fundamental biomedical science to medicine and offers "extraordinary potential", significant problems remain. Because of the

persistent problems of low gene transfer efficiency, specific recommendations of the panel included further development of animal models of disease, enhanced use of preclinical gene therapy approaches in these models, and greater study of stem cell biology in diverse organ systems. (Report and Recommendations of the panel to assess the NIH investment in research on gene therapy).

Clinical applications of gene transfer require that the target cells be efficiently infected under conditions that support both the viability and maintenance of stem cell potential. Based on the culture conditions identified in Chapter 3, the work presented in Chapter 4 describes the development of a supernatant infection protocol that allows efficient gene transfer to lymphomyeloid *in vivo* repopulating cells that should be readily adaptable for clinical purposes. Previous studies, confirmed by results presented in this thesis, have demonstrated that the presence of a stromal layer improves gene transfer and maintenance of long-term repopulating cells (Nolta et al., 1994). FL may, at least partially, substitute for stroma in the maintenance of repopulating cells (Dao et al., 1997), however, for clinical purposes a cell-free system is preferable. To-date most of the clinical studies of retroviral gene transfer have been carried out using cell-free supernatant but with disappointing results (Brenner et al., 1993b; Dunbar et al., 1995). Retroviral supernatant infection in combination with fibronectin and the inclusion of FL allows efficient gene transfer with improved recovery of hematopoietic cells and thus may have significant impact on the development of newer clinical protocols. One of the interesting features of this study was that the gene transfer efficiency to CFC was not predictive of gene transfer to LTC-IC or CRU, which partially explains the discordance seen in previous clinical studies between gene transfer efficiency to CFC *in vitro* and *in vivo* results.

One of the more obvious future applications of this work is to enhance the power of human autograft marking type studies. This would continue to be an important source of information for proposed therapeutic gene transfer protocols. However better marking data could also be valuable in its own right in terms of improving disease management (Brenner et al., 1993b; Deisseroth et al., 1994). The stage is now set to pursue more sophisticated studies using multiple vectors to compare various purging strategies in the same patient. All the clinical marking studies have used neo^r as the reporter gene and there is increasing evidence that introduction of the neo gene into cells may not be completely benign. Data from a number of studies have demonstrated a discrepancy between the number of transduced cells detectable at the progenitor level compared to the number of cells marked in the circulation (Brenner et al., 1993a; Thomas et al., 1991; Dunbar et al., 1996). Possible explanations for this phenomenon include direct toxicity of the neo gene product in maturing hematopoietic cells, immune reactivity against the neo gene product in differentiating cells. Indeed there is some evidence that the latter may be a more widespread problem not only limited to the neo gene (Riddell et al., 1996; Tripathy et al., 1996). However, in some circumstances this could actually have therapeutic benefits. For example if an immune response was generated against vector-encoded tumor cell antigens, this may prove useful in the treatment of human malignancies.

Chapter 5 describes the development and application of a selectable gene marker system. As discussed in Chapter 1, this approach offers several potential advantages, particularly due to the fact that the gene transfer could simply be evaluated by multiparameter flow cytometry. Moreover, the method allows an efficient and nontoxic sorting of the transduced cells so that the fate of the transduced populations and their progeny can easily be

detected both *in vivo* and *in vitro* even at the single cell level. The use of a virtually pure transduced HSC population may also be an important tool for understanding the biology of hematopoietic reconstitution after BM transplantation. This approach has been used by Pawliuk et al (1996) using CD24 (the human homologue of HSA) as a selectable marker to examine the recovery of hematopoietic cell numbers in engrafted mice. In this study they demonstrated that despite the almost complete recovery of BM cellularity and day 12 CFU-S, donor-derived CRU regeneration remained incomplete independent of the origin and dose of the transplant. Such studies will become increasingly important to study whether similar incomplete regeneration occurs following autologous transplants, in particular, whether *ex vivo* expanded cells have the ability to contribute to long-term engraftment. The HSA selection approach has also been applied to test the therapeutic potential of vectors for the treatment of Gaucher's disease. In these studies a bicistronic retroviral vector encoding HSA and a glucocerebrosidase cDNA was used to infect transformed B-cell lines from Gaucher patients. Retrovirally-infected transduced B cell lines showed a slight increase in their level of glucocerebrosidase; however FACS selection of HSA^+ B cells increased glucocerebrosidase expression 5-fold as compared to untransduced cells (Medin et al., 1996).

The use of such a selectable marker is not confined to hematopoietic tissues. We have also used the HSA vector to develop a protocol that allows different subpopulations of primary human breast epithelial cells to be infected and isolated (Bardy et al., 1997). Using this strategy it should be possible to use HSA containing vectors to introduce a variety of functional genes into normal breast epithelial cells *in vitro* and then assess their potential contribution to the subsequent transformation of these cells. The HSA approach was additionally used in this study to demonstrate the effects of different LTR's, diminished

expression of the LTR-driven HSA gene over time was observed in cells infected with a Moloney based HSA vector. On the other hand, use of the MSCV-HSA.NEO overcame this problem suggesting that the modifications to the MSCV LTR allows sustained expression in several different cell types.

More recently, the green fluorescent protein (GFP) has emerged as an additional reporter molecule for non-invasive methods of monitoring gene expression (Persons et al., 1997; Cheng et al., 1997). One of the advantages of this system is the chromophore in GFP is intrinsic to the primary structure of the protein so no additional substrates or co-factors are necessary. This approach has now been used to selectively isolate hematopoietic cells from both murine and human sources. However, the relative immunogenicity of the GFP as compared to the described cell surface proteins is currently unknown.

In summary, these studies have provided methods to quantitate human *in vivo* repopulating cells and to demonstrate efficient gene transfer to these cells. In addition, a novel cell selection marker system has been described that should allow isolation of populations of pure transduced cells. It will be of great interest to combine the technologies described in all three Chapters of the thesis to reconstitute immunodeficient mice with 100% provirally marked cells and thus follow the patterns of reconstitution in these animals.

Chapter 7 References

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