DETECTION, CHARACTERIZATION, AND GENETIC MODIFICATION OF ACUTE MYELOID LEUKEMIA (AML) STEM CELLS

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

In

THE FACULTY OF GRADUATE STUDIES

Genetics Programme

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

March, 1999

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Department of _Genetics Programme_

The University of British Columbia
Vancouver, Canada

Date _April 12, 1999_

ABSTRACT

The first goal of the current project was to establish reliable assays for the routine detection and quantitation of leukemic stem cells. The development of such assays would, firstly, provide definitive proof for the existence of a primitive cell analagous to a normal pluripotent hematopoietic stem cell within the AML population, and secondly, provide means by which to characterize and manipulate these cells in order to gain an understanding of the mechanisms of leukemogenesis. It was demonstrated that a leukemic longterm culture-initiating cell (LTC-IC) could be routinely detected under appropriate conditions, and also that most AML samples contain a mixture of normal and abnormal LTC-IC. The assay was shown to be quantitative, and calculation of the frequencies of both leukemic and normal LTC-IC in AML peripheral blood samples showed that the frequency of both is considerably higher than the frequency of LTC-IC in normal peripheral blood, suggesting that normal LTC-IC are mobilized as a part of the leukemic process. In addition, differences in the responses of normal and leukemic cells to various cytokines known to be active on normal LTC-IC were revealed.

A second and more reliable assay for stem cells is the animal repopulation assay. The ability of a small subset of cells within AML samples to repopulate immunodeficient NOD/SCID mice was demonstrated, again confirming the existence of a rare stem cell in AML that is responsible for the maintenance of the leukemic clone. This cell was quantitated and its high proliferative capacity and self-renewal ability were demonstrated. Like the LTC system, this assay system provides a model in which to assess the manipulation of human AML cells, both for evaluation of therapeutic strategies and for dissecting the mechanisms of leukemogenesis.

The second major goal of this work was to perform retroviral-mediated gene transfer into AML stem cells. It was found that colony-forming cells and LTC-IC were easily transduced under a variety of conditions, but that the NOD/SCID repopulation ability was impaired during the in vitro period required for transduction. When a variety of culture conditions, including the presence or absence of several cytokine combinations, were tested, in all cases this ability was lost within 24 hours. Strategies for the selection of transduced cells were also investigated. Transfer of the murine heat stable antigen (HSA) gene, a cell surface molecule, enabled the enrichment of transduced cells using an immunomagnetic separation technique. It was also demonstrated that retroviral vectors pseudotyped with a gibbon ape leukemia virus were more efficient than those pseudotyped with the amphotropic envelope from the Moloney murine leukemia virus at transducing AML cells.

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^{3}H	tritium
4-HC	4-hydroperoxycyclophosphamide
$^\alpha$MEM	alpha modified Eagle's medium
AML	acute myeloid leukemia
AML-CFC	acute myeloid leukemia colony forming cells
BFU-E	burst forming unit erythroid
BSA	bovine serum albumin
CBF	core binding factor
CFC	colony forming cell
CFU-E	colony forming unit erythroid
CFU-GEMM	colony forming unit granulocyte, erythrocyte, macrophage megacaryocyte
CFU-GM	colony forming unit granulocyte macrophage
CFU-S	colony forming unit spleen
cGy	centiGrey
Ci	Curie
CML	chronic myeloid leukemia
CRU	competitive repopulating unit
DAPI	4,6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
DIG	digoxigenin
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxynucleoside triphosphate
dTTP	deoxytyrosine triphosphate
dUTP	deoxyuridine triphosphate
EDTA	ethylene diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
Epo	erythropoietin
FAB	French American British
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
FL	flt-3 ligand
FLS	forward light scatter
GALV	gibbon-ape leukemia virus
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
G-CSF	granulocyte colony stimulating factor
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
Gy	Grey
HERV-H	human endogenous retrovirus -H
HFN	Hanks balanced salt solution with 2% FCS

HMF	human marrow feeders
HSA	heat stable antigen
HXM	hypoxanthine, xanthine, mycophenolic acid medium
IL-1$^\beta$	interleukin 1 beta
IL-3	interleukin 3
IL-6	interleukin 6
IMDM	Iscove's modified Dulbecco's medium
Lin-	lineage negative
LTC-IC	longterm culture initiating cell
LTR	long terminal repeat
m.o.i.	multiplicity of infection
MeCell	methylcellulose
MMLV	Molony murine leukemia virus
MSCV	murine stem cell virus
NBCS	newborn calf serum
NBM	normal bone marrow
neo	neomycing phosphotransferase gene
NOD/SCID	nonobese diabetic/severe combined immunodeficient
NOD/SL-IC	NOD/SCID leukemia initiating cell
pA	polyadenylation signal
PBS	phosphate buffered saline
PBS-/+	primer binding site for minus/plus strand DNA synthesis
pgk	phosphoglycerate kinase
Ph	Philadelphia chromosome
PI	propidium iodide
PNM	phosphate nonidet P-40 buffer with skim milk powder
ψ	packaging signal
RARalpha	retinoic acid receptor alpha
RB	retinoblastoma
RT-PCR	reverse transcriptase polymerase chain reaction
SA	splice acceptor
SAM	sheep antimouse immunoglobulin
SC-IC	suspension culture initiating cell
SCID	severe combined immunodeficient
SD	splice donor
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SF	steel factor
SMMHC	smooth muscle myosin heavy chain
TAE	tris acetate EDTA buffer
TE	Tris EDTA buffer
TGF$^\beta$	transforming growth factor beta
TNF$^\alpha$	tumor necrosis factor alpha
TPO	thrombopoietin
TR	texas red
YAC	yeast artificial chromosome

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Donna Hogge, for providing me with the opportunity to do this work, and for her many years of excellent guidance and support. I would also like to thank Gitte Gerhard, for her amazing technical and organizational skills in the lab, as well as her sunny disposition, and many great squash games. Thank you also to other members of the Hogge lab, Guan, Michaela, Hiro, and Kathy, for providing a friendly and productive lab atmosphere. I thank the members of my supervisory committee, Dr. Dagmar Kalousek, Dr. Blake Gilks, Dr. Keith Humphries, and Dr. Heather Sutherland, for encouraging me throughout the years, and keeping me on track. I would also like to thank Dr. Sutherland and Dr. Allison Blair, for their collaborations and discussions regarding AML and the NOD/SCID mouse model. I am also very grateful to the Medical Research Council of Canada, and to Roman Babicki, for providing me with financial support.

I would also like to thank my parents, for always supporting me in whatever I chose to do, and always encouraging me to do my best. Finally, a big thanks to the many people, both inside and outside the Terry Fox Lab, for all the good times we had, whether it be backpacking through grizzly bear country, or going for coffee around the corner: Paul, Ron, Doug, Ying, Trish, Heather, Mark, Karina, Sarah, and Cristina. I would like to especially like to thank Paul Kowalski, for putting up with me through the good times and the bad, for always encouraging me, and without whom my stay in Vancouver would have been a lot less fun.

CHAPTER ONE: INTRODUCTION

1.1 Hematopoiesis

Most types of mature blood cells are short-lived, and therefore must be continuously produced throughout the life of an animal. The many different mature blood cell types which result are highly varied and specialized, and yet they all arise from a common set of rare cells called the "pluripotent hematopoietic stem cells". These cells have extensive proliferative capacity, and the process of blood cell production occurs via a hierarchical progression of cell division and differentiation events over many cell generations, ultimately leading to the large and heterogeneous collection of mature cells found in blood. The stem cells are also capable of self-renewal, giving rise to new pluripotent stem cells. Therefore each stem cell is capable of founding and maintaining a large and diverse, clonal population of blood cells (reviewed in Metcalf 1989; Eaves 1996).

Because the hematopoietic process involves a very large degree of amplification, a slight perturbation of the system at the early stages of the hierarchy could lead to a very large perturbation of the total cell population. Thus, in order to maintain hemostasis, as well as to have a regulated response to stresses such as infection or hemorrhage, the whole system must be very tightly regulated. This occurs through a balance of proliferation, differentiation and cell death, which determines the final rate of mature cell output. This in turn is controlled by a variety of factors. The site of hematopoiesis from late in fetal development and throughout the remaining life of the animal is the bone marrow. Within the bone marrow is a network of fibroblasts and endothelial cells, as well as macrophages, lymphocytes and adipocytes, known as the "stromal microenvironment" (Lichtman 1984). Regulation of hematopoiesis involves intimate cellular interactions between developing blood cells and these stromal elements, as well as with soluble cytokines, either locally produced by the bone marrow stroma or present in

the circulation. A network of more than 20 cytokines that regulate hematopoiesis exists, which includes both positive regulators such as colony-stimulating factors and interleukins, and negative regulators such as transforming growth factor-beta (TGF-β) and tumor necrosis factor (TNF). In addition, different cytokines perform different functions in the regulation of differentiation and proliferation at different levels of the hierarchy, with some operating on more differentiated, lineage committed cells, like colony-stimulating factors, erythropoietin and thrombopoietin, key regulators of granulocyte/macrophage, erythroid and megakaryocyte/platelet production, respectively, while others are early-acting cytokines, such as stem cell factor and flt-3 ligand (Lyman and Jacobsen 1998). To complicate things further, some cytokines act at multiple levels of the hematopoietic hierarchy, and they often synergize with each other to elicit or enhance a particular response. Hematopoietic cell-stromal cell interactions are also believed to play a poorly-understood role in the whole process, via direct cell-to-cell contact through adhesion molecules or production of extracellular matrix molecules, as well as production of transmembrane forms of various cytokines by the stromal cells (Almeida Porada and Ascensao 1996). Cytokines exert their effects by binding to specific receptors on their corresponding hematopoietic cell targets, after which a variety of intracellular events occur via the modification of key regulatory proteins in the cytoplasm. These then affect decisions controlling proliferation and differentiation, including changes in gene expression and reactivity to other factors. The role of cytokines in the regulation of hematopoiesis is reviewed in Metcalf (1989) and Alexander (1998).

To summarize, the hematopoietic system is composed of pluripotential self-renewing stem cells, committed progenitor cells, and mature cells, arranged in a hierarchy as illustrated in Figure 1.1. The hierarchy is divided into these three compartments to allow cells to be more

easily defined, but in fact the whole process is a continuum of a very large number of cell division and differentiation steps, all of which are regulated by a complex series of interactions with the extracellular matrix, the marrow stromal cells, and cytokines. The end result is the highly regulated, continous production of the mature blood cell types to maintain an exact balance with those that are continually reaching the end of their short lifespan and dying, as well as the ability to respond rapidly to hematopoietic stresses.

Disruption of the regulation of hematopoiesis at levels of proliferation , differentiation, and/or cell death can lead to a variety of pathogenic syndromes. For example, myeloproliferative syndromes, such as chronic myeloid leukemia (CML), result from abnormal growth expansion, and myelodysplasia results from a block in differentiation. Both of these syndromes can progress to acute leukemia, in which there is a combination of both uncontrolled proliferation and an inability to differentiate normally to mature blood cells (reviewed in Sawyers et al. 1991). Thus the initiation and progression of acute leukemia results from alterations in the normal homeostatic mechanisms used to regulate the production of blood cells. It was the overall goal of this thesis to develop methods whereby cells from acute myeloid leukemia (AML) samples at different levels of the hematopoietic hierarchy could be detected, characterized, and manipulated, thus leading to means by which differences in the regulation of normal and leukemic hematopoiesis could be discovered.
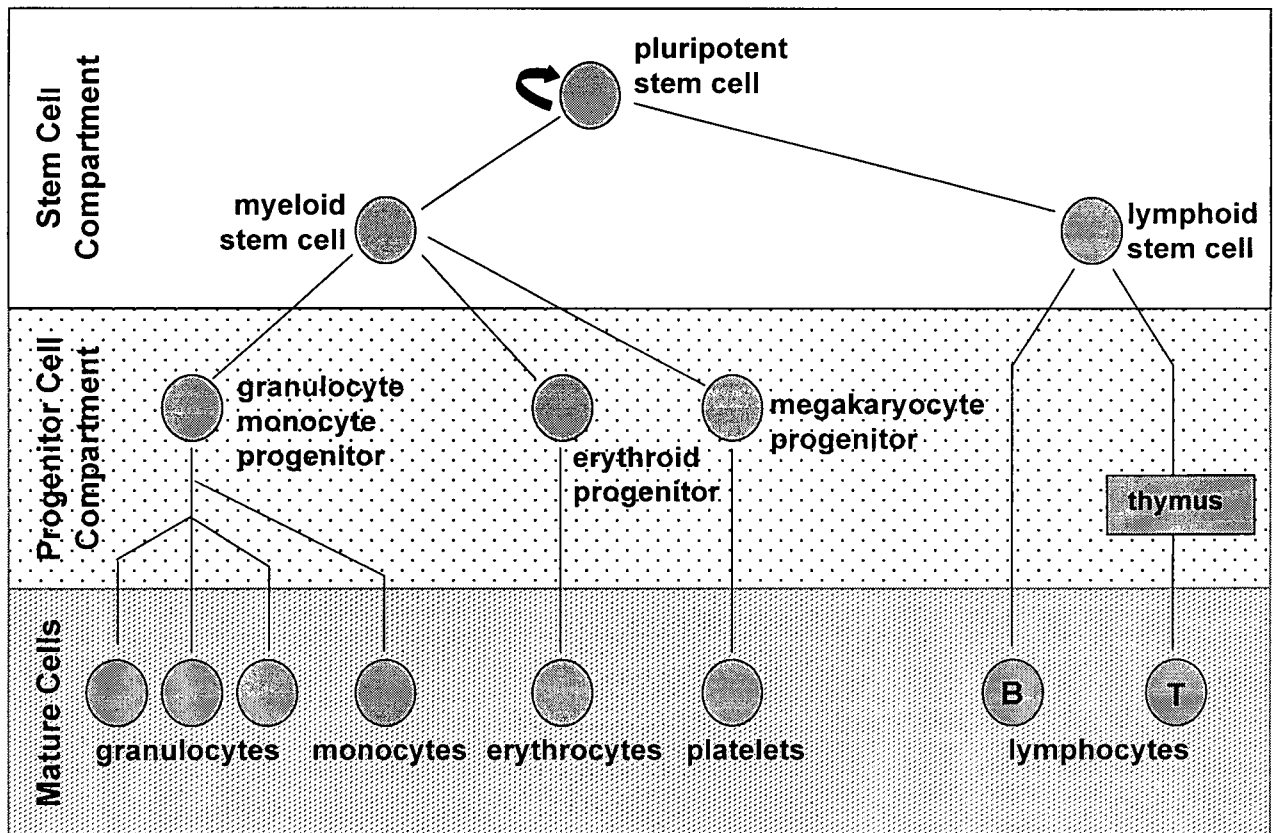
Figure1.1. The hematopoietic hierarchy.

1.2 Acute Myeloid Leukemia

1.2.1 General Characteristics

Acute myeloid leukemia (AML) is a malignancy of blood and bone marrow cells characterized by an accumulation of immature leukemic blast cells and a failure of normal marrow function. This results in the diminished production of normal erythrocytes, granulocytes and platelets. These deficiencies lead to the hallmark manifestations of this disease, namely, weakness, fatigue and pallor as a result of anemia, infection as a result of granulocytopenia, and hemorrhage as a result of thrombocytopenia (Williams et al. 1990b). If left untreated, AML will ultimately result in bone marrow failure and death of the patient.

AML is in fact a group of diseases, which have been classified into a number of subtypes, based on the morphology and cytochemistry of the cells which appear. The most widely-accepted classification is designated the French-American-British (FAB) system, and the FAB subtypes are listed in Table 1.1

Table 1.1. FAB Classification of the Acute Myeloid Leukemias

Description	FAB Type
Acute myeloblastic leukemia with minimal differentiation	M0
Acute myeloblastic leukemia without maturation	M1
Acute myeloblastic leukemia with maturation	M2
Acute promyelocytic leukemia	M3
Acute promyelocytic leukemia (microgranular variant)	M3v
Acute myelomonocytic leukemia	M4
Acute myelomonocytic leukemia with abnormal eosinophils	M4Eo
Acute monocytic leukemia - poorly differentiated	M5a
Acute monocytic leukemia – differentiated	M5b
Acute erythroleukemia	M6
Acute megakaryocytic leukemia	M7

From Schumacher 1990 and Stasi et al 1998.

In addition to the morphological and cytochemical characteristics used to divide AML into the subcategories described in Table 1.1, there are a variety of other features with which particular categories of AML can be associated. The major one is the presence of cytogenetic abnormalities, many of which have been associated with particular FAB subtypes, as well as with patient prognosis. These abnormalities and their associations are described in Table 1.2. The juxtaposition of two genes which are normally on 2 separate chromosomes which is caused by translocation events frequently results in the production of a fusion gene which, when transcribed and translated, results in the production of an abnormal hybrid protein, or in altered expression of one of the genes. Identification and cloning of the fusion genes produced by various translocations has facilitated their detection with PCR-based technologies, allowing

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highly sensitive detection of residual disease in patients in remission. In patients with promyelocytic leukemia with t(15;17), detection of associated fusion gene transcripts by reverse transcription polymerase chain reaction (RT-PCR) was highly correlated with subsequent relapse, whereas those patients who tested negative by this technique remained disease-free at the time of publication (3 months to 5 years post-remission induction; Lo Coco et al. 1992; Huang et al. 1993). Similar techniques have been used for detection of fusion transcripts in remission bone marrow from patients with t(8;21) and inv(16) (Nucifora et al. 1993; Laczika et al. 1998). In the latter study, a competitive RT-PCR technique was quantitative and thus could be used to monitor the extent of residual disease at various stages during and after postremission chemotherapy and bone marrow transplant.The identification of many of the genes which participate in these events, and the possible mechanisms whereby they are involved in leukemogenesis, are described in section 1.2.3.

Table 1.2. Cytogenetic Abnormalities in AML, and their Associations with FAB Types and/or Prognosis

Prognosis	Cytogenetics	FAB Type
Good	abnormal 16q22, especially inv(16)(p13q22)	M4Eo
	t(8;21)(q22;q22)	M2
	t(15;17)(q23;q21)	M3
Intermediate	normal	
Poor	abnormal 11q23, eg. t(9;11)(p21;q23)	M4 or M5
	t(6;9)(p23;q34)	M2 or M4
	-5/del5q	
	-7/del7q	
	+8	
	complex karyotypes	

From Hogge 1994.

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1.2.2   Growth Deregulation in AML

The responses of AML cells to various cytokines when placed in liquid suspension culture have been extensively analyzed in attempts to gain insight into the mechanism(s) of growth deregulation in this disease. There is considerable patient-to-patient heterogeneity, but in general AML progenitors are responsive to some or all of GM-CSF, G-CSF, IL-3, IL-6, SF and FL, with a greater response usually occurring when two or more of these cytokines are provided in combination (Kelleher et al. 1987; Miyauchi et al. 1987; Vellenga et al. 1987; Hoang et al. 1988; Andreef et al. 1992; Piacibello et al. 1995). However, in spite of the requirement for cytokines to obtain maximum proliferation of leukemic clonogenic cells in vitro, autonomous proliferation can also be observed (Griffin et al. 1986). This observation prompted several groups to investigate the expression of various cytokine genes in leukemic cells. Young et al (1987) showed that GM-CSF transcripts could be detected in leukemic cells in half of samples tested, whereas in normal hematopoiesis, GM-CSF expression was limited to activated T lymphocytes. The same group subsequently showed the expression of G-CSF and M-CSF in a significant proportion of AML samples, and in many cases, all three cytokines were expressed in the same sample (Young et al. 1988). The expression of cytokines by leukemic cells has been confirmed at the protein level by the presence of colony-stimulating activity in their culture media, and by specific immunologic assays (Oster et al. 1988; Murohashi et al. 1989). It has since been shown that AML cells can also express IL-3 (Oster et al. 1988), IL-6, TNF-α and IL-1β (Oster et al. 1989). The latter three factors do not stimulate colony growth directly, but IL-6 acts as a costimulator to enhance colony-stimulating factor activity, and IL-1β and TNFα induce colony-stimulating factor production by endothelial cells (Griffin et al. 1987; Oster et al. 1989), thus the expression of these cytokines by AML cells could provide a paracrine loop in

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vivo to support leukemia growth. Expression of functional receptors for G-CSF, GM-CSF and IL-3 have also been demonstrated in leukemic cells (Budel et al. 1989; Park et al. 1989), as have, more recently, the receptors for the early-acting hematopoietic cytokines SF and FL (Broudy et al. 1992; Carow et al. 1996). Thus it appears that both autocrine and paracrine circuits of cytokine stimulation occur in AML, thereby removing one normal mechanism of growth control. However, transgenic mice constitutively expressing the GM-CSF gene and mice reconstituted with bone marrow that had been retrovirally transduced with either the GM-CSF or IL-3 cDNAs, develop myeloproliferative syndromes but not acute leukemia (Johnson et al. 1988; Chang et al. 1989; Wong et al. 1989), indicating that autocrine growth stimulation alone is not sufficient to induce leukemia, but other genetic events that block differentiation must also occur (Lowenberg and Touw 1993).

In addition to an increase in proliferation and a block in differentiation, a third mechanism whereby AML cells may acquire their malignant phenotype is via delayed cell death. In normal hematopoietic cells, withdrawal of growth factors results in programmed cell death (apoptosis), suggesting that cytokines promote cell survival by suppression of this process (Williams et al. 1990a; Brandt et al. 1994). AML cells also undergo apoptosis when cultured under serum-free conditions (Bendall et al. 1994; Murohashi et al. 1997), and this phenomenon can be prevented by the addition of cytokines including one or more of G-CSF, GM-CSF, IL-3, SF and FL (Lisovsky et al. 1996; Murohashi et al. 1997), or by the presence of bone marrow adherent layers (Bendall et al. 1994).

To summarize, three phenomena are associated with the malignant phenotype in the cells of AML: acquisition of an ability to proliferate independently of normal extrinsic growth control mechanisms, acquisition of an anomaly in differentiation commitment, and avoidance of apoptosis. All three of these phenomena could be attributed to genetic changes influencing

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cytokine signalling, including cytokine production, either autocrine or by the local environment, expression of cytokine receptors, and responses to cytokine-receptor coupling at various levels of the associated signal transduction pathways. Studies aimed at elucidating the molecular events in AML cells which lead to the malignant phenotype are under way, as described below.

1.2.3   Genetic Changes in AML

The occurrence of nonrandom chromosome translocations in AML may give clues to the specific mechanisms whereby these cells acquire their malignant phenotype. In many of the cases of AML where translocations occur, the genes which are present at the translocation breakpoints, and the fusion genes which are produced by the translocation event, have been identified, and many of these have proven to be genes that are known to be or have homology to transcription factors; for example retinoic acid receptor $^\alpha$ (RAR$^\alpha$) on 17q21 (de The et al. 1991; Kakizuka et al. 1991), AML-1 on 21q22 (Miyoshi et al. 1991; Erickson et al. 1992), CBF$^\beta$ on 16q22 (Liu et al. 1993), and MLL on 11q23 (Tkachuk et al. 1992).

The identification and cloning of fusion genes, and subsequent expression of these genes in cell lines or animal models has provided methods for the investigation of the molecular mechanisms of leukemogenesis. For example, expression of the PML/RAR$^\alpha$ protein produced by t(15;17) in the myeloid precursor cell line U937 caused these cells to lose their ability to differentiate in response to vitamin D$_3$ and TGF-$^\beta$1, enhanced their sensitivity to retinoic acid, and led to a reduction in apoptotic cell death, thus providing an in vitro system which recapitulates the acute promyelocytic leukemia phenotype in which this translocation occurs (Grignani et al. 1993).

In another example, an AF9/MLL fusion analagous to that produced by the t(9;11) was

produced in murine embryonic stem (ES) cells by homologous recombination. Chimeric mice carrying the fusion gene developed AML, but not other types of tumors, thus providing an in vivo experimental model for the study of tumor development and progression in leukemias involving t(9;11) (Corral et al. 1996).

Perhaps the best example of how identification of translocation-initiated fusion genes have provided clues regarding the mechanisms of leukemogenesis is the identification of the core binding factor (CBF)$^\beta$ gene on 16q22, which is fused to the smooth muscle myosin heavy chain (SMMHC) gene (MYH11) on 16p13 in the inv(16) abnormality (Liu et al. 1993). CBF$^\beta$ is normally a subunit, together with CBF$^\alpha$, of a transcription factor, CBF (Ogawa et al. 1993; Wang et al. 1993). The rearrangement results in the production of a 5'- CBF$^\beta$-MYH11-3' fusion mRNA encoding a CBF$^\beta$/SMMHC chimeric protein (Liu et al. 1993). Interestingly, another AML-associated translocation, t(8;21), involves the CBF$^\alpha$ gene, known as AML1, which is fused to the ETO gene, function unknown (Erickson et al. 1992). Normal CBF has been shown to be an important transcriptional activator of genes involved in mammalian hematopoiesis (Castilla et al. 1996). CBF$^\alpha$ contains a DNA-binding domain, and heterodimerization with CBF$^\beta$ enhances its DNA binding affinity (Ogawa et al. 1993; Wang et al. 1993). The effect of expression of the CBF$^\beta$/MYH11 fusion gene in vivo was assessed by generating mouse embryonic stem cells heterozygous for this gene (Castilla et al. 1996). Chimeric mice were leukemia-free, but the cells carrying the fusion did not contribute to their hematopoietic tissues. Heterozygous embryos lacked definitive hematopoiesis and developed fatal hemorrhages mid-gestation. A similar phenotype was obtained when a similar experiment was done using the AML1/ETO fusion (Yergeau et al. 1997), and this phenotype was also similar to that seen in CBF$^\alpha$- and CBF$^\beta$-deficient mice (Westendorf et al. 1998). Due to the

fact that this phenotype was expressed in embryos which were heterozygous, the mechanism whereby the CBF$^\beta$/SMMHC protein elicits this phenotype was hypothesized to be through the dominant suppression of normal CBF function, resulting in a block in the differentiation of hematopoietic stem cells (Castilla et al. 1996). Further support for this hypothesis was obtained by the demonstration that, upon transient transfection of NIH-3T3 cells with green fluorescent protein (GFP)-tagged proteins, CBF$^\beta$/SMMHC sequestered CBF$^\alpha$ into cytoskeletal filaments, instead of colocalizing to the nucleus, as was seen with normal CBF$^\beta$ and CBF$^\alpha$ (Adya et al. 1998). The AML1/ETO fusion is also proposed to dominantly suppress normal CBF function, based on the similarity of the phenotype of AML/ETO +/- embryos to the CBF$^\beta$/MYH11 +/- embryos, and to CBF$^\alpha$ -/- embryos (Yergeau et al. 1997). Thus the identification and cloning of these and other translocation-induced fusion molecules, and expression of these genes in in vitro and in vivo models, has provided and should continue to provide important clues regarding the mechanisms of leukemogenesis in AML.

Many chromosomal abnormalities are numerical (i.e. trisomies or monosomies), or involve deletions of large chromosomal regions, making the identification of the critical genes involved very difficult. Indeed, such abnormalities may be secondary to the initial transforming event, which could occur through mutations which are undetectable as cytogenetic abnormalities. In fact, a series of such mutations, which would be expected to occur in oncogenes or tumor suppressor genes, would be very likely to be involved in both the initiation and/or progression of leukemogenesis, even when obvious cytogenetic rearrangements are present. For example, 10 to 20% of patients with AML exhibit point mutations in the N- or K-ras genes (Nakagawa et al. 1992; Cline 1994), and point mutations in the FMS (M-CSF receptor) gene which disrupt a negative regulatory site, and thus presumably up-regulate the response of the receptor to ligand

binding, were found in approximately 17% of AML samples (Ridge et al. 1990). Also, inactivation of the tumor suppressor gene retinoblastoma (RB) has been shown to occur in approximately 30% of cases of AML (Kornblau et al. 1992; Tang et al. 1992), and loss of RB expression was associated with shortened survival of AML patients (Kornblau et al. 1992). The p53 tumor suppressor gene, which has been implicated in the pathogenesis of many malignancies, is occassionally mutated in AML (approximately 7% of samples; Fenaux et al. 1992), and loss of the tumor suppressor gene DCC, the inactivation of which is believed to be a critical event in the development of colorectal cancers, has also been implicated in leukemogenesis (Porfiri et al. 1993). It is likely that many more oncogenes and tumor suppressor genes are involved in leukemogenesis, and models such as those described above will be required to dissect the exact molecular mechanisms by which malignant transformation occurs.

The identification of specific genes involved in the process of leukemogenesis, whether it be translocation-associated fusion genes, mutated oncogenes, or inactivated tumor suppressor genes, has led to the idea that therapeutic strategies which target these genes could be developed. This is discussed further in section 1.8.5.

1.3   *Functional Assays for Cells at Different Stages of the Hematopoietic Hierarchy*

Due to the extremely heterogeneous nature of AML with respect to morphology, clinical course, and genetics, as described in section 1.2, it has been difficult to find any consistent properties which may elucidate the underlying cause(s) of this disease. In order to identify and define the properties of leukemic cells which lead to unregulated expansion and blocks at various stages of differentiation, assays which allow the detection and manipulation of cells at

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different stages within the hematopoietic hierarchy would be useful. Such assays have been developed and well-characterized for normal hematopoietic cells, as described below, and in this thesis have been developed and optimized to allow the identification and characterization of analagous cell types in AML samples.

The majority of cells present in the bone marrow and blood of a normal individual are terminally differentiated, and their distinctive cellular morphologies make them the easiest to monitor. The higher up in the hierarchy, however, the more rare the cell, necessitating the development of a variety of techniques to detect, measure and characterize the progenitor and stem cells. These assays are performed by placing cells under conditions where their differentiation potential (lineages present among the daughter cells generated) and their proliferative potential (number of daughter cells generated) can be assessed (reviewed in Eaves and Eaves 1997).

1.3.1   Clonogenic Cells

When normal hematopoietic cells from various sources are plated under appropriate conditions in semisolid medium, colonies will form which contain cells of the various myeloid lineages, either alone or in combination. The cells that give rise to colonies are referred to as clonogenic progenitors, colony-forming cells (CFC), or colony-forming units (CFU), and represent the directly clonogenic progenitor cell compartment of the hematopoietic hierarchy. There is considerable heterogeneity in the size and composition of the colonies produced, reflecting the heterogeneity in the types of cells present within the clonogenic cell compartment. To achieve growth of colonies representative of the different cell types present, a source of appropriate cytokines must be provided. This has traditionally been done by supplementing the cultures with serum and medium conditioned by either phytohemaglutinin-

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stimulated leukocytes, or the 5637 bladder carcinoma cell line, as well as exogenously added

erythropoietin (Iscove et al. 1971; Gregory and Eaves 1977; Fauser and Messner 1978; Hoang

and McCulloch 1985). More recently, the availability of recombinant, purified cytokines has

allowed the use of more defined media, in which the appropriate cytokines are added

individually (Eaves and Eaves 1997). The colonies which result are then individually scored

based on the lineage(s) present, as well as their size. The greater the colony size and content of

different lineages, the more primitive the progenitor cell from which the colony was derived.

The different categories of progenitors, and the cytokines which stimulate their growth, are

described in Table 1.3. Sutherland et al (1991a) report the average frequencies of various

colony types from 87 normal human bone marrow samples as being 67, 56, 62, and 1 per $2X10^5$

nucleated cells for CFU-E, BFU-E, CFU-GM and CFU-GEMM, respectively. This works out

to a total CFC frequency of approximately 1 per 1000 nucleated cells.

Table 1.3. Normal Clonogenic Progenitors Detected in Semisolid Assays

Lineage	Colony Name	Description	Cytokines*
Granulopoietic/ Monocytic	CFU-GM (mature)	<500 cells; granulocytes, macrophages, or both; rapidly cycling	G-CSF, GM-CSF, IL-3, IL-6, SF, FL
	CFU-GM (primitive)	>500 cells; granulocytes, macrophages, or both; slowly cycling	
Erythropoietic	CFU-E	1-2 clusters of erythroblasts	Epo, SF, IL-3, IL-6, GM-CSF
	BFU-E (mature)	3-8 clusters of erythroblasts	
	BFU-E (intermediate)	9-16 clusters of erythroblasts	
	BFU-E (primitive)	>16 clusters of erythroblasts	
Megakaryo-cytic	CFU-Mk (mature)	1 cluster with >2 cells	IL-3, IL-6, GM-CSF, SF, TPO
	CFU-Mk (primitive)	multiple, larger clusters	
Multipotent	CFU-GEMM	combination of granulocytes, macrophages, megakaryocytes and erythroid cells; large	SF, IL-6, IL-3, Epo, FL
Multipotent	CFU-blast	<100 cells; undifferentiated	SF, IL-3, others?

*Cytokines which stimulate the growth of the given colony type in semisolid assays.

CFU=colony forming unit; BFU=burst-forming unit; GM=granulocyte/macrophage; E=erythroid; Mk=megakaryocyte; GEMM=granulocyte/erythrocyte/macrophage/ megakaryocyte.

G-CSF=granulocyte colony-stimulating factor; GM-CSF=granulocyte-macrophage colony-stimulating factor; Epo=erythropoietin; IL-3/6=interleukin-3/6; SF=steel factor; FL=flt-3 ligand; TPO=thrombopoietin.

Compiled from information presented in Eaves and Eaves 1997, Leary et al. 1987a, Hannum et al. 1994, Leary et al. 1987b, Tsuji et al. 1991.

When AML blood or bone marrow samples are placed into clonogenic cell assays, in most cases small clusters or colonies of cells with blast-like morphology form; however, only a minority of the total cells (1 in 10^2 to 1 in 10^4) are capable of proliferating to form colonies in this system (AML-CFC; Moore et al. 1973; Dicke et al. 1976; Buick et al. 1977). Growth of AML-CFC from most patients also requires exogenous colony-stimulating factors, such as conditioned media (Buick et al. 1977; Minden et al. 1978; Welte et al. 1985) or combinations of recombinant human cytokines, such as G-CSF, GM-CSF and IL-3 (Griffin and Lowenberg 1986; Vellenga et al. 1987). The cells in the colonies which form morphologically resemble the blasts from the patients from which they were derived and, when chromosomal abnormalities are found in the patient's cells, they are also present in the colonies (McCulloch et al. 1979). The AML-CFC have been shown to have a high thymidine suicide index, indicating a high proportion of cells in active cell cycle (Minden et al. 1978).

The ability of a small subset of AML cells to form colonies indicates the existence of a clonogenic cell compartment from which the bulk of nonproliferating blast cells are derived. Exponential growth of AML-CFC could be maintained in liquid suspension cultures for extended periods of time (Nara and McCulloch 1985), but whether this growth represented simply AML-CFC renewal, or the recruitment of new blast progenitors from more primitive leukemic precursors was not clear. Evidence for the latter came with the illustration that AML cells can be physically separated into subpopulations with different proliferative capacities based on cell surface antigen expression (Sutherland et al. 1996). It therefore seemed likely that a hierarchy exists in AML samples similar to that of the normal hematopoietic hierarchy, with a primitive "leukemic stem cell" being responsible for the maintenance and expansion of the leukemic clone. Therefore assays which allow the measurement of more primitive cell types than CFC will be required to understand this disease.

1.3.2   In Vitro Assays for Cells More Primitive than CFC

Most colony forming cells are not true stem cells as evidenced by their lack of self-renewal ability and their commitment to a single cell lineage. A cell which is capable of giving rise to multiple lineages of CFC must be a precursor to cells within the committed colony forming cell compartment. Cells from normal individuals which are capable of giving rise to multiple lineages of CFC in vitro for extended periods of time have been identified using the so-called longterm culture (LTC) system. Dexter et al (1977) developed a method for long-term growth of mouse bone marrow cells in liquid medium, in which the production of progenitors is dependent upon the presence of a marrow-derived adherent population containing macrophages, endothelial cells and adipocytes. Gartner et al (1980) modified this system to develop a method for the LTC of human bone marrow cells. This essentially involves placing unseparated human bone marrow into suspension culture containing a combination of horse and fetal calf serum and hydrocortisone at high cell density. Over the initial 2 to 3 weeks in culture the stromal elements will form an adherent layer with which the primitive hematopoietic cells become associated. Over several weeks of culture with weekly removal and replacement of one half of the culture media, the continued generation of hematopoietic progenitors and their differentiation into mature progeny occurs, in the absence of any exogenously provided growth factors (Coulombel et al. 1983). The adherent stromal layer appears to mimic the in vivo hematopoietic microenvironment, as indicated by the presence of the majority of progenitor cells in the adherent layer after 7 to 8 weeks in culture (Coulombel et al. 1983).

The disadvantage of the technique described above was that human hematopoiesis in vitro could only be established with whole bone marrow samples. This was overcome by the use of pre-established feeders, made by subculturing the stromal layers from primary longterm

cultures, designated human marrow feeders, or HMF. This then allowed other populations of

hematopoietic cells to be assessed for their ability to initiate hematopoiesis in vitro, including

peripheral blood and various subpopulations of cells purified on the basis of their cell surface

phenotype (Eaves et al. 1986; Sutherland et al. 1989; Udomsakdi et al. 1992c). The continuous

production of multiple lineages of CFC for an extended period of time indicates the presence of

a more primitive, multipotent progenitor cell, designated a longterm culture-initiating cell

(LTC-IC). Further evidence that LTC-IC are more primitive than CFC came from strategies

designed to enrich for or purify stem cells. When human bone marrow cells are treated with

chemotherapeutic drugs, such as 4-hydroperoxycyclophosphamide (4-HC) or 5-fluorouracil (5-

FU), no CFC remain, but LTCs initiated with the treated cells give longterm production of

progenitors equivalent to that of control LTCs (Siena et al. 1985; Winton and Colenda 1987;

Berardi et al. 1995). Furthermore, separation by flow cytometry of cells into various

subpopulations based on the expression of different cell surface antigens showed that CFC and

LTC-IC can be separated from one another, with LTC-IC being present in a rare subpopulation

which is both $CD34^+$ and lacking in expression of CD38 and lineage-specific antigens

(Sutherland et al. 1989; Baum et al. 1992; Terstappen et al. 1991; Huang and Terstappen 1994).

A very useful characteristic of the LTC system is that the clonogenic cell output, routinely

assessed from cultures 5 or more weeks old, is linearly related to the input cell number over a

wide range of cell concentrations, allowing the frequency of LTC-IC in a given cell population

to be measured by limiting dilution analysis (Sutherland et al. 1990b). It has been found that,

when normal bone marrow cells are assessed in this way on pre-established HMFs, the

frequency of LTC-IC is approximately 1 per $2X10^4$ cells. Furthermore, the proliferative

capacity of individual LTC-IC could be ascertained from cultures initiated by limiting numbers

of cells. This was found to range from 1 to 30 CFC produced per LTC-IC, with a mean of 4,

and the generation of both erythroid and myeloid progeny from individual LTC-IC provided a

further indication of the multipotency of these cells (Sutherland et al. 1990b). Although LTC-

IC have many characteristics which suggest that they may represent hematopoietic stem cells

(they are largely quiescent, as demonstrated by their resistance to chemotherapeutic drugs, they

give rise to multiple lineages of CFC for extended periods of time in vitro, they can be

physically separated from CFC, and they lack expression of differentiation-associated

antigens), it remains controversial whether they do in fact represent a true lymphomyeloid

hematopoietic stem cell.

It has since been discovered that various murine stromal cell lines can give support for

longterm human hematopoiesis equivalent to endogenous marrow stromal cells (Sutherland et

al. 1990a; Otsuka et al. 1991; Sutherland et al. 1991b). Furthermore, by engineering murine

stromal cell lines to constitutively produce human cytokines, it has been possible to enhance the

sensitivity of detection of these progenitors and increase their output of CFC progeny (Otsuka

et al. 1991, Sutherland et al. 1993, Hogge et al. 1996). One group of investigators has found

that optimal feeders for normal LTC-IC detection consist of a combination of murine M2-10B4

fibroblasts (a cloned line of mouse BM origin; Lemoine et al. 1988) engineered to produce high

levels of both human G-CSF and IL-3, mixed 1:1 with Sl/Sl fibroblasts (an embryonic cell line

derived from Sl/Sl mice, which are defective in the production of SF; Sutherland et al. 1993)

engineered to produce high levels of soluble SF. These "mixed feeders" have been found to

stimulate the production of up to 20-fold more CFC in LTC of cells from normal bone marrow,

mobilized peripheral blood or umbilical cord blood, relative to the same cells cultured on

HMFs. This increase was due to an overall increase in the plating efficiency, as well as an

increase in the proliferative capacity of individual LTC-IC, as determined by limiting dilution

analysis. There was also a significant enhancement of LTC-IC maintenance on these feeders

compared to HMFs (Hogge et al. 1996). These developments have allowed the standardization of LTC conditions for normal human hematopoietic cells. The LTC system has thus proven valuable in gaining information about a very primitive hematopoietic cell type. In addition to allowing determination of the frequency and proliferative capacity of these cells, their cytokine responses and the kinetics of cytokine-stimulated entry into S phase have also been characterized (Ponchio et al. 1995). The identification of an analagous cell type in AML could therefore provide a means by which to identify differences in such characteristics between normal and leukemic cells, and thus provide some insight into the mechanisms underlying the disease.

A variety of studies have been done in which leukemic samples have been placed in LTC. When cells from CML patients are placed into LTC, a cell that is functionally analogous to normal LTC-IC, but which is Philadelphia-chromosome positive (Ph^+), is detected (Barnett and Eaves 1996). However, cytogenetically normal LTC-ICs are also present at detectable levels in the marrow of many CML patients, at least some of which have been shown not to belong to the malignant clone, and which are therefore presumably normal (Barnett and Eaves 1996). The frequency of these residual, normal LTC-IC in CML patients is highly variable from one patient to the next.

Very little is known about cells analogous to LTC-IC among the malignant cells of patients with AML. Previous efforts to characterize primitive AML progenitors in the LTC system had suggested that they were often poorly maintained under standard conditions (Firkin et al. 1990; Coulombel et al. 1985). CFC assays done by Coulombel et al (1985) on 13 AML bone marrow samples at the time of diagnosis showed normal progenitor numbers to be reduced in most cases, and the presence of colonies and clusters with abnormal "blast" morphology to be present in most. When these samples were placed in LTC, 9 of 13 showed an emergence of

apparently normal CFC after several weeks, and abnormal colonies could no longer be found after 4 weeks of LTC in 8 of these 9. In the other 4 cases, blast progenitors were maintained in LTCs of two of them, and no progenitor activity of any kind was present in the other two. In a similar study by Firkin et al (1990), of 15 AML samples placed in LTC, an increase in differentiation to normal morphology took place in 9, while in the other 6 cases, blast morphology was maintained. In 3 of the 9 cases where morphologically normal cells emerged, reversion from an abnormal to a normal karyotype was also seen. It was therefore concluded by these authors that leukemic cells from many (although not all) AML patient samples could not be maintained under LTC conditions. Conversely, in other studies of AML in LTC, the emersion of morphologically normal cells was also observed, but these were found to be of leukemic origin based either on the presence of Auer rods in the differentiated cells (Iland et al, 1987), or the presence of cells with leukemia-associated abnormalities (Scholzel and Lowenberg 1985). When Singer et al (1988) placed AML cells from 2 AML patients who were heterozygous for X-linked glucose-6-phosphate dehydrogenase, in both cases, again, morphologically normal CFC emerged, but in one case these were shown to express the G-6-P-D type of the leukemic blast cells. It therefore appears that normalization of colony growth is by itself not a sufficient criterion for determination of whether CFC growing in LTC of AML cells are derived from normal or leukemic precursors.

These experiments were all done at a time when LTCs were established by plating unprocessed bone marrow aspirates and allowing them to form their own adherent layers (Coulombel et al. 1983). The more recent use of preformed feeder layers, either HMF or mixed feeders, has since allowed the standardization and manipulation of LTC conditions. This, combined with increased information regarding cytokine responsiveness of leukemic cells and the availability of recombinant human cytokines, led to the hypothesis that appropriate

conditions for LTC of AML cells could be discovered. In addition, the advent of fluorescent in situ hybridization (FISH) techniques that allow the recognition of chromosomal abnormalities in interphase as well as metaphase cells would enhance the ease with which leukemic progenitors could be discriminated from their normal counterparts (Trask 1991). Therefore experiments were initiated to determine if a sensitive and quantitative assay for AML-LTC-IC could be developed. Chapter 3 describes the establishment of such an assay, and some of the characteristics of the leukemic cells that it detects.

1.3.3   In Vivo Repopulating Cells

Early studies in mice indicated that adult mouse marrow contained cells capable of forming macroscopic, multilineage colonies in the spleens of transplanted recipients (Till and McCulloch 1961; McCulloch et al. 1964). In a fashion analagous to what is seen with CFC, these colony forming units-spleen (CFU-S) can be divided into subsets based on the time taken for the colonies to form and on their size and composition. For example, day 7-8 CFU-S contain a few hundred thousand cells, whereas after longer periods, successively larger colonies which may contain more than 10^6 cells appear. Most of the day 11 CFU-S contain multiple lineages of maturing cells, and some are capable of self-renewal as indicated by the formation of secondary CFU-S after injection into secondary recipients (Schofield and Dexter 1985). In spite of these characteristics, which indicate that CFU-S are cells with a high proliferative capacity and multilineage potential, they are not believed to represent a population of true pluripotent stem cells. This is due to the identification of lympho-myeloid repopulating cells that can be physically separated from CFU-S, suggesting the existence of a pre-CFU-S cell (Ploemacher and Brons 1989).

An alternative in vivo assay that has allowed the identification and quantitation of a true

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pluripotent murine hematopoietic stem cell is the detection of cells with the potential to reconstitute all hematopoietic lineages in lethally irradiated transplant recipients. This assay has two important features; first, by coinjecting test cells with a sufficient number of hematopoietic cells that are compromised in their longterm reconstituting potential, the survival of the lethally irradiated mice is ensured even when the population of test cells is small; second, the use of a limiting dilution strategy enables quantitation of the repopulating cells (Szilvassy et al. 1990). The frequency of these cells, designated competitive repopulating units, or CRU, is defined using Poisson statistics as the reciprocal of the total number of cells that allows 63% of transplanted recipients to show a sustained output of test-cell derived progeny in the regenerated hematopoietic tissues (Coller and Coller 1986). The only other requirement is the need for an appropriate genetic marker to allow the test cells to be distinguished from the host and coinjected compromised cells, such as male vs female, or different isotypes of particular antigens. The frequency of CRU in the bone marrow of normal adult mice is 1 per 10^4 nucleated cells (Szilvassy et al. 1990).

The availability of various strains of immunocompromised mice has made it possible to develop an assay similar to the mouse CRU assay for human cells. This involves injecting irradiated immunocompromised mice intravenously with human hematopoietic cells, and after a sufficient period of time, assessing them for the presence of human lympho-myeloid repopulation. Initially, such experiments were performed by injecting human bone marrow into sublethally irradiated severe combined immunodeficient (SCID) mice, which are homozygous for a mutation which leads to a lack of functional T and B lymphocytes (Bosma et al. 1983). These mice were then treated with human cytokines (SF, IL-3 and GM-CSF), and the presence of multiple human myeloid and lymphoid lineages could subsequently be demonstrated (Lapidot et al. 1992). Similar results were obtained when human cord blood cells were injected

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into SCID mice, except that cytokine treatments of the mice were not required to achieve significant levels of engraftment (Vormoor et al. 1994). It has since been shown that higher engraftment levels can be achieved when nonobese diabetic (NOD)/SCID mice are used as recipients (Lowry et al. 1996; Pflumio et al. 1996). These mice are more severely immunocompromised than SCID mice, and were developed by backcrossing SCID mice onto the NOD/Lt strain, which is characterized by a functional deficit in NK cells, absence of circulating complement, and defects in the differentiation and function of antigen-presenting cells (Shultz et al. 1995). Thus the NOD/SCID strain provides a model in which there are multiple defects in innate immunity as well as an absence of T and B cell function. Longterm engraftment of these mice with both myeloid and lymphoid lineages indicates the presence of pluripotent hematopoietic stem cells.

By using a limiting dilution technique in the NOD/SCID model similar to that described for murine CRU, it has been possible to determine the frequencies of the analagous cell type in normal human hematopoietic samples. The frequencies of human CRU have been found to be 1 in $9.3X10^5$ cord blood cells, 1 in $3X10^6$ adult bone marrow cells, and 1 in $6X10^6$ mobilized peripheral blood cells (Wang et al. 1997). Furthermore, the self-renewal of human CRU in vivo has been illustrated by the engraftment of secondary mice (Cashman et al. 1997a).

Once animal transplantation models became available, it was then possible to determine whether the established phenotypes for cell populations highly enriched in LTC-IC would be equally enriched for CRU. Civin et al (1996) showed that $CD34^+CD38^-$ human bone marrow cells initiated longterm multilineage human hematopoiesis in a human-fetal sheep in vivo model, whereas $CD34^+CD38^+$ cells generated only short-term engraftment. Bhatia et al (1997) found similar results with $Lin^- CD34^+CD38^-$ cells in NOD/SCID mice, and were able to calculate that the frequency of CRU in this population is 1 CRU per 617 cells. More recently

there have been reports of in vivo repopulating activity in a very small population of cells which is CD34⁻ and negative for lineage markers, as well as for the antigens Thy-1 and HLA-DR, which are both expressed on CD34⁺ CRU (Zanjani et al. 1998; Bhatia et al. 1998). Whether there are any functional differences between CD34⁻ and CD34⁺ CRU remains to be determined. Meanwhile, the isolation of CD34⁺CD38⁻ cells remains the most common technique for obtaining populations of hematopoietic cells which are highly enriched for CRU, a cell believed to represent the true pluripotent hematopoietic stem cell.

Immunodeficient mice will also support the growth of malignant progenitors from patients with CML and AML (Lapidot et al. 1994; Sirard et al. 1996; Blair et al. 1997; Bonnet and Dick 1997; Wang et al. 1998). Sirard et al (1996) obtained consistent multilineage engraftment of cells from patients with either chronic or blast phase CML in SCID mouse bone marrow. The Philadelphia (Ph) chromosome was present in a proportion of CFCs from mouse BM engrafted with chronic phase CML (32% by RT-PCR; 13% by metaphase analysis). Cells from blast crisis patients engrafted more rapidly than chronic phase cells and gave rise to exclusively Ph+ progenitors in mouse bone marrow. Wang et al (1998) then showed that higher levels of engraftment of CML cells could be obtained in NOD/SCID mice than in SCID mice, and that the proportions of leukemic cells in these were also higher. Thus NOD/SCID mice provide an in vivo model for the quantitation and characterization of the normal and leukemic stem cells present in CML patients.

As with CML, xenotransplant experiments with AML were first reported in SCID mice by Lapidot et al (1994), who showed that this resulted in "a pattern of dissemination and leukemic cell morphology similar to that seen in the original patients". It was again found that NOD/SCID mice were superior for the engraftment of AML cells, in that lower cell doses were required to give equivalent levels of engraftment (Bonnet and Dick 1997). The AML cells with

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the potential to initiate leukemia in SCID or NOD/SCID mice (NOD/SCID leukemia-initiating cells or NOD/SL-IC) have been shown to be rare progenitors that are largely CD34^{++}, CD38^{-} (Lapidot et al. 1994; Blair et al. 1997; Bonnet and Dick 1997) and Thy-1^{-} (Blair et al. 1997). These results suggested that NOD/SCID animals could be used for the detection and further characterization of very primitive leukemic progenitors that may be analogous to those which maintain the leukemic clone in patients.

Although it was clear from these studies that AML cells would grow successfully in the appropriate mouse strains, the variability of the growth potential of various AML samples was not so apparent. In addition, little was known about factors that may influence growth of human AML cells in these animals, or how various classes of malignant progenitors were maintained over time in the mice. Chapter 4 describes studies undertaken to answer several of these questions, and to further characterize NOD/SL-IC.

1.4 *Genetic Modification of Normal and Leukemic Stem Cells*

The genetic modification of leukemic stem cells would have two potential applications. First, it could allow the development of gene therapy strategies to overcome the disease in patients, and second, it could provide, in combination with the functional assays described above, an extremely valuable tool to facilitate dissection of the molecular mechanisms involved in leukemogenesis.

A major issue to be resolved before this can be done is the best method for gene delivery. Based on the observation that many DNA and RNA viral life cycles include insertion of the viral genome into host cell DNA, a variety of virally-based gene transfer methodologies have been developed (Friedmann 1989). A large number of different viruses have been studied for their ability to act as vectors for gene transfer, including adenoviruses, adeno-associated

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viruses, foamy viruses, HIV, herpes viruses and non-complex retroviruses (Medin and Karlsson 1997). The latter have generally been the virus of choice for gene transfer to hematopoietic stem cells due to their ability to stably integrate into the host-cell genome without incurring cellular toxicity. The rest of this discussion will therefore focus on the use of these vectors for gene transfer to normal and leukemic stem cells.

1.4.1   Retroviral Vectors

Retroviruses contain an RNA genome within a protein core, surrounded by an envelope that is studded with viral glycoproteins and derived from the membrane of the previous host cell (Varmus 1988). The viral particle also contains within it viral enzymes (reverse transcriptase, integrase and protease) which direct the reverse transcription of the viral genome, and integration of the resulting DNA into the host cell genome (Varmus 1988). The integrated DNA form of a retrovirus (the provirus) consists of two long terminal repeats (LTRs), between which are the gag, pol and env genes. The transcription of these genes is under the control of a promoter located in the 5'LTR (Miller 1990). The gag gene encodes the core proteins, the pol sequence encodes the reverse transcriptase, protease and integrase enzymes, and the env gene encodes the envelope glycoproteins (Varmus 1988). Also contained within the viral genome are the signals required for reverse transcription, integration, transcription, RNA processing, and packaging of RNA into progeny viral particles.

To make such retroviruses suitable for use as vectors for gene transfer, all of the viral protein coding regions can be removed and replaced with the DNA sequences to be transferred to the host cell. This can be done without affecting the ability of the viral RNA to be encapsulated into infectious viral particles, provided that the packaging signal and signals for viral integration (i.e. the LTRs) are left intact, and that all of the retroviral proteins required for

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assembly are provided in trans. The latter is done using "packaging cells". These are cell lines that have been stably transfected with "helper virus" sequences, that is, the gag, pol and env genes, but which are lacking the signals required for packaging, reverse transcription, and integration, thus preventing the production of replication-competent retroviruses. As a further safety mechanism, the gag-pol and env genes are in separate transcriptional units, making the probability of infectious helper virus formation via recombination extremely small (Miller 1990). Transfection of the packaging cell line with the vector of choice then results in the packaging of the recombinant vector into an infectious particle. The host range and cellular tropism of this is determined by the envelope gene in the packaging cell line. Different envelope glycoproteins recognize and bind to different cell surface receptors, thus the virus can only infect a cell which expresses the appropriate receptor. Once the recombinant retrovirus has entered a cell and integrated into the host cell genome, it is thereafter incapable of propagating itself, due to the absence of the proteins required for viral assembly. Thus the end result is the transfer of the desired DNA sequence into the target cell and stable integration of that sequence into the genomic DNA, with no further harmful effects on the cell. The site of insertion of the retrovirus into the cell genome is random, so that each individual transduced cell and all of its progeny contain the provirus at a unique site in the genome. This allows the fate of individual cells to be tracked throughout subsequent assays and manipulations.The only restrictions to the infection of a target cell by a retroviral vector are that it must be expressing the appropriate receptor, and that it must be actively replicating at the time of infection (Miller et al. 1990). The latter is believed to be due to a requirement for nuclear envelope breakdown to allow entry of the viral integration complex into the nucleus (Roe et al. 1993). A schematic representation of a packaging cell line is shown in Figure 1.2.

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Packaging Cell Line:
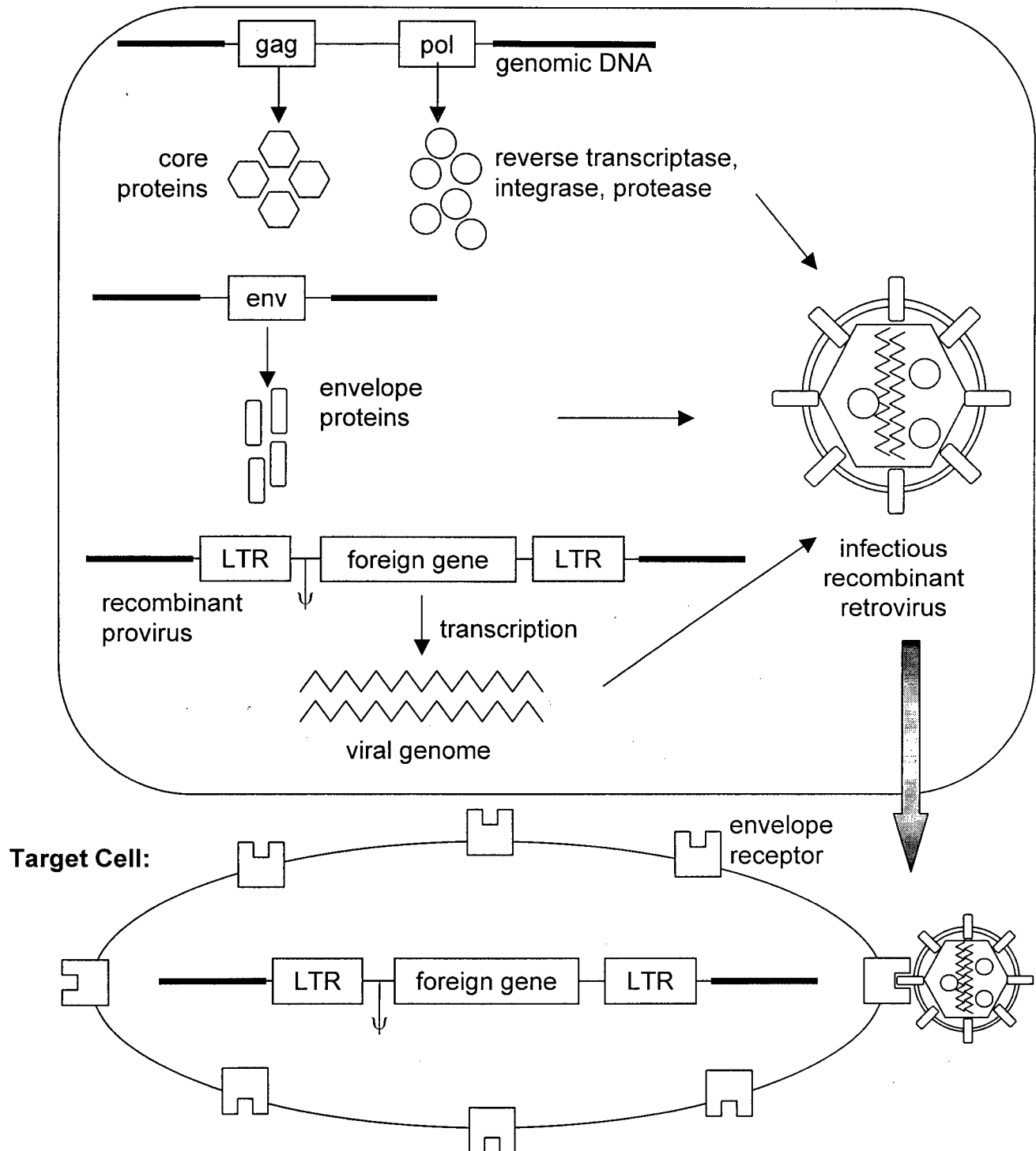
Figure1.2. Schematic representation of a retroviral packaging cell line. The cell line contains the gag-pol and env genes on two separate transcriptional units, as well as the proviral form of the recombinant viral vector. The gag, pol and env genes provide the components required for assembly of an infectious recombinant retroviral particle, which can then infect target cells expressing the appropriate envelope receptor. The virus can then no longer replicate due to the absence of the gag, pol and env gene products in the target cell.

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1.4.2   Retroviral Marking Studies in Mice

Initial attempts to retrovirally transduce bone marrow repopulating cells were done in the murine system. Williams et al (1984) initially described the use of retrovirus vectors to transfer the neomycin-resistance gene (neo) into bone marrow-derived hematopoietic progenitors, as assessed by the presence of CFU-S in lethally irradiated recipients which contained the foreign gene. Approximately 15 to 20% of CFU-S could be successfully transduced. Serial transplantation of single, transduced CFU-S cells into secondary recipients and subsequent clonal analysis of the spleens and bone marrow of these animals indicated successful infection of stem cells with self-renewal ability and multipotentiality. The ability to uniquely mark individual hematopoietic stem cells based on random retroviral integration sites has allowed many studies to be done in which the fate of individual stem cells can be followed in vivo. Dick et al (1985) and Keller et al (1985) also reconstituted mice with retrovirally neo-transduced bone marrow, and clonal analysis of DNA from spleen, bone marrow and thymus indicated insertion of the vector into primitive pluripotent stem cells capable of producing both myeloid and lymphoid progeny, as well as more committed stem cells apparently restricted to either the myeloid or lymphoid lineages. Lemischka et al (1986) showed that in the majority of recipients of gene-marked stem cells, few stem cell clones (usually 1 or 2) account for the majority of the mature hematopoietic cells at any given time. However, periodic sampling of recipients over time indicated that normal hematopoiesis results from the sequential activation of different stem cell clones, rather than from an averaged contribution of the entire stem cell pool (Lemischka et al. 1986). Such gene marking studies were subsequently used to aid in the development of procedures for the purification of primitive cells. Szilvassy et al (1989) used a purification strategy in combination with subsequent retroviral marking to demonstrate the long-term

lympho-myelopoietic repopulating ability of the purified cells. This technique has also been used to demonstrate expansion in vitro of pluripotent hematopoietic stem cells, as evidenced by the presence of the same retrovirally marked clone in multiple mice injected with cultured cells (Fraser et al. 1990). Thus retroviral-mediated gene transfer to murine long-term repopulating stem cells has been successful, and has provided scientists with a valuable tool for following the fate of individual stem cells in vivo under various conditions, as well as after in vitro manipulation.

1.4.3   Genetic Modification of Normal and Leukemic Human Stem Cells

One of the goals of the work described in Chapter 5 was to use the engraftment of NOD/SCID mice with retrovirally marked AML cells to perform similar clonal analysis-type studies as those described above for murine cells, and thus gain further information regarding the proliferative capacity and engraftment ability of NOD/SL-IC. The ability to genetically modify AML cells would also provide a means for developing gene therapy strategies, as well as of introducing into the leukemic stem cells gene products, such as cytokines, cytokine receptors, or oncogenes, which might affect properties of these cells in functional assays, and thereby provide clues to the regulatory processes involved. Therefore, in addition to clonal analysis studies, the goal of the work in Chapter 5 was also to investigate various techniques for the optimization of gene transfer to leukemic cells and for the purification of transduced cells (see section 1.4.4 for more details on the latter).

A large amount of work has been focused on the retroviral transduction of normal hematopoietic stem cells. Standard protocols for this involve the exposure of the cells to virus, either by coculturing the target cells in the same dishes as the packaging cell lines, or by suspending the target cells in supernatants that have been conditioned by packaging cell lines.

In most cases, the hematopoietic cells are exposed to cytokines, both before and during the transduction period, in an attempt to induce the target stem cells into cycle. Early results were highly encouraging, with CFC and LTC-IC being successfully transduced using such techniques (Hughes et al. 1989; Hughes et al. 1992). However, high level transduction has not been achieved in human gene marking trials (Brenner 1996; Dilloo et al. 1996) and human cells capable of longterm reconstitution of NOD/SCID mice have until recently proven resistant to retroviral transduction (Larochelle et al. 1996). This is likely due to the requirement for target cells to be in cycle for retroviral integration to occur (Miller et al. 1990; Roe et al. 1993). Since hematopoietic stem cells are largely quiescent (Young et al. 1996) they must be induced into cell cycle for transduction to be successful. Several strategies have been used in attempts to stimulate stem cells into cycle in vitro, while simultaneously retaining their longterm repopulating ability. Nolta et al (1995) did find that if they transduced bone marrow CD34$^+$ cells in the presence of stromal cells, efficient transduction of immune-deficient beige/nude/xid (bnx) mouse repopulating cells could be obtained, but in the absence of stroma, even with the cytokines IL-3, IL-6 and SF present, the transduced cells failed to engraft. Subsequent clonal analysis of the engrafted cell populations illustrated that myeloid and T-lymphoid populations in reconstituted mice had been derived from a common precursor, confirming that pluripotent human hematopoietic stem cells had been transduced (Nolta et al. 1996). The same group has since shown that addition of FL to suspension cultures with IL-3, IL-6 and SF can substitute for stroma in the maintenance of primitive repopulating cells (Dao et al. 1997). The difference between the success described in these studies and the failure described by others may be due to the difference in the strains of recipient mice used (bnx versus NOD/SCID). Recently, two studies have also reported conditions for successful transduction of human CRU as assessed by their ability to repopulate NOD/SCID mice. It had previously been shown that LTC-IC and

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CRU from CD34$^+$CD38^{lo} human cord blood cells could be amplified in short-term cultures containing high concentrations of FL, SF, IL-3, IL-6 and G-CSF (Conneally et al. 1997). When CD34$^+$ cord blood cells were cultured for 3 days in serum-free medium containing these cytokines, followed by two 24-hour incubations in virus-containing medium with the same cytokines, reproducibly high levels of gene transfer to CRU (approximately 30%) were obtained (Conneally et al. 1998). Marandin et al (1998) reported similar results upon transducing CD34$^+$CD38^{lo} human cord blood cells in the presence of stroma and 6 cytokines (IL-3, IL-6, SF, FL, GM-CSF and TPO) for 7 days. Multilineage engraftment with retrovirally transduced cells was achieved, with 12 to 33 % of total CD45$^+$ cells and 10 to 30% of bone marrow-derived CFC containing the transgene, 5 weeks post-injection. Thus careful selection of transduction conditions which both induce stem cells into cycle and maintain the potential of those stem cells for multilineage repopulation combined with the use of cord blood rather than bone marrow as a source of stem cells has lead to successful gene transfer to NOD/SCID repopulating cells. The use of cord blood may in fact be the key to these successes, as it has been observed that hematopoietic stem cells from different stages of ontogeny have different functional properties: in cytokine stimulated cultures, CD34$^+$lin$^-$ fetal liver and cord blood cells have a higher turnover rate, as assessed by ^{3}H-thymidine incorporation, than their counterparts in adult bone marrow, and the number of CD34$^+$ cells produced was much larger in fetal liver or cord blood cultures compared to the bone marrow cultures (Lansdorp et al. 1993). Thus some intrinsic property of cord blood cells which confers them with a higher replicative potential than adult bone marrow cells may be responsible for the ability to achieve gene transfer to CRU from this population, and it may be necessary to achieve an understanding of this phenomenon before gene transfer to adult bone marrow-derived CRU can be successful.

1.4.4   Strategies for the Purification of Transduced Cells

By using a transgene which encodes a selectable marker, cells which have been transduced

can be separated from those which have not. Traditionally, this has been done by using

transgenes which confer drug-resistance to the host cell. Subsequently, exposure of these cells

to the drugs at concentrations which would normally kill them would allow selective survival

and growth of the transduced cells. However, this method has several disadvantages, including

the requirement for the transduced cells to remain under selection for an extended period of

time before the differential survival of the positive cells is assured and can be quantitated

(Conneally et al. 1996). It is also possible for metabolic cooperation between transduced and

nontransduced cells to occur through the presence of the gene product as a soluble factor in the

tissue culture medium (Bayever 1990). Thus a selectable marker for gene transfer which can be

rapidly assessed and which provides a phenotype that cannot be transferred to nontransduced

cells in the same culture dish is required. A variety of such markers have recently been

developed, in which a gene encoding a cell surface molecule is included in the retroviral vector,

allowing transduced cells to be identified and purified using specific antibodies. For example,

the human nerve growth factor receptor (NGFR), which is not normally expressed in

hematopoietic cells, has been used as a reporter to measure the efficiency of gene transfer into

various hematopoietic cell subpopulations by multiparameter flow cytometry, and to follow

transgene expression during differentiation in vitro (i.e. in CFC and LTC-IC assay systems;

Valtieri et al. 1994; Cheng et al. 1998). Furthermore, post-transduction purification of NGFR$^+$

cells by FACS has subsequently led to high levels of transduced erythroid and myeloid

progenitors in 6 week longterm cultures, and in repopulated human bone grafts in SCID mice

(Cheng et al. 1998). Similar experiments have been done using the human multidrug resistance

gene (MDR1) and the CD24 (heat stable antigen; HSA) gene. The former is a transmembrane

protein that functions as an efflux pump for many cytotoxins, including several chemo-

therapeutic agents, and whose expression is consistently low in hematopoietic cells. As with

NGFR, the transfer of this gene has allowed gene transfer efficiencies to be easily assessed by

flow cytometry (Ward et al. 1994), and preselection by FACS of MDR-expressing murine

hematopoietic cells prior to transplantation led to increased engraftment of circulating cells that

contained and expressed MDR (Richardson and Bank 1995). Similar results were obtained in

the murine system using human CD24 as a reporter and purification marker, and in human cells

using the murine CD24 homolog, HSA (Pawliuk et al. 1994; Conneally et al. 1996). CD24 is a

small glycosyl phosphatidylinositol-linked glycoprotein that is expressed on the surface of a

large number of hematopoietic cell types (Kay et al. 1990; Hough et al. 1994). Its function is

not well-understood, although it has been shown to be involved in murine T-cell development,

as well as having a costimulatory role in murine antigen-presenting cells and other cell-

adhesion-mediated events (Kadmon et al. 1992; Liu et al. 1992; Hahne et al. 1994; Sammar et

al. 1994). Antibodies to the mouse and human homologs are not cross-reactive, thus expression

of CD24 from one species in cells of the other allows specific antibody staining of transduced

cells. When murine bone marrow cells were transduced with a retroviral vector carrying the

human CD24 gene, and CD24^{+} cells were purified by FACS, functional analysis showed that

these cells included CFC, CFU-S, and CRU (Pawliuk et al. 1994). When the murine CD24

(HSA) and neo genes in the same vector were transduced into human hematopoietic cells,

transduced cell populations were significantly enriched by FACS for HSA^{+} cells; G418-

resistant erythroid and myeloid CFC were increased from 11 and 12% in the initial CD34^{+} cells

to 100 and 77%, respectively, in the CD34^{+}, HSA^{+} fraction, and G418-resistant LTC-IC were

increased from $\leq$7% to 86% (Conneally et al. 1996). The ability to purify transduced cells would provide a better starting population for subsequent experiments, whether they be clonal analysis studies, assessment of the effects of particular genes on cell behavior, or gene therapy strategies. Chapter 5 describes the results of experiments in which AML cells were transduced with an HSA-expressing vector, and purified using an immunomagnetic purification strategy.

1.4.5 *Potential Gene Therapy Strategies for the Treatment of AML*

The identification of genes which are involved in the transformation of hematopoietic cells to cause AML, as described in section 1.2.3, combined with the availability of retroviral-mediated gene transfer technology, opens up many possible gene therapy strategies for the treatment of AML patients. In the cases where translocation events lead to the production of oncogenic fusion genes, it may be possible to inhibit the effect of these genes by transducing cells with a vector that produces breakpoint-specific antisense sequences. This approach has been put into practice in CML, in which the t(9;22)(q34;q11) causes the juxtaposition of the bcr and abl genes, resulting in the production of the oncogenic BCR/ABL fusion gene product (Rowley 1990). When CML blast cells were exposed to synthetic 18-mer oligodeoxynucleo-tides complementary to the BCR/ABL junction, leukemic colony formation was suppressed by 60 to 90%, whereas colony formation from normal marrow clonogenic cells was unaffected (Szczylik et al. 1991). Another strategy that has been suggested for the gene therapy of leukemia is a "chemoprotection" strategy, in which normal hematopoietic stem cells are transduced with a vector carrying a drug-resistance gene. Transplant of a leukemia patient with these cells would then allow extensive chemotherapy to kill all residual leukemic cells in the patient, while sparing the normal bone marrow. This would have the added advantage that drug treatment would select for growth of the transduced cells in vivo (Sorrentino et al. 1992). To

test this hypothesis, Sorrentino et al (1992) transplanted mice with multidrug resistance (MDR1)-transduced cells, then treated the mice with taxol. A substantial enrichment for transduced cells was observed. Zhao et al (1997) have suggested a strategy which combines BCR/ABL antisense expression with drug resistance. By using a vector with both of these sequences to transduce remission bone marrow prior to autologous transplant, post-transplant chemotherapy could be used to eliminate persistent disease, while transduced normal hematopoietic cells from the transplant would be protected. Any residual leukemic stem cells in the autograft that were also inadvertently transduced and thus drug-resistant would be rendered functionally normal by the presence of the BCR/ABL antisense sequence. This strategy was tested by transducing BCR/ABL-expressing cell lines with a vector carrying the dihydrofolate reductase gene, which confers methotrexate resistance, and antisense sequence directed at the BCR/ABL breakpoint. After selection in methotrexate, BCR/ABL mRNA and protein levels were reduced by 6- to 10-fold, and restoration of the normal phenotype of these cells, i.e. slower growth in the presence of IL-3, apoptosis upon IL-3 withdrawal, and restored expression and function of adhesion receptors, was seen. Also, the tumorigenicity of the transduced cell line in C3H mice was reduced by 3 to 4 logs. 20 to 30% transduction efficiencies of primary $CD34^+$ CML cells were obtained; these cells were rendered methotrexate-resistant, and BCR/ABL mRNA levels were reduced by 10-fold (Zhao et al. 1997). This therefore appears to be a very promising strategy for gene therapy of CML if used as an adjunct to autologous transplant. AML with t(8;21), inv(16), t(15;17) and t(9;11), plus other, rarer translocations whose fusion partners have also been identified, make up a significant proportion of all AML samples, thus the use of a similar strategy could be used to treat a large number of AML patients.

An alternative strategy for AML gene therapy is known as immunotherapy. Cytokines play

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an important role in communication in the immune network. It has therefore been hypothesized that engineering tumor cells to secrete cytokines may enhance antitumor immunity, either by enhancing presentation of tumor-specific antigens or by enhancing the activation of tumor-specific lymphocytes. Injection of cytokine-transduced tumor cells that have been irradiated to prevent their proliferation have been used as "cancer vaccines" in a variety of animal models (Tepper and Mule 1994). For example, Gautam et al (1998) transduced a tumorigenic myeloid progenitor cell line with the TNF$^{\alpha}$ gene. These cells induced antileukemic activity against coinjected non-transduced cells. In a similar study, Hsieh et al (1997) transduced a T leukemia cell line with the GM-CSF gene, whereupon injection into syngeneic mice elicited protective immunity in the animals. Dunussi-Joannopoulos et al (1998) showed a similar phenomenon with GM-CSF-transduced murine AML cells. Another strategy for the creation of tumor vaccines is to transduce tumor cells with costimulatory molecules. These are molecules which are expressed on antigen-presenting cells, and which are required to give a second, costimulatory signal to activate T cells after recognition of an antigenic peptide by the T cell receptor (TCR) has occurred. In the absence of costimulation, TCR engagement induces antigen-specific anergy, and many tumor cells evade immune rejection due to the absence of expression of costimulatory molecules (Gimmi et al. 1993). Two potent costimulatory molecules are B7.1 and B7.2, which both interact with CD28 on the T cell (Greenfield et al. 1998). Thus a strategy for creating a tumor vaccine would involve the transduction of tumor cells with the gene for one of these molecules. This strategy could be particularly useful in AML, as it as been shown that B7.1 is not expressed in AML samples (Hirano et al. 1996). Hirst et al (1997) used a retroviral vector to transfer B7.1 into primary AML cells. After immunoselection to obtain populations of 30 to 60% transduced cells, they were able to

demonstrate the ability of these transduced AML cells to stimulate allogeneic T cells in vitro. Also, murine acute leukemia cells that are genetically modified to express B7.1 can become immunogenic and be used effectively as vaccines (Dunussi-Joannopoulos et al. 1996; Hirano et al. 1997). It has been reported that coexpression of the cytokine IL-12 and the costimulatory molecule B7.1 on antigen-presenting cells leads to a synergistic effect on the activation of T cells in vitro (Kubin et al. 1994; Murphy et al. 1994). Thus it is possible that more effective tumor vaccines may be created in the future by using vectors coexpressing cytokines and costimulatory molecules to transduce tumor cells.

1.5   Rationale and Thesis Objectives

As described in the previous sections, well characterized assays for the routine detection of leukemic stem cells have not been extensively developed. The first main objective of my thesis was to optimize the assay systems commonly used for the detection and characterization of normal stem cells, specifically the LTC system and the NOD/SCID mouse model, for the detection and characterization of analagous cell types in AML samples. Previous efforts to characterize primitive AML progenitors in the LTC system had suggested that they were poorly maintained under standard LTC conditions (Coulombel et al. 1985, Firkin et al. 1990). The availability of improved, genetically engineered feeder layers and recombinant cytokines, information regarding the cytokine-responsiveness of AML cells, and access to probes which could be used to easily discriminate between normal and leukemic cells by fluorescent in situ hybridization (FISH), led to the hypothesis that appropriate conditions for the detection of leukemic LTC-IC could be developed. It would also be necessary to demonstrate that the relationship between input cell number and output colony number in this system is linear,

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allowing limiting dilution analysis of AML cell populations to determine both the frequency and proliferative capactiy of leukemic LTC-IC. Chapter 3 describes in detail the conditions assessed, and the results obtained, and demonstrates that AML-LTC-IC can be routinely detected under appropriate culture conditions, and that this assay can be used to determine the frequency and proliferative capacity of AML-LTC-IC.

The NOD/SCID mouse model has allowed the development of an assay for a high proliferative potential, multilineage repopulating cell among normal hematopoietic cells, which is believed to represent the true pluripotent hematopoietic stem cell. The NOD/SCID mouse has been shown to support the growth of AML cells in some cases, but the growth potential of different AML samples is variable. The availability of a large number of cryopreserved AML samples obtained at diagnosis made a broad survey of their ability to engraft NOD/SCID mice possible, and correlations between engraftment ability and FAB subtype, cytogenetics, or other characteristics of these samples could then be identified. The results of such a survey, and the correlations found, are described in Chapter 4. Once the reproducibility of the model was established, it was possible to perform limiting dilution analysis to determine the frequency of NOD/SL-IC in AML samples, and clonogenic cell assays, LTC-IC assays and FISH provided the tools for assessing the proliferative capacity of individual NOD/SL-IC, as well as to confirm the leukemic nature of these extremely primitive cells. Furthermore, secondary transplant experiments could be performed to evaluate the self-renewal ability of NOD/SL-IC. The results of such assays are also described in Chapter 4.

The second main goal of this thesis was to genetically modify leukemic stem cells by retroviral-mediated gene transfer, and to investigate techniques whereby the proportion of transduced cells within a given population of target cells could be maximized, by purification of transduced cells and/or by increasing gene transfer efficiency. The availability of established

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protocols for the transduction of normal hematopoietic cells provided a starting point for optimization of this procedure. The assays described above for LTC-IC and NOD/SL-IC, combined with immunological and DNA techniques for detection of the transgene in target cells could then be used to determine whether leukemic stem cells had been successfully transduced. Chapter 5 describes the results of experiments designed to assess gene transfer efficiency into NOD/SL-IC, as well as the subsequent clonal analysis of engrafted cell populations that this makes possible. In addition, the use of a retroviral vector carrying a transgene for a cell surface molecule which allowed the purification of transduced cells using an immunomagnetic separation technique was explored. Chapter 5 describes the results of gene transfer to AML-CFC and LTC-IC obtained under a variety of retroviral transduction conditions. In addition, a comparison of transduction efficiencies of AML cells by a vector packaged with two different envelopes are was also performed. Based on the results of these experiments, a strategy for achieving optimal gene transfer to AML stem cells was proposed.

CHAPTER TWO : MATERIALS AND METHODS

2.1   Patient Cells

Peripheral blood cells were obtained from patients with newly diagnosed AML undergoing examination for diagnostic purposes after informed consent and with the approval of the Clinical Research Ethics Board of the University of British Columbia. Normal bone marrow cells were surplus cells obtained from normal (allogeneic) bone marrow transplant harvests. Mononuclear cells were separated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation and cryopreserved in Iscove's modified Dulbecco's medium (IMDM; StemCell Technologies, Vancouver, Canada) with 50% fetal calf serum (FCS) and 10% dimethylsulfoxide. Frozen cells were thawed at $37^{\circ}C$, washed in Alpha MEM (StemCell Technologies) with 5% FCS, and cell viability was determined by trypan blue exclusion. As part of their clinical evaluation, all patient samples had morphological, histochemical, and flow cytometric studies performed on their leukemic blasts with subsequent classification according to French American British (FAB) subtypes (Bennett et al. 1985). Cytogenetic analysis was performed on the initial diagnostic bone marrow samples.

2.2   AML-CFC Assays

To quantitate AML-CFC numbers, either freshly thawed cells, cells harvested from suspension cultures, cells harvested from longterm cultures, or cells from mouse bone marrow were plated at a concentration of 0.2 to 1×10^5 cells/mL in methylcellulose (MeCell) medium (0.92% MeCell, 30% FCS, 2mM L-glutamine, 10^{-4} M β-mercaptoethanol, 1% bovine serum albumin (BSA) in IMDM) with some or all of the following recombinant human cytokines: 3 U/mL erythropoietin (Epo; StemCell Technologies), 10 ng/mL granulocyte-macrophage colony

stimulating factor (GM-CSF; Novartis, Basel, Switzerland), 10 ng/mL interleukin-3 (IL-3; Sandoz), 50 ng/mL steel factor (SF; purified in the Terry Fox Lab from supernatants of COS cells transfected with the cytokine cDNA), and 50 ng/mL Flt-3 ligand (FL; Immunex, Seattle, WA). Cultures were scored for the presence of clusters (5-20 cells) and colonies (>20 cells) 14 days later.

2.3 Longterm Cultures

Human marrow feeders (HMF) were obtained as previously described (Eaves et al. 1991) by subculturing cells from 2- to 6-week-old confluent normal bone marrow adherent layers established in primary LTC. These were trypsinized by washing flasks twice with phosphate buffered saline (PBS; StemCell Technologies), incubating with 0.25% trypsin (StemCell Technologies) at 37°C for 10 minutes, then washing cells from the flask with media containing 5% FCS. The cells were irradiated with 15 Gy of 250 kilovolt (peak) x-rays and then seeded into new collagen-coated tissue culture dishes at 3 x 10^4 cells/cm^2. Sl/Sl feeders consist of Sl/Sl fibroblasts originally obtained from Sl/Sl mouse embryos (Sutherland et al. 1993). Sl/Sl-J-IL-3 fibroblasts were obtained by transduction of Sl/Sl fibroblasts with a human IL-3 cDNA-containing retrovirus as previously described (Hogge et al. 1996). These cells produce bioactive human IL-3 at a concentration of 16 ng/mL as determined by the ability of their growth medium to stimulate ^{3}H-thymidine incorporation into MO7e cells, an IL-3-dependent cell line (Otsuka et al. 1991). Mixed feeders are a 1:1 mixture of M2-10B4 cells, a cloned line of mouse bone marrow origin (Lemoine et al. 1988) engineered to produce human IL-3 and human G-CSF at 4 ng/mL and 190 ng/mL, respectively, and Sl/Sl cells engineered to produce human IL-3 and human SF at 1 ng/mL and 4 ng/mL, respectively (Hogge et al. 1996). Sl/Sl, Sl/Sl-J-IL-3

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and mixed feeders were all irradiated with 80 Gy before plating in collagen-coated tissue culture dishes at 3 X10^4 cells/cm^2 for use in LTC. AML cells were suspended in Myelocult LTC media (StemCell Technologies) with 10^{-6} M solucortef (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) and seeded onto the various feeders at different cell concentrations as required by experimental design. Cultures were maintained at 37°C in 5% CO_2 and received weekly one-half media changes. In LTCs which were supplemented with SF or FL, the weekly half-medium change contained the cytokine of interest at twice the final concentration (50 ng/mL). In addition, a second dose of the cytokine was added midweek between feedings to yield the same final concentration. After 5 weeks both nonadherent and adherent cells were obtained from LTC (the latter by trypsinization) (Coulombel et al. 1983), pooled, and plated in methylcellulose as described above. The number of CFC per million cells initially plated in LTC could then be calculated as follows:

$$\frac{\text{\#CFC/dish MeCell}}{\text{\# cells plated in MeCell}} \quad X \quad \frac{\text{\# cells/LTC at harvest}}{\text{\#cells plated in LTC at time 0}} \quad X\ 10^6\ = \frac{\text{CFC/10}^6\text{ cells}}{\text{plated at time 0}}$$

In this way CFC output could be standardized to allow comparison of different culture conditions.

2.4 Determination of LTC-IC Frequencies

Light density AML cells were plated at four cell concentrations (10^4, 3 x 10^4, 10^5 and 5 x 10^5 cells per well), with 25 to 30 replicate wells per concentration, in 96-well flat-bottomed Nunclon microwell plates (Nunc, Roskilde, Denmark) containing either HMF or mixed feeders and 100 μL LTC media per well. After 5 weeks of culture with weekly half-media changes, all nonadherent and adherent cells from each well were harvested and plated in individual

methylcellulose assays. The frequency of LTC-IC in the starting cell population was calculated from the frequency of negative wells (wells containing no detectable CFC) at each cell concentration, using Poisson statistics and the weighted mean method, as previously described (Sutherland et al. 1990b). Once the frequency of LTC-IC in a given sample was known the average number of CFC produced per LTC-IC could be determined by dividing the number of output CFC by the number of input LTC-IC. The range of CFC produced per individual LTC-IC could also be determined by looking at CFC output numbers from those cell concentrations at which 2/3 of the wells were negative for colony growth. At this concentration, it is >85% likely that positive cultures were initiated by a single LTC-IC, and thus the number of CFC produced is assumed to be the number produced by a single LTC-IC (Coller and Coller 1986).

2.5   Fluorescent In Situ Hybridization (FISH)

2.5.1   Slide Preparation

Individual colonies were plucked from methylcellulose assays using finely drawn glass pipets into hypotonic solution (0.075 M KCl). After 10 minutes, they were transferred to multiwell glass slides (Celline Assoc., New Field, NJ), then fixed in 3:1 methanol:acetic acid. Although DNA probes were selected to recognize karyotypic abnormalities in the leukemic cells in interphase, in some cases colonies were synchronized to obtain metaphases. This was done by adding 2 drops of 3 μM fluorodeoxyuridine, 120 μM uridine in IMDM per dish to block DNA synthesis. The dishes were incubated at 37°C for 16 hours, and then the DNA synthesis block was released by addition of 2 drops per dish of 300 μM thymidine in IMDM. 4 hours later, 4 drops/dish of colcemid were added, and 1 hour later colonies were plucked onto

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multiwell glass slides for FISH, as described above (Fraser et al. 1987).

Bone marrow smears from mice were prepared for FISH by snipping the end off the femur and smearing the cut end on a glass slide. This slide was then fixed in 3:1 methanol:acetic acid for 10 minutes and allowed to air dry. Cytospins of cells were prepared by suspending cells in alpha-MEM with 50% FCS and spinning onto glass slides using a Cytospin 2 centrifuge (Shandon, Pittsburgh, PA). Slides were again fixed in 3:1 methanol:acetic acid for 10 minutes prior to FISH.

2.5.2 Probes

Probes for FISH included several probes specific for centromeric repeat sequences, namely the plasmid pRY3.4 for chromosome Y (gift of Dr. Y.W. Kan, University of California, San Francisco, CA), the plasmid D8Z2 for chromosome 8 (ATCC, Rockville, MD), and the QuintEssential 13-specific probe from Oncor (Gaithersburg, MD). Also used was a yeast artificial chromosome (YAC) clone containing 550 kb of human DNA encompassing the breakpoint on 16p13 which occurs in the inv(16) rearrangement (CEPHy904e02854; Max-Planck Institut, Berlin, Germany), and the MLL(11q23) probe from Oncor, which spans the breakpoint on chromosome 11 in the t(9;11) rearrangement. The pRY3.4 plasmid and the D8Z2 plasmid were labeled by nick translation with either digoxigenin (DIG; Boehringer-Mannheim, Mannheim, Germany), biotin (Life Technologies, Gaithersburg, MD), or spectrum-green (Vysis, Downers Grove, IL). The reaction mixture contained 10 μg of plasmid DNA with 1X nick translation buffer (5 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.1 mM β-mercaptoethanol, 10 μg/mL BSA, 30 μM each of dATP, dGTP, and dCTP), 1X enzyme mix (0.07 U/μL DNA polymerase I (Life Technologies), 0.00075 U/μL DNAse I (Life Technologies), 5 mM Tris, pH 7.5, 0.5 mM magnesium acetate, 0.01 mM phenylmethylsulfonyl fluoride, 0.1 mM β-

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mercaptoethanol, 5% glycerol, 10 μg/mL BSA), 30 μM DIG-dUTP (Boehringer-Mannheim),

biotinylated dUTP (Life Technologies) or spectrum green-dUTP (Vysis), and water to give a

total volume of 100 μL. This reaction mixture was incubated at 16°C for 90 minutes, after

which the DNA was precipitated by the addition 10 μL of 3 M sodium acetate and 250 μL of

ice cold 100% ethanol, and incubation on dry ice for 15 minutes. The DNA was pelleted, dried,

and resuspended in 30 μL of water. 5 μL were run in a 1% agarose gel next to 0.5 μg of $^\lambda$phage

DNA digested with HindIII (Life Technologies), and the concentration of the probe was

estimated by comparison with this known standard.

The human DNA contained in the chromosome 16 YAC was amplified by inter-Alu PCR,

using the following primers:

5'-TCCCAAAGTGCTGGGATTACA-3' and 5'-CTGCACTCCAGCCTGGG-3' (Lengauer et

al. 1992). The PCR reaction contained 200 ng of YAC DNA, 1X PCR buffer (Life

Technologies PCR Buffer containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl), 50 μM of each

deoxynucleoside triphosphate (dNTP; Life Technologies), 3 mM MgCl$_2$, 25 pmol of each

primer, 2.5 units of Taq DNA Polymerase (Life Technologies), and water to give a final

volume of 50 μL. PCR was performed in an Ericomp TwinBlock thermal cycler. 30 cycles of

96°C for 1 minute, 37°C for 30 seconds, and 72°C for 6 minutes were performed. An aliquot (5

μL) of the PCR product was gel electrophoresed to assess the success of the PCR reaction. The

PCR products appear as a smear in the gel, due to the variable distances between the Alu

elements in the human genomic DNA.The PCR product was then labeled with DIG by nick

translation as described above for plasmid probes, using 45 μL of PCR product per reaction.

The QuintEssential chromosome 13 probe and the MLL(11q23) probe, both from Oncor, were

labeled with DIG by the manufacturer. In the case of samples which were analyzed for the –Y

abnormality, a double hybridization was done with biotin-labeled pRY3.4 and DIG-labeled D8Z2, the latter serving as an internal control to ensure, in the absence of a Y signal, that the FISH procedure had been successful.

2.5.3   Slide Pretreatment, Hybridization and Visualization of Fluorescent Signals

Microscope slide preparations of plucked colonies, cytospins, or bone marrow smears were pretreated in 2X SSC (1X SSC = 0.15 M NaCl, 0.015 M NaCitrate, pH 7.0) at 37°C for 30 minutes, denatured in 70% formamide in 4X SSC at 72°C for 2 minutes and then dehydrated for 2 minutes each through a series of 70%, 80%, 90% and 100% ethanol. The chromosomes Y and 8 centromere probes, at a concentration of 2 ng/μL in 78% formamide, 14% wt/vol dextran sulphate, 2.4X SSC and 100 μg/mL salmon sperm DNA, were denatured at 75°C for 5 minutes and then applied to the slides. The inv(16) probe was hybridized at a concentration of 20 ng/μL in 71% formamide, 14% wt/vol dextran sulphate, 3X SSC, and 40 μg/mL human Cot-1 DNA (Life Technologies). The probe mixture was denatured at 75°C for 5 minutes, then incubated at 37°C for 30 minutes to allow annealing of repetitive DNA sequences before application to the slides. The QuintEssential chromosome 13 probe and the MLL(11q23) probe from Oncor were placed at 37°C for 5 minutes and applied to the slides. In all cases, 3 μL were applied to each well of multiwell slides, or 10 μL were applied to plain glass slides. Slides were then coverslipped, sealed with rubber cement, and incubated at 37°C overnight.

In the case of plasmid probes, after removal of coverslips the hybridized slides were washed for 15 minutes each in 50% formamide in 4X SSC, 2 changes of 55% formamide in 4X SSC, 2X SSC, and 2 changes of 0.1X SSC, all at 45°C with agitation. For the inv(16) probe and the Oncor chromosome 13 and MLL probes, slides were washed in 50% formamide in 4X SSC

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at 43°C for 15 minutes, then in 2X SSC at 37°C for 8 minutes. For spectrum green-labeled

probes, the slides were immediately counterstained and viewed. DIG-labeled probes were

detected by further incubation of slides with 8 μg/mL sheep anti-DIG-fluorescein

isothiocyanate (FITC) antibody (Boehringer-Mannheim) in 4X SSC with 1% BSA. 100 μL of

the antibody solution was applied to the slide, and a coverslip-sized piece of parafilm was

applied to spread the antibody evenly over the slide. After incubation at 37°C for 1 hour, the

slides were washed three times at room temperature for 10 minutes, once in 4X SSC, once in

4X SSC, 0.1% Triton-X-100, and once in PN buffer (0.1 M NaH_2PO_4/Na_2HPO_4, pH 8.0, 0.1%

Nonidet-P40). The signal was then amplified by a further 1-hour, 37°C incubation of the slide

with 30 μg/mL rabbit anti-sheep-FITC (Vector Labs, Burlingame, CA) in PNM buffer (PN

buffer with 5% nonfat dry milk powder) followed by four 5-minute washes, three at room

temperature in PN buffer and one in 4X SSC with 0.1% Triton-X-100 at 40°C. Biotin-labeled

probes were detected by incubation of the slide for 45 minutes at 37°C with 25 μg/mL avidin-

Texas Red (TR; Vector Labs) in 4X SSC with 1% BSA followed by 10-minute room

temperature washes in 4X SSC, 4X SSC with 0.1% Triton-X-100, and PN buffer. 10 μg/mL of

biotin-anti-avidin (Vector Labs) in PNM was then added for 1 hour at 37°C followed by three

5-minute room temperature washes in PN buffer. After a second incubation with avidin-TR,

slides were again washed three times in PN buffer and finally in 4X SSC with 0.1% Triton-X-

100 at 40°C. Slides were counterstained either by coverslipping with 0.5 μg/mL propidium

iodide (PI) in antifade (200 mM 1,4-diazabicyclo-[2,2,2]-octaine, 2 mM Tris, pH 8.0, in 90%

glycerol) or by staining with 250 ng/mL 4,6-diamidino-2-phenylindole (DAPI) for 10 minutes

followed by coverslipping with antifade. Slides were viewed on a Zeiss Axioplan fluorescence

microscope equipped with double and triple bandpass filters to allow simultaneous

visualization of FITC and PI or FITC, TR and DAPI signals (Omega Optical Inc., Battleboro, VT).

Individual colonies were scored as either positive or negative for the particular cytogenetic abnormality. In cases where colonies were small, and therefore only a few cells could be detected on the slide, a minimum of 5 cells with a clear signal were required for that colony to be scored. In addition, in all cases the same hybridization signals had to be present in at least 80% of the cells scored from a particular colony to allow it to be classified as normal or abnormal.

2.6   *Transplantation of AML Cells into NOD/SCID Mice*

NOD/SCID mice were bred and maintained under sterile conditions in the British Columbia Cancer Research Center Joint Animal Facility according to protocols approved by the Animal Care Committee of the University of British Columbia. 6-to-8-week-old mice were irradiated with 350 cGy from a ^{137}Cs source 24 hours prior to injection of the required number of patient AML cells via the tail vein. After various periods of time, mice were sacrificed by CO_2 inhalation. Blood cells were harvested by cardiac puncture. Bone marrow was removed from the four long bones of the limbs by flushing with Alpha MEM with 5% FCS. Spleen cells were obtained by mincing and flushing separated cells free with media. These cells were then assessed for levels of human cell engraftment as described in sections 2.7 and 2.9, and in some cases were also used for CFC and LTC-IC assays, FISH and histology. When human cytokines were administered to mice they were diluted in sterile PBS with 0.1% BSA and injected intraperitoneally 3 times per week. Cytokines used were human IL-3 at a dose of 6 μg per mouse per injection, and human SF at a dose of 10 μg per mouse per injection.

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2.7   Flow Cytometry to Determine Mouse Engraftment Levels

Cells from mouse tissue were suspended in 7% ammonium chloride (StemCell Technologies) and placed on ice for 20 minutes to lyse red blood cells, then spun down and resuspended in Hanks balanced salt solution (StemCell Technologies) with 2% FCS (HFN) and 5% human serum for blocking of human Fc receptors. Cells were incubated on ice for 30 minutes with an antimouse IgG Fc receptor monoclonal antibody (2.4G2; SyStemix, Palo Alto, CA) for blocking of nonspecific binding to mouse Fc receptors. Half of each sample was then incubated for 30 minutes on ice with fluoresceinated anti-CD45, a human-specific pan-leukocyte marker (prepared in the Terry Fox Laboratory from ATCC clone #HB10508) to detect human cells, and the other half with fluoresceinated mouse IgG1 (Becton Dickinson Immunocytometry Systems, San Jose, CA) as an isotype control for nonspecific immunofluorescence. Finally, cells were stained with 2 μg/mL PI in HFN, then resuspended in 0.4 mL of HFN per sample. Flow cytometric analysis was performed on a Becton Dickinson FACScan or FACSort flow cytometer. A gate was set to exclude at least 99.9% of cells labeled with the isotype control, and the %CD45$^+$ cells was then determined using the same gate after excluding nonviable cells (see Figure 2.1). In experiments where bone marrow cells from control (uninjected) mice were analyzed with anti-CD45-FITC, the mean $\pm$ SEM %CD45$^+$ cells for 30 such animals was 0.09$\pm$0.01. The highest level of apparently CD45$^+$ cells detected in one of these mice was 0.33%. In some experiments, CD45$^+$ cells were sorted into media and used for CFC and LTC-IC assays, FISH, or histology.

For calculation of the total number of human AML cells present per mouse it was assumed that the number of cells recovered from the marrow of 4 long bones represented approximately

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25% of the entire mouse marrow (Boggs 1984), and that more than 90% of the AML blasts in the animal were present in the marrow. The total number of AML blasts in a mouse at the time of analysis would thus be the %CD45$^+$ cells in mouse marrow multiplied by the number of cells recovered from 4 long bones divided by 0.25.
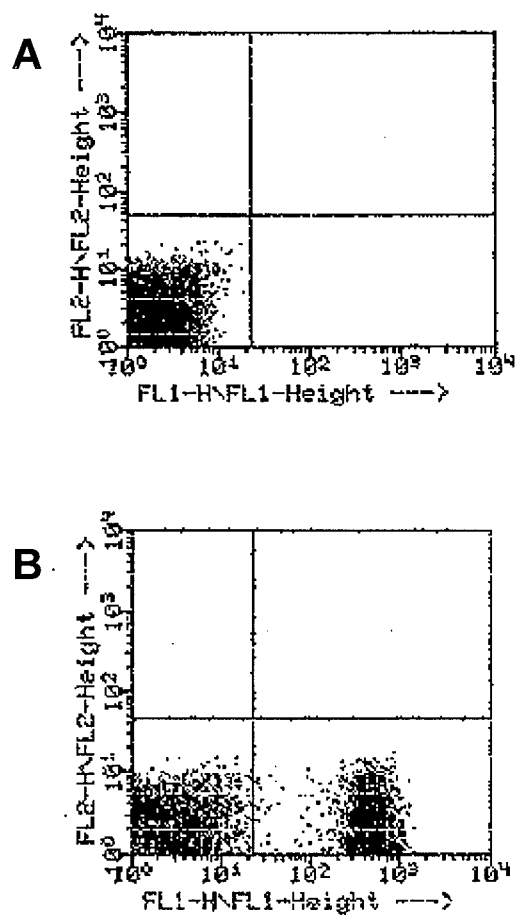
Figure 2.1. CD45 FACS analysis on cells from a NOD/SCID mouse 4 weeks after injection with cells from patient 6. (A) IgG1-FITC-labeled cells from mouse bone marrow. The gate is set to exclude at least 99.9% of cells labeled with this isotype control. (B) CD45-labeled cells from the same mouse. The bone marrow from this mouse contained 49.7% CD45$^+$ cells.

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2.8   Histology

Bone marrow smears or cytospins from mice were prepared as described in section 2.5.1, and fixed in 1:3 methanol:acetone for 20 minutes. Slides were then submerged in 1:1 May-Grunwald stain in water for 15 minutes, then in 1:9 Giemsa stain in water for 12 minutes. After 3 to 4 rapid washes in water, slides were let stand in water for 3 minutes, then allowed to dry upright. Coverslips were mounted with permount.

2.9   DNA Analysis to Determine Mouse Engraftment Levels

In some cases the proportion of human cells in mouse tissue was determined by DNA analysis. Cells from mouse tissues suspended in media were centrifuged and resuspended in 2 mL of DNA lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulphate (SDS), 200 μg/mL proteinase K (Life Technologies)) and incubated at 37°C overnight. Two equal-volume phenol extractions and 2 equal volume chloroform:isoamyl alcohol (24:1) extractions were performed, and then DNA was dialyzed for 48 hours, with 3 changes of dialysis buffer (10 mM Tris, 1 mM EDTA, pH 7.5). DNA concentrations were then determined by measuring the optical density at 260 nm. For Southern blotting, 10 μg of DNA were digested with 100 units of EcoRI (Life Technologies) at 37°C, overnight, and gel electrophoresed in 1% agarose in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The gel was then stained with 1μg/mL ethidium bromide in TAE for 20 minutes, viewed under ultraviolet (UV) light and photographed to show equal loading of the lanes. The gel was then soaked in 0.2 N HCl for 10 minutes for depurination, rinsed briefly in deionized water, then denatured in 1.5 M NaCl, 0.5 N NaOH for 45 minutes and again rinsed briefly in deionized water. Finally, the gel was neutralized in 1 M Tris, pH 7.4, 1.5 M NaCl, once for 30 minutes and a second time for

15 minutes. A piece of ZetaProbe nylon membrane (Bio-Rad, Richmond, CA) was cut to exactly the size of the gel, moistened in deionized water, then immersed in 20X SSC for at least 5 minutes. A reservoir with 20X SSC and a wick consisting of of 2 layers of Whatman 3MM filter paper soaked in 20X SSC, with ends submerged in the SSC in the reservoir were prepared. The gel was placed face down on the wick, then the membrane was placed on the gel. Two layers of Whatman 3MM filter paper trimmed to the size of the gel and soaked in 20X SSC were placed on top of the filter, and then a stack of paper towels, also trimmed to the size of the gel, was added and a weight placed on top. This was left overnight, after which the membrane was rinsed in 4X SSC and UV crosslinked in a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The blot was then placed in a Hybaid jar with prehybridization solution (0.9 M NaCl, 10% formamide, 1% SDS, 0.2 mM EDTA, 2% nonfat dry milk powder, and 0.5 mg/mL denatured salmon sperm DNA) and rotated for 4 to 6 hours at 60°C in a Hybaid Micro-4 oven (InterSciences, Markham, Ontario). Blots were hybridized with a ^{32}P-labeled EcoRI fragment of the human endogenous retroviral sequence (HERV-H) as a human-specific probe. This DNA sequence is known to be present at approximately 1000 copies in the human genome (Mager and Freeman 1987). This probe was labeled using the Random Primers DNA Labelling System (Life Technologies). This involved denaturing 75 ng of probe DNA by boiling for 5 minutes, then adding 2 μL of each of 0.5 mM dATP, dGTP, and dTTP, 15 μL of random primer buffer (0.67 M Hepes, 0.17 M Tris-HCl, 17 mM $MgCl_2$, 33 mM β-mercaptoethanol, 1.33 mg/mL BSA, 18 OD_{260} units/mL oligodeoxyribonucleotide primers (hexamer fraction), pH 6.8), 5 μl (50 μCi) of ^{32}P-dCTP and 3 units of Klenow fragment, and incubating at room temperature for 1-2 hours. 5 μL of stop buffer (0.2M Na_2 EDTA, pH 7.5) were added to the reaction, and TE (10 mM Tris, 1mM EDTA, pH 7.5) to bring the volume up to 200 μL. 5 mL of a Sephadex-G50

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slurry (5% wt/vol Sephadex-G50 (Pharmacia) in TE with 0.01% sodium azide) was placed in a

QuikSep spin column (Isolab Inc., Akron, OH), and centrifuged at 1000 rpm in a Beckman

model TJ-6 centrifuge (Beckman, Palo Alto, CA) for 5 minutes, then 2500 rpm for 5 minutes

to pack the sephadex into the column. To remove unincorporated dNTPs, the 200 μL of labeled

probe solution was added to the top of the column, which was then centrifuged at 2500 rpm for

5 minutes, with a collection tube to collect the flowthrough containing the labeled probe. The

cpm of the probe was then measured using a BioScan/QC2000 (InterSciences) and the entire

probe preparation (range of 4 to 6 X 10^7 cpm) was added to hybridization solution (0.9 M NaCl,

20% formamide, 1% SDS, 0.2 mM EDTA, 2% nonfat dry milk powder, 10% dextran sulphate,

and 0.5 mg/mL salmon sperm DNA), which was then used to replace the prehybridization

solution in the Hybaid jar. The blot was then rotated at 60°C overnight. The hybridization

solution was removed from the jar and the blot was washed by filling the jar approximately

half-full with 2X SSC, 0.5% SDS and rotating at 60°C for 45 minutes, twice. The blot was then

exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY) at -80°C to visualize

hybridization signals. To estimate the proportion of human cells in mouse tissue, a dilution

series of human genomic DNA mixed with mouse DNA, ranging from 0.1% to 100% human

DNA, was digested and run on the same gel as the samples to be evaluated and the intensities

of hybridization signals from the samples compared with that from the known mixtures. This

technique could be used to detect as little as 0.1% human DNA in genomic DNA samples.

2.10 Determination of NOD/SL-IC Frequencies

The frequency of progenitors that initiate AML cell growth in mice (NOD/SL-IC) was

estimated by limiting dilution analysis. Cohorts of up to 8 mice each were injected with 5

different doses of AML cells from the same patient sample (10^5, 3 x 10^5, 10^6, 5 x 10^6, 10^7). Because of concerns that the seeding efficiency of the AML cells would be reduced when very small numbers of cells were injected, 2 x 10^6 irradiated (15 Gy) normal human bone marrow cells were coinjected with leukemic cell doses of 1 x 10^5 and 3 x 10^5. Eight weeks after injection, mouse bone marrow was analyzed for %CD45$^+$ cells, and the frequency of NOD/SL-IC calculated from the frequency of negative mice in each group using Poisson statistics (Coller and Coller 1986). In cases where engraftment levels were <1% by FACS analysis, DNA analysis was also carried out to determine whether small numbers of human cells were present.

Once the frequency of NOD/SL-IC in a given sample was known, the average number of cell progeny per NOD/SL-IC could be calculated. This was done by ascertaining the number of AML blasts, AML-CFC or AML-LTC-IC present in the mouse at week 8 post-injection, and dividing this number by the number of NOD/SL-IC injected into the mouse on day 0 as determined by limiting dilution.

2.11 Serial Transplantation of NOD/SL-IC

A small number of experiments were performed in which primary mice were injected with 1 x 10^7 cells, and 3 to 4 weeks later they were sacrificed and their bone marrow cells divided between 3 secondary recipients. The bone marrow of these secondary recipients was then analyzed for engraftment 8 to 12 weeks later.

2.12 Engraftment Ability of AML Cells After Liquid Suspension Culture

In order to determine the effect of various culture conditions on the ability of AML cells to

engraft NOD/SCID mice, AML cells were thawed and placed in suspension culture in Petrie

dishes in IMDM with 20% FCS, containing various combinations of cytokines at the following

concentrations: IL-3, 20 ng/mL; SF and FL, 100 ng/mL; thrombopoietin (TPO; Genentech, San

Francisco, CA), 50 ng/mL; hyper-IL-6 (HIL-6; a gift from Dr. Stefan Rose-John, Johannes

Gutenberg –Universat, Mainz, Germany), 10 ng/mL. Cells were harvested from these cultures

after 4, 12 and 24 hours of culture and injected into irradiated NOD/SCID mice as described in

section 2.6. After 8 weeks, mouse bone marrow was harvested and assessed for the presence of

human cells by CD45-FACS, as described in section 2.7.

2.13   Vectors for Gene Transfer

2.13.1   MSCV-Neo

The MSCV-Neo vector was kindly provided by Dr. R. Hawley, University of Toronto,

Toronto, Canada, and is illustrated in Figure 2.2. It is an engineered retrovirus derived from the

murine embryonic stem cell virus, with sequences engineered as described in Hawley et al

(1994) for optimized packaging, high viral titer, and minimized chance of replication-

competent retrovirus production. It contains the neomycin phosphotransferase (neo) gene,

conferring resistance in mammalian cells to G418. The neo gene has been placed under the

transcriptional control of the murine phosphoglycerate kinase (pgk) promoter, for constitutive

expression in a wide range of cells types (Hawley et al. 1994).

2.13.2   MSCV-HSA-Neo

The MSCV-HSA-Neo vector contains the 271 bp murine heat-stable antigen (HSA) coding

region inserted upstream of the pgk-neo cassette of the MSCV-Neo virus described above

(Conneally et al. 1996). HSA is a small glycosyl phosphatidyl-inositol-linked glycoprotein that is expressed on the surface of a large number of hematopoietic cell types, and recognized by the monoclonal antibody M1/69 (Kay et al. 1990). Insertion of the HSA gene in this position places it under the transcriptional control of the 5' LTR promoter, as illustrated in Figure 2.2.

2.13.3 MSCV-EGFP-Neo

The MSCV-EGFP-Neo vector contains the humanized red-shifted green fluorescent protein (EGFP; Clontech, Palo Alto, CA) inserted upstream of the pgk-neo cassette of the MSCV-Neo virus such that it is under the transcriptional control of the 5' LTR promoter, as illustrated in Figure 2.2 (Hennemann et al. in press).
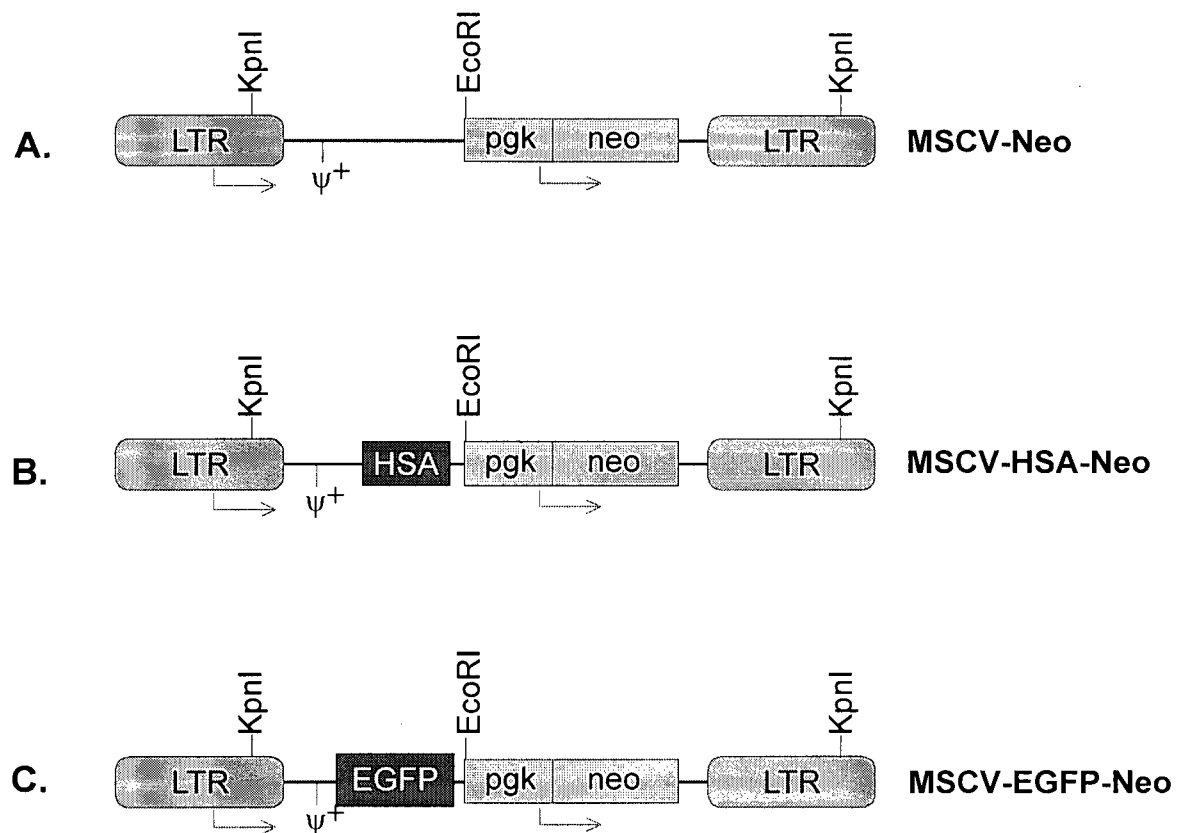
Figure 2.2. Schematic representation of retroviral vectors used.
(A) MSCV-Neo, which contains the neomycin phosphotransferase gene under the transcriptional control of the murine phosphoglycerate kinase promoter (Hawley et al. 1994). (B) MSCV-HSA-Neo, which has the HSA gene inserted upstream of the pgk-neo cassette, such that its transcription is promoted by the 5' LTR (Conneally et al. 1996). (C) MSCV-EGFP-Neo, which has the EGFP gene inserted upstream of the pgk-neo gene, such that its transcription is promoted by the 5 'LTR (Cheng et al. 1997). Arrows indicate transcription initiation sites, and Ψ^+ indicates the packaging signal.

2.14 Retroviral Packaging Cell Lines

2.14.1   GP+envAm12

GP+envAm12 is an NIH 3T3-derived cell line with the viral *gag* and *pol* genes and the amphotropic env gene from the Moloney murine leukemia virus (MMLV) separated on two different expression vectors, and the packaging signals and the 3' LTRs removed. Packaging of viral particles with the amphotropic envelope allows them to infect most mammalian cells, which express the amphotropic env receptor, Ram-1 (Miller et al. 1994; Orlic et al. 1996)

2.14.2   PG13

This cell line, also NIH 3T3-derived, expresses the MMLV *gag* and *pol* proteins and the gibbon ape leukemia virus (GALV) *env*, again on separate expression vectors to reduce the potential for helper virus production (Miller et al. 1991). The different *env* protein results in a packaged viral particle which has a host range including rat, hamster, bovine, cat, dog, monkey, and human cells (Miller et al. 1991). The receptor for the GALV *env* is GLVR-1, and has been reported to be expressed at higher levels in hematopoietic cells than the amphotropic receptor, Ram-1 (Orlic et al. 1996; Kiem et al. 1997).

2.15 Retroviral Producer Cell Lines

To make a cell line which is producing viral particles carrying the vector of choice, the retroviral packaging cell lines described above must be transfected with the vector DNA.

2.15.1   Am12-MSCV-Neo

GP+envAm12 cells transfected with the MSCV-Neo vector yielded the producer cell line

Am12-MSCV-Neo (Conneally et al. 1996). This producer line was cultured in Dulbecco's

modified Eagle's medium (DMEM; StemCell Technologies) with 10% heat-inactivated

newborn calf serum (NBCS; Bio-Whittaker, Walkersville, MD), 25 $^\mu$g/mL mycophenolic acid

(Gibco-BRL, Burlington, Ontario, Canada), 250 $^\mu$g/mL xanthine (Sigma Chemicals, St. Louis,

MO), and 15 $^\mu$g/mL hypoxanthine (Sigma). This media is referred to as HXM. In addition, 200

$^\mu$g/mL hygromycin B (Calbiochem, La Jolla, CA) and 1 mg/mL G418 (Life Technologies)

were added for selection of the helper sequences and the retroviral vector sequences,

respectively.

2.15.2 *Am12-MSCV-HSA-Neo and PG13-MSCV-HSA-Neo*

The GP+envAm12 cell line was also transfected with the MSCV-HSA-Neo vector to yield

a cell line producing packaged particles containing this vector. Initially, such a producer line

previously created in the Terry Fox Laboratory (Conneally et al. 1996) was used for these

studies, but consistently low titers led me to remake this producer line myself. This was done

by first transiently transfecting BOSC 23 cells with the vector DNA. BOSC 23 cells are 293T

cells, a human embryonic kidney cell line, into which constructs that express retroviral

packaging functions have been stably introduced. The result is an ecotropic virus packaging cell

line which produces high titers of infectious retrovirus within 72 hours after $CaPO_4$-mediated

transfection (Pear 1993). The media on the BOSC 23 cells, which were maintained in DMEM

with 10% FCS in 60 mm diameter tissue culture dishes, was changed to 4 mL of DMEM with

10% FCS and 25 $^\mu$M chloroquine. 7.5 $^\mu$g of vector DNA (MSCV-HSA-Neo cloned into the

plasmid pUC) was mixed with 2 M $CaCl_2$, to give a final concentration of 0.25 M $CaCl_2$ in a

volume of 0.5 mL. 0.5 mL of 2X Hepes buffered saline (50 mM Hepes, pH 7.05, 10 mM KCl,

12 mM dextrose, 280 mM NaCl, 1.5 mM Na_2HPO_4) was added dropwise, while simultaneously

bubbling air through the solution. This solution was then immediately added to the dish of BOSC 23 cells. After 8 hours of incubation at 37°C, the media was changed to DMEM with 10% FCS. After 48 hours, with one media change at 24 hours, the supernatant from the BOSC 23 cells was harvested and spun at 1500 rpm for 5 minutes to remove any cells. Polybrene was added to give a concentration of 4 μg/mL and 1 mL of the supernatant was added to each of 3 60 mm diameter tissue culture dishes containing subconfluent GP+envAm12 cells. Leftover BOSC 23 supernatant was frozen at -80°C. Dishes were then incubated for 4 hours at 37°C, and then 3 mL of DMEM with 10% NBCS were added. 48 hours later, frozen BOSC 23 supernatant was thawed and the transfection procedure was repeated, and another 48 hours later it was repeated again. Cells were split in the interim when required. 48 hours following the final transfection, G418 was added to the media at a concentration of 1 mg/mL, and cells were maintained in this media for two weeks, resulting in a polyclonal population of G418-resistant Am12-MSCV-HSA-Neo producer cells. Once established, this cell line was maintained in DMEM with 10% NBCS, with occasional selection in HXM with G418 and hygromycin B.

A PG13-MSCV-HSA-Neo producer cell line was made in the same way as the Am12-MSCV-HSA-Neo cell line, using PG13 cells as the target for BOSC 23 supernatant transfection. Titers of the polyclonal population were low, so clones were isolated, and the clone with the highest titer was selected for use in the experiments described here (see section 2.16 and Table 2.1 for titers). This cell line was maintained in DMEM with 10% NBCS, with occasional G418 selection.

2.15.3   Am12-MSCV-EGFP-Neo and PG13-MSCV-EGFP-Neo

The GP+envAm12 and the PG13 cell lines were also transduced with the MSCV-EGFP-Neo vector. This was done in the Terry Fox Laboratory, as described by Hennemann et al (in

press). Briefly, BOSC 23 cells were $CaPO_4$-transfected with the vector DNA, and the supernatant was then used to repeatedly infect the Am12 and PG13 cell lines. Single clones were isolated, and the highest-titer clones were selected. These cell lines were maintained in DMEM with 10% NBCS, with occasional G418 selection.

2.16 Titer Determinations

2.16.1 Retroviral Titer Assay by End-Point Dilution

NIH 3T3 cells (for titering Am12-packaged viruses) or Rat1 cells (Van Roy et al. 1986; for titering Am12- or PG13-packaged viruses, since GALV-pseudotyped vectors cannot transduce the murine NIH 3T3 cells) were plated in 60 mm tissue culture dishes at 1×10^5 cells per dish in DMEM with 10% FCS. At the same time, the media on the viral producer cell line was changed to IMDM with 20% FCS. 24 hours later, the media from the producer cells was harvested and a series of dilutions set up as follows: 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}, in DMEM with 10% FCS. 1 mL of each dilution was plated in duplicate in the dishes containing the NIH 3T3 or Rat1 cells, which were then incubated at 37°C for 4 hours before adding a further 4 mL of DMEM with 10% FCS. 24 hours later the media was changed to DMEM with 10% FCS and 1 mg/mL G418. The cells were then incubated at 37°C for 12 days, with a media change every 3 to 4 days, always including 1 mg/mL G418. Media was then removed from the dishes and cells were fixed with 2 mL methanol per dish for 15 minutes at room temperature. Methanol was removed and dishes were allowed to air dry before addition of 3 mL of 5% Giemsa in water. Dishes were left at room temperature for 1 hour, then rinsed with water and allowed to air dry. Colonies were counted and the titer was calculated as follows: pfu/mL = # colonies/dish X dilution factor.

2.16.2   Retroviral Titer Assay by Flow Cytometry

This titer method could only be used for the vectors expressing either HSA or EGFP, as both of these transgenes are detectable by flow cytometry. Rat1 cells were plated in 35 mm diameter tissue culture dishes at 2×10^5 cells per dish. The media on producer cells to be titered was changed to IMDM with 20% FCS. 24 hours later, the supernatant from the producer cells was added to the dishes of Rat1 cells from which the media had been removed. 1 mL of each of 3 different concentrations of supernatant were plated: undiluted, 1:10 and 1:100, each in duplicate. 2 dishes were also plated with just media, as negative controls. 2 spare dishes of Rat1 cells were trypsinized and the "time 0 number of cells per dish" was determined. After incubation for 4 hours at 37°C, 1 mL of media (IMDM with 10% FCS) was added and cells were incubated for a further 48 hours. Media was then removed and the Rat1 cells were harvested by trypsinization and stained for HSA if the vector used was MSCV-HSA-Neo. This was done by spinning cells down and resuspending in HFN, dividing the sample in half, and to one half adding IgG1-FITC as a negative control, and to the other half adding M1/69, a monoclonal antibody specific for murine HSA (Kay et al. 1990). After 30 minutes on ice, cells were spun down, stained with 2 μg/mL propidium iodide in HFN, spun down again and resuspended in HFN. Flow cytometric analysis was performed on a Becton Dickinson FACScan or FACSort flow cytometer. A gate was set to exclude at least 99.9% of cells labeled with IgG1, and the %HSA$^+$ cells was then determined using the same gate after excluding nonviable cells. The titer could then be determined as follows:

$$\text{pfu/mL} = \text{time 0 \# cells/dish} \times \frac{\text{\%HSA}^+}{100} \times \text{dilution factor}$$

In the case of the cell lines producing the MSCV-EGFP-Neo vector, the same protocol was followed, except that the cells did not need to be stained with an antibody following

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trypsinization. Cells were simply harvested, washed, stained with PI and resuspended in HFN

prior to flow cytometric analysis. A gate was set to exclude at least 99.9% of the cells from the

dishes which received only media (no supernatant), and the % EGFP$^+$ cells was then determined

using the same gate after excluding nonviable cells. The calculation of titer was the same as

above, substituting %HSA$^+$ cells with %EGFP$^+$ cells. An example of a titer determination for

PG13-MSCV-EGFP-Neo is shown in Figure 2.3. The titers determined using both methods

described above for the various producer cell lines used in these studies are shown in Table 2.1.

Table 2.1. Mean Titers of Retroviral Producer Cell Lines, Determined by the Endpoint Method or the Flow Cytometry Method

Producer Line	Pfu/mL Supernatant	
	Endpoint Titer (n*)	Titer by Flow Cytometry(n*)
Am12-MSCV-Neo	1.0×10^6 (1)	ND
Am12-MSCV-HSA-Neo	9.9×10^5 (6)	1×10^5 (1)
Am12-MSCV-EGFP-Neo	1.7×10^6 (2)	5.4×10^5 (3)
PG13- MSCV-HSA-Neo	1.6×10^4 (1)	2.0×10^5 (2)
PG13- MSCV-EGFP-Neo	3.4×10^5 (2)	2.1×10^5 (4)

*n=number of titers performed
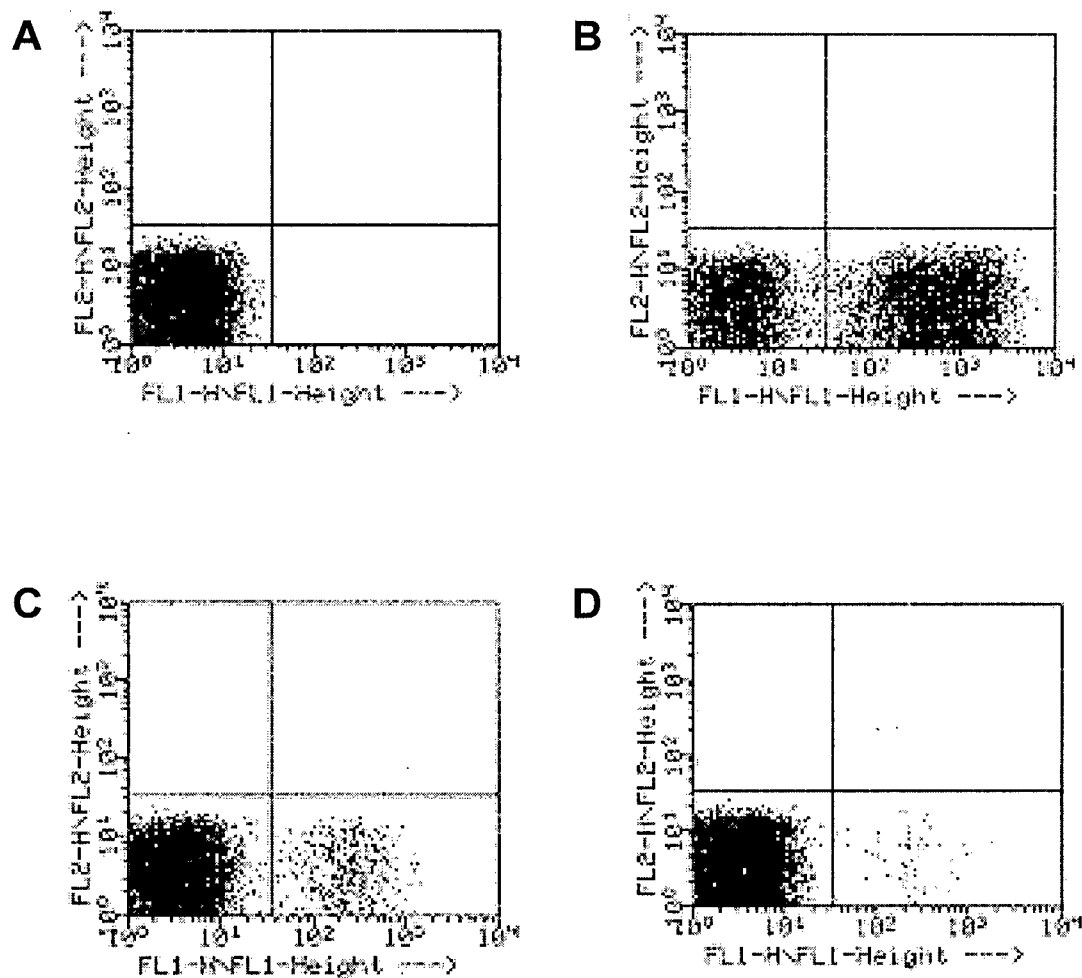
Figure 2.3. Titer determination of producer line PG13-MSCV-EGFP-Neo by flow cytometry. Rat1 cells were transduced with 1 mL of viral supernatant at 3 different concentrations: undiluted, 1:10 and 1:100, then analyzed by flow cytometry for %EGFP$^+$ cells. (A) Mock transfected cells, 0% EGFP$^+$. (B) Undiluted supernatant, 59% EGFP$^+$. (C) 1:10 supernatant, 6% EGFP$^+$. (D) 1:100 supernatant, 0.8% EGFP$^+$. Time 0 cell number was 8.6X0^5, mean titer was 5.7X10^5.

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2.17 Gene Transfer by Cocultivation

AML patient cells to be transduced with MSCV-Neo were first stimulated to proliferate by incubation in IMDM supplemented with 20% FCS, 10 ng/mL IL-3, and 50 ng/mL SF for 48 hours at a concentration of 10^6 cells/mL, with a media change after 24 hours. The cells were then harvested, washed, and plated directly into 150 mm diameter tissue culture dishes containing subconfluent irradiated (1500 cGy) producer cells, at a density of $5X10^5$/mL in medium with the same combination of serum and cytokines as above plus 4 μg/mL polybrene (Sigma). Media was changed at 24 hours, and at 48 hours the AML cells were removed from the adherent producers by gentle agitation and washing of the dishes. Finally, the cells were resuspended at a density of $5X10^5$/mL in fresh medium and incubated for a further 24 hours. Cells were then harvested, washed, and resuspended in Alpha MEM with 5% FCS at an appropriate concentration for injection into mice. An aliquot of cells was set aside for DNA analysis.

For experiments in which AML cells were transduced with MSCV-HSA-Neo by coculture, the prestimulation step was removed, and cells were cocultured with producers for various periods of time, as described in Chapter 5. The 24 hour post-stimulation step was also removed.

2.18 Gene Transfer with Viral Supernatant

Viral supernatants containing MSCV-HSA-Neo were made by changing media in 80-90% confluent dishes of viral producers to IMDM with 20% FCS. Medium was then harvested 24 hours later and filtered through a 0.22 μm syringe filter and used immediately. AML cells were thawed and resuspended at approximately $5X10^5$ cells/mL in viral supernatant with IL-3, SF, FL and protamine sulphate at final concentrations of 20 ng/mL, 100 ng/mL, 100 ng/mL and 5

$^{\mu}$g/mL, respectively. The cells were then plated in Petrie dishes which had been coated with 4 $^{\mu}$g/cm^2 of fibronectin (Sigma). The cells were removed from the dishes after 7-8 hours, spun down and resuspended in fresh viral supernatant with cytokines and protamine sulphate. They were harvested after a total of 24 hours of incubation and some cells were removed for DNA analysis. The rest were used for CFC and LTC-IC assays and, in the case of MSCV-HSA-Neo-transduced cells, immunomagnetic purification (described in section 2.20).

Viral supernatants containing MSCV-EGFP-Neo were made in the same way. Cells transduced with this vector were first prestimulated for 48 hours in IMDM with 20% FCS, plus IL-3, SF, and FL as above. They were then transduced with 2 changes of viral supernatant, supplemented with the same cytokines and protamine sulphate, for 24 hours each, prior to harvest and assessment of gene transfer.

2.19 Separation of AML Cells into CD34$^+$CD38$^-$, CD34$^+$CD38$^+$ and CD34$^-$ Fractions

AML cells were thawed, washed in HFN, and viable cell counts were done. Cells were suspended in HFN to give a final concentration of 5×10^7 cells/mL, and divided into 100 $^{\mu}$L aliquots. To each aliquot 4 $^{\mu}$L of anti-CD34-FITC (8G12; a gift from Dr. Peter Lansdorp, Terry Fox Laboratory) and 2 $^{\mu}$L of anti-CD38-phycoerythrin (Becton-Dickinson) were added, and they were incubated on ice for 30 minutes. Each aliquot was then washed with HFN, then PI in HFN to exclude nonviable cells, and finally, resuspended in 1 mL of HFN. CD34$^+$CD38$^-$, CD34$^+$CD38$^+$ and CD34$^-$ fractions were sorted using a FACStar$^+$ (Becton Dickinson) equipped with a 5 W argon laser and a 30 mW helium laser.

2.20 Evaluation of Gene Transfer by DNA Analysis

2.20.1 Southern Blotting to Determine Transduction Efficiency and % Engrafted Tissues Marked

Genomic DNA was extracted from cells directly following transduction, and from the tissues of mice at various time points following injection of transduced cells, as described in section 2.9. To determine the proportion of retrovirally marked cells in these samples, 10 μg of the genomic DNA was digested with 100 units of KpnI, which excises the entire sequence between the two LTRs of the inserted vector (see Figure 2.2). A dilution series containing DNA from the human MO7e cell line (Avanzi et al, 1988) retrovirally transduced with a vector containing the human IL-3 and neo genes (Mo7e-J-IL-3) and containing a single copy of the proviral DNA diluted into mouse DNA was digested in parallel, and samples were run in a 1% agarose gel, Southern blotted and probed with a ^{32}P-labelled neo-specific sequence as described in section 2.9. By comparison with the dilution series of MO7e-J-IL-3 cells in mouse DNA, the % retrovirally marked cells in the sample could then be estimated. The limit of sensitivity with this method was approximately 5% neo+ DNA in untransduced DNA.

2.20.2 Southern Blotting to Assess Clonality

If a DNA sample from a mouse tissue showed a high level of engraftment with retrovirally transduced cells, the next step was to determine the clonality of the engrafted cell population. To do this, the genomic DNA was digested with EcoRI, Southern blotted, and probed with the neo probe as described above. There is a single EcoRI site within the retroviral sequence (see Figure 2.2), therefore the size of the fragment detected with the neo probe will be unique to each individual retroviral integration site, allowing different clones arising from individual

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retrovirally transduced cells to be distinguished.

2.20.3   PCR to Determine Transduction Efficiency and % Engrafted Tissues Marked

In cases where transduction efficiencies or mouse engraftment levels were too low to allow detection of the neo gene by Southern blotting, PCR was carried out. Neo-specific primers were synthesized in our center using a PCR-Mate 391 DNA synthesizer (Applied Biosystems, Mississauga, Ontario, Canada). The sequences of these were as follows: 5'-CAAGATGGATTGCACGCAGG-3' and 5'-TTGAGCCTGGCGAACAGTTTC-3', with a predicted PCR product size of 514 base pairs. 200 ng of genomic DNA was used in a 50^{μ}l reaction mixture containing 1X PCR buffer, 0.5mM of each dNTP, 3mM magnesium chloride, 25 pM of each primer, and 2.5 units of Taq DNA polymerase. Before test DNA was added, the reaction mixture was treated with HhaI (10 U for 1 hour at 37°C; Life Technologies), which cleaves the neo sequence, to minimize the possibility of contamination by exogenous neo sequences (Hughes et al, 1989). The HhaI was inactivated by incubation at 75°C for 10 minutes prior to addition of test DNA and PCR. Samples were denatured at 95°C for 5 minutes, then amplified with a programmable heating block (Ericomp, San Diego, CA) set for a cycle of 95°C for 30 seconds, 56°C for 1 minute and 72°C for 1 minute. In order to make the technique semiquantitative, an experiment was performed using MO7e-J-IL-3 genomic DNA, which is known to carry the target neo sequence. 6 identical reactions were set up as described above, and were removed one at a time after 18, 20, 22, 24,26 and 28 cycles of PCR. These samples were gel electrophoresed in a 1% agarose gel. The gel was denatured in 1.5M NaCl, 0.5N NaOH for 30 minutes and neutralized in 1M Tris, pH 7.4, 1.5M NaCl for 30 minutes. The gel was then placed face-down on a piece of plastic wrap on a flat surface. A piece of ZetaProbe nylon membrane was wet in deionized water, soaked in 20X SSC for 5 minutes, then placed on

top of the gel. 2 layers of 3MM Whatman paper soaked in 20X SSC were placed on top of the membrane, and then a thick layer of paper towels, all trimmed to the size of the gel. The edges of the plastic wrap were then folded up to enclose the whole stack, a weight was placed on top and the DNA was allowed to transfer overnight. The blot was then rinsed in 4X SSC and UV crosslinked. The prehybridization was carried out in 10 mL 6X SSC, 1X Denhardt's solution (0.02% wt/vol ficoll, 0.02% wt/vol polyvinylpyrollidone, 0.02% wt/vol BSA), and 1% SDS for 2 to 4 hours at 65°C. The neo probe was labeled with ^{32}P as described previously for the HERV-H probe, and 10^{7} cpm were added to the prehybridization solution in the jar. The probe was allowed to hybridize overnight at 65°C. The blot was then washed with 2 to 3 changes of 3X SSC, 1% SDS at 65°C for 30 minutes each, and exposed to film. Densitometry was performed on the resulting autoradiograph and the values obtained were plotted as shown in Figure 2.4. Thereafter, all PCR reactions performed on test samples were done in parallel with a dilution series of MO7e-J-IL-3 DNA and removed after 21 cycles, as this was demonstrated to be well within the linear phase of the amplification. A final 5 minutes at 72°C was added to the end of the cycles. PCR products were gel electrophoresed, Southern blotted and probed with ^{32}P-labelled neo probe as described above. The % neo+ DNA in a given sample could then be estimated by comparison with the dilution series that was run in parallel.
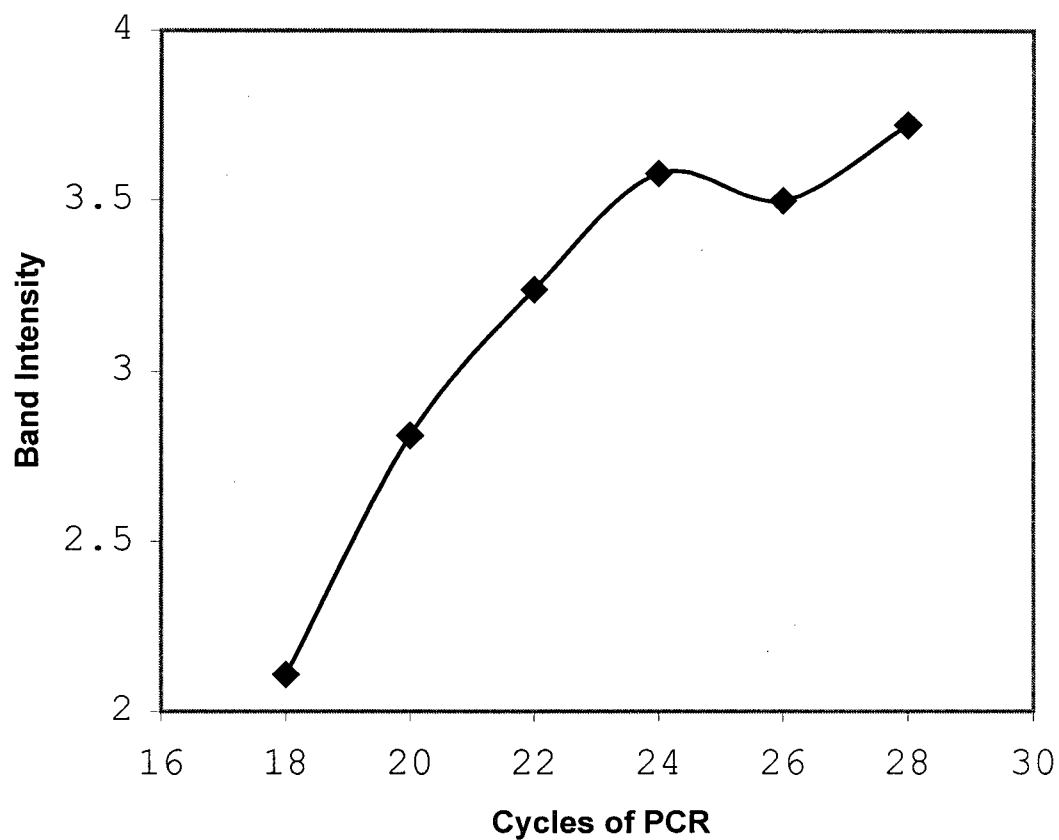
Figure 2.4. PCR cycle series with neo primers to determine linear phase of reaction. PCR
for neo was carried out on DNA from MO7e-J-IL-3 cells for a variety of cycle numbers to
determine the linear phase of the reaction. 21 cycles was determined to be well within the linear
phase and was used for all subsequent semiquantitative PCR reactions, in parallel with a
dilution series of MO7e-J-IL-3 DNA diluted into nontransduced mouse DNA.

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2.21 Magnetic Purification of Cells Transduced with Murine HSA

After transduction with the MSCV-HSA-Neo vector, transduced AML cells could be separated based on their expression of the HSA gene. Cells were harvested and some cells were removed to set up the appropriate assays on the "precolumn" fraction (DNA analysis, CFC and LTC-IC assays). The remainder were spun down and resuspended in HFN with 5% human serum to give a final concentration of 1 to 5 X 10^7 per mL. 2 μL of biotinylated-M1/69 (StemCell Technologies) were added per mL of cells, and they were incubated on ice for 30 minutes. The cells were spun down, resuspended at the same concentration in HFN with 5% human serum, and 100 μL of bi-specific anti-biotin/anti-dextran tetrameric complex (StemCell Technologies) were added per mL. This complex recognizes both dextran and biotin (Lansdorp and Thomas 1990). After 30 minutes on ice, 60 μL of magnetic colloid (dextran-iron;StemCell Technologies) were added per mL of cells, which were then incubated for a further 30 minutes on ice. The end result is the labelling of cells which are expressing HSA with dextran-iron, as illustrated in Figure 2.5. Meanwhile, a 0.3- or 0.5-inch StemSep™ magnetic column (for <8x10^7 and >8x10^7 cells, respectively) was set up as illustrated in Figure 2.6. PBS was pumped through in reverse at 0.3 mL/minute to fill the column, then the column was washed 3 times with HFN at 1 mL/minute. The labeled cells were added and pumped into the column, which was then washed 3 times with HFN, also at 1 mL/minute. The flowthrough fraction from these steps was collected. The column was then removed from the magnet, and cells were flushed from the column with a syringe containing 5 mL of IMDM with 5% FCS. This is the HSA$^+$-enriched fraction. The precolumn, HSA$^+$ and flowthrough fractions were all subjected to various assays, including DNA analysis for neo to determine gene transfer efficiency, CFC and LTC-IC assays, and flow cytometry to determine the %HSA$^+$ cells.
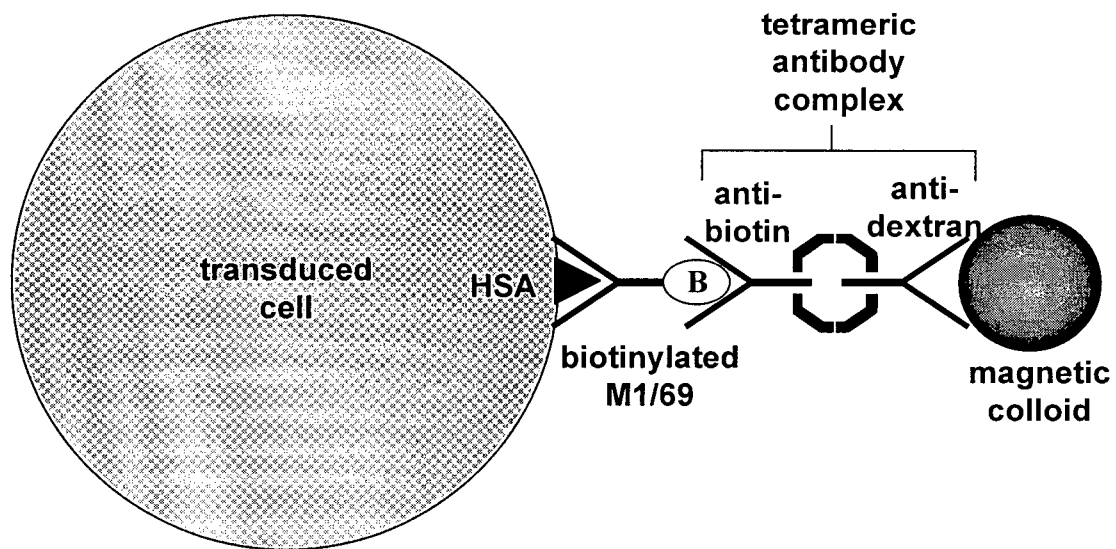
Figure 2.5. Immunomagnetic labeling of cells transduced with murine HSA. Cells expressing HSA are labeled with M1/69, an HSA-specific monoclonal antibody, which has been biotinylated. Colloidal magnetic dextran-iron particles are then bound to the labeled cells using a bispecific antibody complex which recognizes both dextran and biotin (From StemSep™ Operating Instructions, StemCell Technologies, Inc., June 1998).

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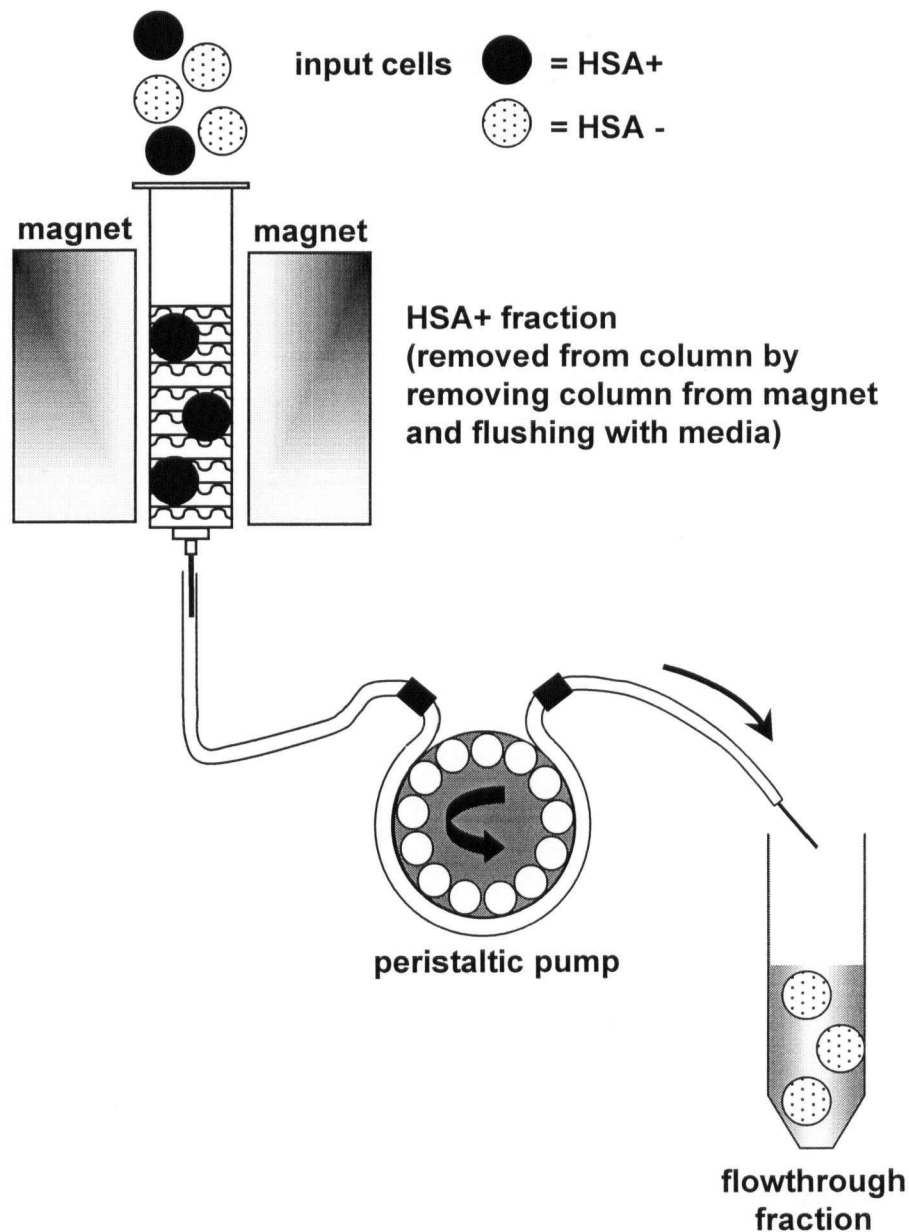
Figure 2.6. Separation of immunomagnetically labeled cells. Labeled cells are pumped into a column of iron mesh placed within a magnetic field. The labeled cells will remain in the column and the unlabeled cells will pass through. The cells in the column can subsequently be removed by removing the column from the magnetic field and flushing with a syringe of media (From StemSep™ Operating Instructions, StemCell Technologies, Inc., June 1998).

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2.22 Flow Cytometery to Determine Transduction Efficiency

Flow cytometry could be used to determine the proportion of transduced cells in a given cell population, both for the HSA and the GFP-containing vectors. In the case of HSA-transduced cells, the precolumn, HSA+ and flowthrough fractions were spun down and resuspended in 0.4 mL of HFN with 5% human serum. Each fraction was then divided into 4 100 μL aliquots, and FITC-labeled antibodies were added to separate aliquots as follows:

M1/69:	100 μL of 1:400 (final dilution 1:800)
IgG1:	4 μL (final dilution of 1:25)
anti-CD45:	4 μL(final dilution of 1:25)
sheep-anti-mouse IgG (SAM):	1 μL(final dilution of 1:100)

M1/69 could be used to detect HSA expression on the surface of cells immediately following transduction (the precolumn fraction). CD45 was used as a positive control and IgG1 was the negative control. After purification, the cells were already labeled with biotinylated M1/69, therefore FITC-labeled M1/69 could not be used to determine the % transduced cells in the HSA$^+$ fraction. Instead, FITC-labeled SAM IgG was used to detect those cells which had already been labeled with M1/69-biotin. The precolumn fraction acted a negative control for this.

Cells were incubated with antibodies on ice for 30 minutes, then stained with propidium iodide and analyzed on a Becton Dickinson FACScan or FACSort flow cytometer. A gate was set to exclude at least 99.9% of precolumn cells, labelled with IgG1or SAM. The % M1/69-positive cells in the precolumn fraction, or % SAM-positive cells in the HSA$^+$ and flowthrough fractions, was then determined using the same gate after excluding nonviable cells.

2.23 PCR on Colonies to Determine Transduction Efficiency to CFC

Colonies which grew from transduced cells, or from LTCs of transduced cells, were plucked into PCR lysis buffer (0.32M sucrose, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100) with 60 ng/μL of proteinase K (Life Technologies). Each colony was plucked into an individual 0.5 mL microtube containing 15 μL of PCR lysis buffer. A different aerosol-resistant tip was used for each colony. The colonies were then incubated at 55°C for 1 hour prior to inactivation of the proteinase K by placing at 95°C for 5 minutes. An individual PCR reaction was then set up for each colony using 1X PCR buffer, 0.5mM of each dNTP, 3mM magnesium chloride, 25 pM of each primer, and 2.5 units of Taq DNA polymerase. The reaction mixture was treated with Hha I, as described in section 2.19.3, to minimize the possibility of contamination by exogenous neo sequences, before adding 5 μL of the lysed colony solution. For CFC that were plated directly following transduction, two primer sets were used, one set for neo and one set for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The latter primer set was a positive control to show that, in cases where there was no neo amplification, there was in fact human DNA present in the PCR reaction. This was necessary due to the frequently small size of the colonies being plucked, making it possible that there could be no DNA present in some of the reactions. The neo primer sequences were shown in section 2.19.3. The GAPDH primers were as follows:

5'-CGGGTTATGCTGGTTTAGGC-3' and 5'-CCCAGCCTTCTCCATGGTG-3'

These primers yielded a PCR product 300 bp in size, which could be easily distinguished from the 514 bp PCR product produced by the neo primers.

When colonies were derived from LTCs of transduced cells, the neo primers could not be used, because the feeders used for longterm cultures contained the neo gene, so there would be

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a possibility of contamination of plucked colony cells with feeder cells, yielding false positives. Therefore an HSA-specific primer set was made, as follows:

5'-TCCTACCCACGCAGATTT-3' and 5'-GAGAGAGCCAGGAGACCA-3'

PCR was then carried out as above, using the HSA primer set and the GAPDH primer set in the same reaction. The HSA primer set yielded a PCR product that was 148 bp in size, and thus could be easily distinguished from the 300 bp GAPDH PCR product. PCR conditions were the same for reactions containing neo and GAPDH primers or HSA and GAPDH primers. Samples were denatured at 95°C for 5 minutes, then amplified with 35 cycles of 95°C for 30 seconds, 54°C for 1 minute and 72°C for 1 minute. PCR products were then run in a 1.2% agarose gel, Southern blotted, and probed with the two corresponding sequences, either neo and GAPDH or HSA and GAPDH. This blotting and hybridization protocol was the same as that described in section 2.19.3. Colonies were assessed as positive if both a neo or HSA band and a GAPDH band were present, and negative if only a GAPDH band was present. If no GAPDH band was present, the colony was not included in the data.

CHAPTER 3 : DETECTION, CHARACTERIZATION AND QUANTITATION
OF AML-LTC-IC

The data presented in this chapter composed the following manuscript:

Ailles L.E., Gerhard B., and Hogge D.E. 1997. Detection and characterization of primitive malignant and normal progenitors in patients with acute myelogenous leukemia using long-term coculture with supportive feeder layers and cytokines. Blood 90:2555-2564.

3.1 Introduction

As described in Chapter 1, normal hematopoietic cells that initiate longterm hematopoiesis when placed in coculture with supportive fibroblast feeder layers are rare progenitors that display many of the characteristics expected of marrow repopulating stem cells. Such longterm culture initiating cells (LTC-IC) are detected and defined by their ability to give rise to committed clonogenic colony forming cells (CFC) after 5 or more weeks in LTC. Previous efforts to characterize primitive AML progenitors in the LTC system had suggested that they were poorly maintained under standard conditions (Coulombel et al. 1985, Firkin et al. 1990). However, these experiments were done at a time when LTCs were established by plating unprocessed bone marrow aspirates and allowing them to form their own adherent layers (Coulombel et al. 1983). The more recent use of preformed feeder layers derived from normal human bone marrow (human marrow feeders, or HMFs; Sutherland et al. 1989), or murine stromal cell lines which are genetically engineered to produced human cytokines, has allowed the standardization and manipulation of LTC conditions for normal cells (reviewed in Chapter 1).

It has been demonstrated that many hematopoietic cytokines stimulate both unfractionated AML blasts and AML-CFC (reviewed in Chapter 1).The use of recombinant cytokines in LTC of AML cells might therefore facilitate improved detection and maintenance of leukemic LTC-IC. To more systematically determine which growth factors or combinations had the greatest activity on AML cells directly isolated from patients, a variety of cytokines were tested in our laboratory, both in short-term proliferation studies and in colony assays. The results of these studies are shown in Appendix A. Nine different cytokines were tested alone and in combination for their ability to stimulate ^{3}H-thymidine incorporation into AML blasts after 3

days in media (IMDM) plus 5% FCS (Table A-1). There was great heterogeneity, both in the baseline proliferative activity in the absence of growth factors and in the extent to which this proliferation could be enhanced (up to 253-fold). Nevertheless, for the 53 different patient samples analyzed, evidence of a significant response (greater than 2-fold over controls) to the single factors IL-3, SF or FL was evident in more than 60%, and more than 90% responded to two or three of these factors in combination. The magnitude of the response was also greater with the cytokine combinations (median fold-increase ^{3}H-thymidine incorporation 2- to 4-fold for the cytokines individually versus 4- to 16-fold for the combinations; Table A-1).

The growth factor responsiveness of AML progenitors was also evaluated in colony assays. In this case the results of the ^{3}H-thymidine incorporation studies were used to select cytokines for testing and comparison to assays using agar-stimulated leukocyte conditioned medium or medium conditioned by the 5637 cell line (Welte et al. 1985) at a final concentration of 10%. Among 50 patient samples tested in preliminary studies, all showed equivalent colony growth, and 19 (38%) showed at least a twofold increase in the number of blast colonies present in assays containing SF, IL-3 and GM-CSF as compared with assays containing either of the two conditioned media. The addition of FL to this cytokine cocktail further increased the number of colonies derived from 14 (28%) of these 50 samples. Interestingly, among 19 AML samples tested in methylcellulose assay containing serum but no other source of cytokines, 9 showed evidence of factor-independent colony growth. However, 5 of these also showed increased colony formation when cytokines were added.

The cytokine-stimulated ^{3}H-thymidine incorporation and blast colony formation data for the 10 patient samples used in the experiments described in this chapter are shown in Tables A-2 and A-3, respectively. All of these samples showed some increased proliferation in response to single cytokines, although in the case of samples 1 and 6 this increase was modest.

Combinations of two or three cytokines enhanced proliferation more than any single factor alone from every patient sample except number 6.

The growth of AML-CFC from these samples was similar to that observed in the larger group of 50 AML specimens. Some evidence of factor-independent growth was noted in six cases, but evidence of increased colony formation in the presence of one or more cytokines was also obtained with every sample except that from patient 6, whose cells also showed a poor response to growth factors as measured by ^{3}H-thymidine incorporation.

The development of improved feeders for LTC, as well as the information regarding cytokine-responsiveness of leukemic cells, led to the hypothesis that appropriate conditions for LTC of AML cells could be discovered. In addition, the advent of fluorescent in situ hybridization (FISH) techniques that allow the recognition of chromosomal abnormalities in interphase as well as metaphase cells would enhance the ease with which leukemic progenitors could be discriminated from their normal counterparts (Trask 1991). Therefore experiments were initiated to determine if a sensitive and quantitative assay for "AML-LTC-IC" could be developed. The patient samples used in these experiments are listed in Table 3.1. It was shown that malignant progenitors that initiate LTC (AML-LTC-IC) can be routinely detected and both their frequency and proliferative capacity measured using limiting dilution analysis techniques. Manipulation of the growth conditions in LTC allowed some interesting differences between the responses of normal and leukemic progenitors to be revealed.

3.2   *Results*

The patient samples used for experiments described in this chapter are listed in Table 3.1.

Table 3.1. AML Patient Samples – Clinical Characteristics

Patient	Age (yr)	Sex	FAB Type	WBC(%blasts)* (X 10⁹/L)	Bone Marrow Cytogenetics [% abnormal]	FISH Probe
1	68	M	RAEBIT	27 (14)	45,X,-Y,inv(16)(p13q22) [92]	Y,8
2	44	M	M1	165 (95)	45,X,-Y,t(8;21)(q22q22) [100]	Y,8
3	64	F	M5A	129 (95)	47,XX,+8,rea(11)(q23) [100]	8
4	69	F	M4	65 (83)	47,XX,+8 [100]	8
5	59	F	M5A	36 (94)	47,XX,-7,+8,+8,t(9;11)(p22;q23) [100]	8
6	69	F	M5B	155 (95)	46,XX,t(9;11)(p22;q23) [100]	11q23
7	77	M	M2	57 (51)	47,XY,+8,t(12;22)(p13;q12)del(20)(q1.2q1.3) [100]	8
8	36	F	M4Eo	18 (42)	47,XX,inv(16)(p13q22).+22 [87]	16p13
9	55	M	M5	106 (95)	46,XY; 48,XY,+8,+8 [58]	8
10	48	F	M4Eo	18 (52)	46,XX,inv(16)(p13q22) [100]	16p13

*WBC, peripheral blood total white blood cell count at diagnosis.

The various longterm culture conditions described in this chapter are described in Table 3.2

Table 3.2. Longterm Culture Conditions

Feeder Layer* (human cytokines produced, ng/mL)	Soluble Cytokines Added (final concentration)
HMF	
Sl/Sl	
Sl/Sl-J-IL-3 (IL-3, 16.5 ng/mL)	SF (50 ng/mL)
Sl/Sl	FL (50 ng/mL)
Sl/Sl-J-IL-3 (IL-3 16.5 ng/mL)	FL (50 ng/mL)
Mixed (IL-3, 2.5 ng/mL; G-CSF, 95 ng/mL; SF, 2 ng/mL)	
Mixed (IL-3, 2.5 ng/mL; G-CSF, 95 ng/mL; SF, 2 ng/mL)	FL (50 ng/mL)

* Feeder layer composition for HMF, Sl/Sl, Sl/Sl-J-IL-3 and Mixed feeders is described section 2.3.

3.2.1 Detection of Malignant Progenitors in LTC Containing HMF or Sl/Sl Feeders

Light density PB or BM cells from nine newly diagnosed AML patients (patients 1-8 and 10, Table 3.1) were cocultured with irradiated HMF or Sl/Sl feeders for 5 weeks with weekly half medium changes. They were then harvested and placed in methylcellulose colony assays. Colony numbers were recorded, then colonies were plucked and analyzed by FISH for the presence of cytogenetic abnormalities that had been identified in the leukemic clone at diagnosis. Figure 3.1 illustrates FISH for the detection of the various cytogenetic abnormalities present in the patient samples used for these studies. The frequency of CFC per 10^6 input light density BM or PB cells, and the proportion of these that were cytogenetically abnormal by FISH, could then be calculated (Table 3.3). The number of colonies assessed by FISH in each group ranged from 10 to 80.
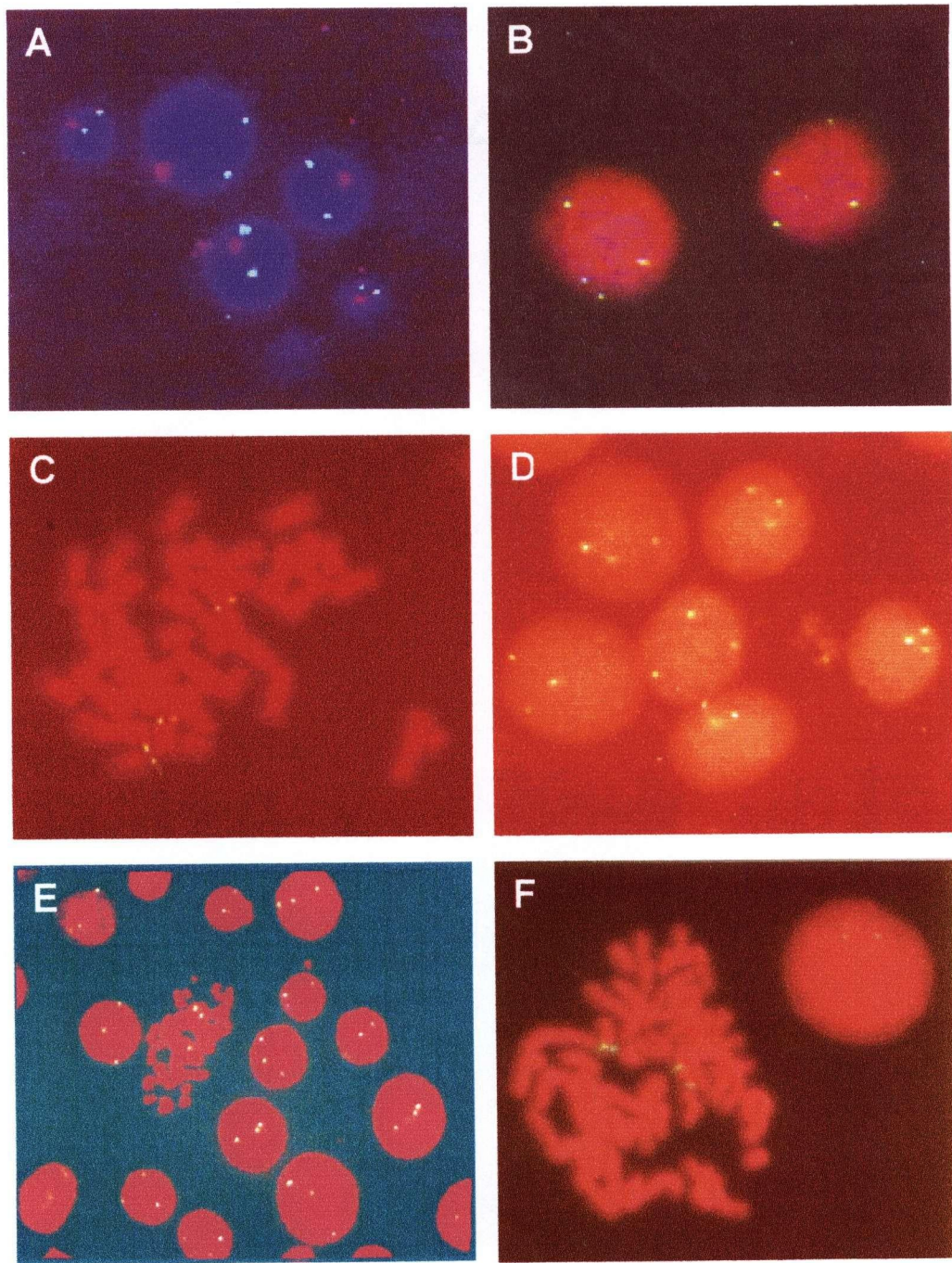
Figure 3.1. FISH analysis of colony cells from LTCs of patients. (A)Colony from patient 1 hybridized simultaneously with the pRY3.4 probe for chromosome Y (red) and the D8Z2 probe for chromosome 8. This colony was positive for the Y chromosome, indicating normal cytogenetics. Cells from an abnormal colony (-Y) would have the 2 green signals, but not the red signal. (B)Cells from patient 5 hybridized with the D8Z2 probe. 4 signals indicate the +8+8 abnormality. (C,D)Colonies from patient 6 hybridized with the MLL(11q23) probe from Oncor. 3 signals are clearly present in both metaphase and interphase cells. (E)Cells from patient 7 hybridized with the D8Z2 probe. 3 signals indicate the +8 abnormality. (F)Cells from patient 10 hybridized with the inv(16) probe. The split signal on one chromosome 16 is easily detectable on the metaphase cell, and 3 signals are visible on the interphase cell.

Table 3.3. CFC in Week 5 LTCs of AML Samples Under Various Growth Conditions

Patient (Tissue)	CFC/10^6 Cells (% Abnormal*)		Fold Change from HMF (% abnormal*)					
	Day 0	HMF	SI/SI	SI/SI JIL3 + SF	SI/SI + FL	SI/SI JIL3 + FL	Mixed	Mixed + FL
1(BM)	165 (40)	54 (25)	2 (6)	3 (7)				
2(BM)	30 (33)	9 (25)	2 (0)$^\phi$	6 (21)				
3(BM)	ND	13 (53)	ND	8 (34)				
3(PB)	3150 (90)	15 (32)	5 (60)	4 (68)			30 (5)$^\phi$	12 (0)$^\phi$
4(PB)$^\Psi$	485 (73)	34 (88)	0.4 (85)	2 (87)	1 (11)$^\phi$	2 (23)$^\phi$		
		55 (71)	1 (43)	1 (52)	0.8 (3)$^\phi$	0.7 (15)$^\phi$		
5(PB)	48100 (100)	307 (100)	0.02 (92)	0.8 (100)	2 (100)	0.6 (88)		
6(PB)	158 (87)	2790 (80)	0.7 (82)	0.5 (69)	0.6 (82)	0.4 (82)		
7(PB)	31260 (95)	129 (0)$^\#$	0.03 (29)	0.7 (60)	0.4 (68)	1 (78)	0.3 (52)	4 (50)
8(PB)	45 (67)	6 (65)	0.5 (44)	3 (33)	3 (35)	16 (29)$^\phi$	3 (28)$^\phi$	59 (38)
10(PB)	225 (50)	3 (21)	2 (0)	25 (4)	185 (13)	547 (0)$^\phi$	11 (0)$^\phi$	429 (8)

* % cytogenetically abnormal colonies detected by FISH

$^\Psi$ Data for two separate experiments in LTC

$^\#$ This sample plated at lower cell densities with HMF in limiting dilution analyses (described in section 3.4) produced CFC which were 55% cytogenetically abnormal

$^\phi$ The difference in the proportion of cytogenetically abnormal CFC between this LTC condition and that of LTC containing HMF achieved a P value <0.05 by chi-square analysis

ND = not done

The frequency of CFC was higher at Day 0 than the frequency in 5-week-old HMF cocultures in every case, except in cultures of cells from patient 6, for which the reverse was true. FISH analysis of colonies plucked from methylcellulose assays showed that, in every case, cytogenetically abnormal colonies could be detected at Day 0, with the proportion of abnormal colonies ranging from 33% to 100% (Table 3.3). In colony assays from HMF cocultures, the proportion of CFC carrying the chromosomal marker was similar to that seen in colonies derived from the same cell sample plated directly into methylcellulose, except for patient 7

cells, with which only CFC that were karyotypically normal by FISH for trisomy 8 were seen in assays from HMF cocultures. However, colonies carrying the +8 abnormality were recovered from 5-week-old cocultures of these cells with Sl/Sl feeders, and also when they were cultured at lower cell densities with HMF in the limiting dilution analyses described in section 3.2.3.

Sl/Sl mouse fibroblast feeders were variable in the extent to which they supported the generation of CFC in 5-week-old cocultures with AML cells. Cocultures with cells from patients 2 and 10 did not support the cytogenetically abnormal CFC, although there was a 2-fold increase in total CFC numbers as compared to HMFs in both cases. Cocultures with cells from patients 5 and 7 showed significant reductions in the total numbers of CFC detected as compared with HMF cocultures. However, cultures with Sl/Sl feeders and cells from the other five patients behaved similarly to cultures with HMF.

3.2.2 Effect of Cytokine Supplements on AML Progenitors in LTC

The effects of modifying the growth conditions in LTC of AML cells were investigated by cytokine supplements in the form of feeders genetically engineered to produce human growth factors and/or biweekly additions of soluble cytokines over the 5-week culture period. The various different conditions tested were shown in Table 3.2. When AML LTCs were supplemented with cytokines, either as biweekly factor additions or with the use of genetically engineered feeders, the results were very heterogeneous, although some patterns were observed (Table 3.3). Engineered feeders previously chosen for their ability to support the detection of normal LTC-IC (ie. mixed feeders producing IL-3, G-CSF and SF) produced a large increase in the output of CFC from cocultures with three of four AML samples tested. However, in all three of these cases (numbers 3, 8 and 10) the proportion of cytogenetically abnormal CFC was significantly reduced as compared with that present in cocultures with HMF. Cocultures with

Sl/Sl feeders or mixed feeders which were supplemented with one or more of the three factors that were most potent in stimulating colony formation or ^{3}H-thymidine incorporation from AML blasts (SF, IL-3 or FL) showed at least a twofold increase in the number of CFC detected from 5-week-old LTC as compared with Sl/Sl or mixed feeder controls with five of the nine patient samples (numbers 2, 5, 7, 8, and 10). In cultures with Sl/Sl feeders supplemented with IL-3 and SF, the numbers of both cytogenetically normal and abnormal CFC increased with all five patient samples so that the relative proportions of each did not change, as compared with control cultures with Sl/Sl feeders alone. The response to FL supplements of Sl/Sl or mixed feeder cocultures was similar to that seen with IL-3 and SF for four patient samples (numbers 5, 7, 8, and 10). However, testing of cells from patient 4 produced a different result. Although the total number of CFC produced changed very little among the different culture conditions with Sl/Sl feeders, the addition of FL ($\pm$ IL-3) to these cultures significantly reduced the output of cytogenetically abnormal CFC from LTC in each of two experiments (see Figure 3.2). This contrasts with the apparent stimulation of AML cells from this patient by FL in short-term assays (Tables A-2 and A-3).

Although Epo was added to CFC assays on cells from 5-week-old LTC, few erythroid colonies were seen and their proportion of the total CFC output did not change significantly under the different culture conditions. Those BFU-E that were analyzed for chromosomal abnormalities by FISH were exclusively normal in assays of cells from patients 2, 3, 4, 6, and 8. However, in assays of cells from LTC of samples from patients 1 and 7, 7 of 23 and 1 of 6 BFU-E, respectively, were cytogenetically abnormal.
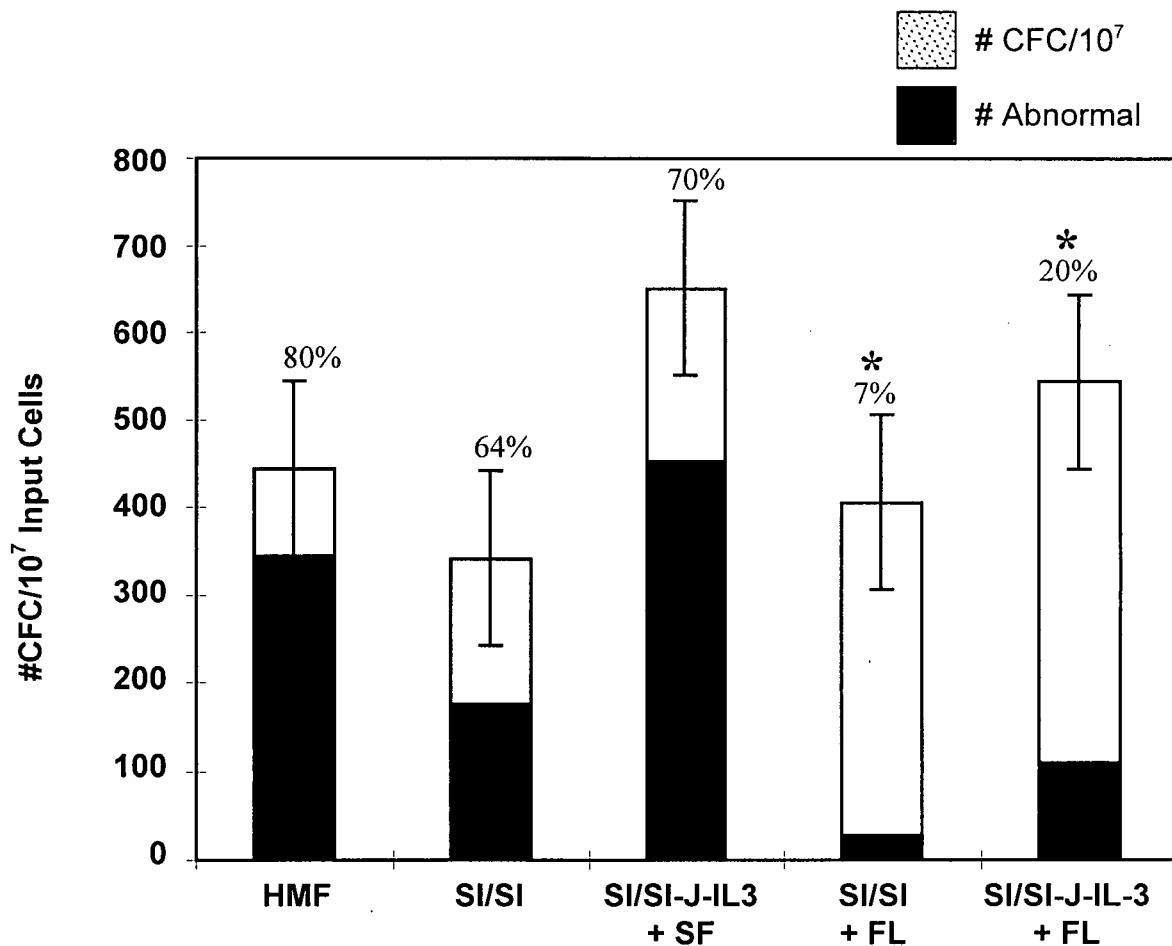
Figure 3.2. Longterm cultures of cells from patient 4. The number of CFC $\pm$ SEM (n=2) that grew per 10^7 cells placed in culture at time 0 are shown in the white bars. The number from the same cultures that were cytogenetically abnormal are shown in the black bars. The percent abnormal is shown at the top of each bar. In cultures that had FL present ($\pm$IL-3) there was a significantly lower proportion of abnormal CFC, even though the total output CFC number did not change significantly (* indicates difference in proportion of abnormal CFC relative to HMF statistically significant, p<0.05, Student t-test).

3.2.3   Analysis of Cells from AML Blood that Initiate LTC at Limiting Dilution

Figure 3.3 shows that when different concentrations of AML cells from five patients were plated on HMF, and in two cases on mixed feeders, the logarithm of the number of CFC recovered from LTC 5 weeks later maintained a linear relationship to the logarithm of the number of input cells. The mean ($\pm$SD) slope of the lines generated (0.85 ± 0.16) is not significantly different from 1, indicative of a linear relationship between the CFC output and the inoculum input. These cultures could therefore be used to quantitate the frequency of LTC-IC among AML PB cells and the average number of CFC produced per LTC-IC. Table 3.3 shows the frequency of LTC-IC calculated for 6 different samples on HMFs, and 2 samples on mixed feeders.

The frequencies varied greatly, from as low as 3 to greater than 100 per 10^6 cells originally placed in LTC. In every case this frequency was considerably lower (10 to 260-fold) than the frequency of AML-CFC in the same sample. The mean ($\pm$SD) number of CFC produced per LTC-IC in cultures containing HMF was 2.1 ± 1.2, which is lower than the value of 4 to 7 CFC per LTC-IC obtained for normal PB or BM LTC-IC analyzed under similar conditions (Udomsakdi et al, 1992c; Hogge et al, 1996). In contrast to previous experience with normal LTC-IC (Hogge et al, 1996), mixed feeders did not significantly enhance the frequency of LTC-IC detected in either patient sample where these were compared with HMF in limiting dilution experiments. However, the CFC output per LTC-IC did increase twofold to threefold on mixed feeders (Table 3.4).

94
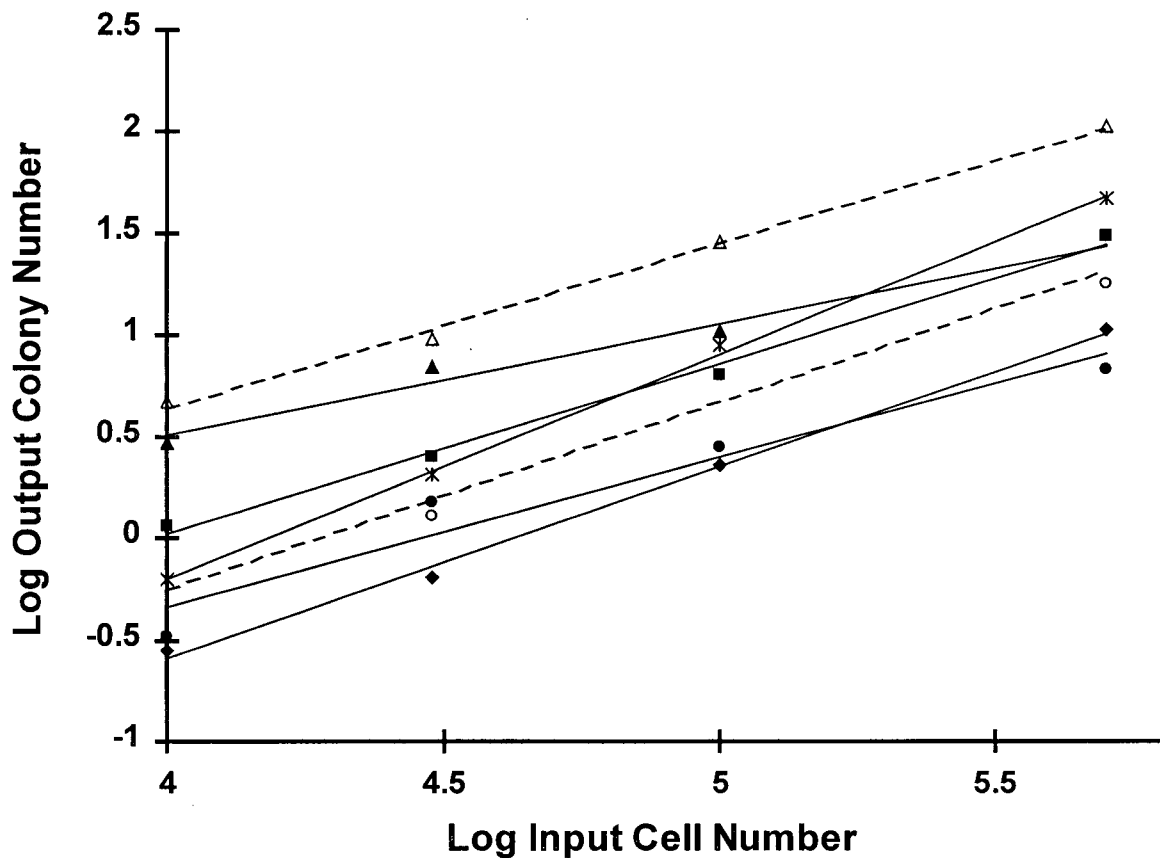
Figure 3.3. Linear relationship between input cell concentration and number of output colonies from LTC of AML cells. Peripheral blood cells from five patients with AML were plated at concentrations varying from 5 X 10^6 to 1 X 10^5 per mL on irradiated feeders in microtitre wells. 25 to 30 duplicate LTCs were plated at each cell concentration. After 5 weeks in culture the total cell content of each well was obtained and plated in methylcellulose assay as described in Chapter 2. The log of the input cell number is plotted against the log of the mean number of colonies produced per duplicate longterm culture at that cell concentration. HMF were used for longterm cultures with each of the five patient samples, patient 3 (◆), patient 4 (■), patient 7 (▲), patient 8 (●) and patient 9 (*). In addition, mixed feeders were used for duplicate cultures with cells from patients 7 (Δ) and 8 (#). The mean (±SD) of the slope of the lines generated is 0.85 ± 0.16.

Table 3.4. Frequency and Proliferative Capacity of LTC-IC in AML Peripheral Blood Determined by Limiting Dilution

Patient	Day 0 CFC/10^6 Cells (% abnormal)	Feeder	LTC-IC/10^6 Cells (% abnormal)	CFC/LTC-IC*
3	3150 (90)	HMF	15 (32)	1.4
4	485 (73)	HMF	50 (64)	1.8
7	31260 (95)	HMF	121 (55)	1.2
		Mixed	116 (73)	2.7
8	45 (67)	HMF	4 (7)	4.4
		Mixed	4 (9)	12.8
9	1138 (0)	HMF	40 (0)	1.7
10	225 (50)	HMF	3 (21)	2.1

*CFC per LTC-IC were calculated as average values by dividing the total number of CFC produced in all of the limiting dilution cultures plated by the number of calculated LTC-IC used to initiate those cultures for each culture condition tested.

A proportion of the CFC detected in both direct methylcellulose assays and in assays of cells from LTC were cytogenetically abnormal by FISH for all patient cell samples except patient 9. As shown in Table 3.1, this patient had a mixture of cytogenetically normal and abnormal cells on initial bone marrow cytogenetic analysis. Although the morphology of colonies detected in the direct methylcellulose assays of peripheral blood cells or assays from LTC was typical of AML-CFC (i.e. small clusters), none showed the +8+8 abnormality.

When less than 31% of replicate 5-week-old mini-LTC from limiting dilution (plated at a given cell density in 96-well plates) contain CFC, the probability that these LTCs were initiated by a single progenitor is greater than or equal to 85% according to Poisson statistics (Coller and Coller 1986). From such assays the range of CFC produced by individual LTC-IC can be determined. Among the experiments with cells from patients 3, 4, 7, 8 and 10 shown in Table

3.4, colonies from methylcellulose assays of 43 mini-LTC plated at this limiting cell density were analyzed by FISH. These results are shown in Table 3.5. Eighteen LTC (42%) produced exclusively cytogenetically abnormal CFC, whereas 23 (53%) generated only cytogenetically normal colonies, and 2 (5%) showed a mixture of normal and abnormal CFC. The number of CFC produced per LTC-IC ranged from 1 to 7 and 1 to 30 for the cytogenetically abnormal and normal LTC-IC, respectively.

The concentration of cytogenetically normal and abnormal LTC-IC in AML PB was calculated using data from Tables 3.1 and 3.4 and compared with the concentration of LTC-IC in normal steady state blood that was previously determined under the same assay conditions (Udomsakdi et al. 1992c). As shown in Table 3.6, in each case the concentration of LTC-IC in AML blood, including those with normal cytogenetics, was at least 10-fold, and in two cases more than 100-fold higher than that detected in normal individuals.

Table 3.5. Colonies Arising from LTCs Initiated with a Single LTC-IC

		# CFC/LTC-IC (range)
Number of cultures producing exclusively cyto-genetically abnormal CFC	18 (42%)	1-7
Number of cultures producing exclusively cyto-genetically normal CFC	23 (53%)	1-30
Number of cultures producing a mixture of cyto-genetically abnormal and normal CFC	2 (5%)	

Table 3.6. Concentrations of Cytogenetically Normal and Abnormal LTC-IC in the Blood of AML Patients

Patient	LTC-IC per mL*	
	Normal	Abnormal
3	1320	619
4	1170	2080
7	3100	3790
8	67	5
9	4240	-
10	43	11
Normal controls n = 23	$2.9^{\pm}0.5^{\Psi}$	

* As detected in cocultures with HMF
Ψ In steady state blood from normal individuals as reported in Udomsakdi et al, 1992c

3.3 Discussion

In spite of the heterogeneity exhibited by AML cells in many aspects of their pathological features and clinical behavior, including cytokine responsiveness, the majority of leukemic blast cells and AML-CFC exhibit some degree of responsiveness to conditions that stimulate the growth of normal hematopoietic cells in vitro (Appendix A). This suggested that a more primitive type of AML precursor cell analogous to normal LTC-IC might also be supported by conditions developed for normal progenitors, as shown for Ph^+ LTC-IC present in the peripheral blood and bone marrow of patients with CML (Eaves et al. 1986; Udomsakdi et al. 1992b).

The data shown in Table 3.3 shows that AML-LTC-IC can be routinely detected in the

peripheral blood and bone marrow of AML patients at diagnosis. When AML samples were grown on HMF ("standard" LTC conditions), the proportion of abnormal CFC arising from these cultures was usually similar to that seen in colonies derived from the input sample on Day 0. Thus culture on HMF does not seem to cause a significant decrease in the proportion of malignant CFC that was present at Time 0, as was previously described under conditions where AML bone marrow samples were allowed to form their own feeder layers (Coulombel et al. 1985). Growth on HMF was therefore used as the "standard"condition to which the others were compared to determine their usefulness in improving AML-LTC-IC detection.

Some differences were observed between the behavior of AML progenitors in LTC and their normal counterparts characterized previously. Firstly, although Sl/Sl fibroblast feeders are equivalent to HMF for the support of normal LTC-IC, they did not allow the detection of AML LTC-IC from patient samples 2, 5, and 7 as well as HMF. Nevertheless, when IL-3 and SF or FL were added, these patient cell samples showed an increase in the output of cytogenetically abnormal CFC to levels at least as high as those obtained with HMF, suggesting that these AML LTC-IC were particularly responsive to one or more of these cytokines and largely failed to produce AML-CFC progeny in their absence.

With four of the AML samples, growth factor supplements to LTC, either as biweekly factor additions or with the use of feeders engineered to produce human cytokines, reduced the proportion of cytogenetically abnormal CFC detected in 5-week-old cultures. In cultures of cells from patients 3, 8 and 10, the predominant effect was the stimulation of normal CFC output by mixed feeders. This finding was not unexpected, as it was previously shown that these feeders producing IL-3, SF and G-CSF enhance normal CFC output from LTC up to 20-fold as compared with control feeders (Hogge et al. 1996). It is therefore of interest that AML LTC-IC failed to show such a response to these feeders in any of the four cases tested. One

possible explanation for this finding is that high concentrations of G-CSF, which was the major

difference between cocultures with mixed feeders and the other conditions tested, have little

effect on the production of CFC progeny by AML LTC-IC as compared with the stimulation

that is seen with normal LTC-IC. In cultures from patient 4 the presence of FL appeared to

suppress the production of malignant CFC from LTC. The mechanism whereby this might

occur remains to be investigated. Most AML blasts have been shown to express both flt-3 and

the SF receptor, c-kit, and to respond to these cytokines in short-term assays (Appendix A;

Piacibello et al. 1995; Carow et al. 1996; Broudy et al. 1992). Because LTC conditions

supplemented with either of these factors, with or without IL-3 or mixed feeders, increased the

output of cytogenetically abnormal CFC from cultures of five of the AML samples tested, it

appears that at least some AML LTC-IC may share these characteristics.

In two cases some malignant LTC-IC were shown to have erythroid potential. One of these

samples was from a patient with myelodysplasia that had transformed to AML. Multilineage

differentiative potential of the malignant clone in some patients with AML has been described

previously as being associated with a "smouldering" or "preleukemic" phase and occurring

more typically in elderly patients (Fialkow et al. 1981). This, in combination with the ability to

routinely detect leukemic LTC-IC, suggests that the hierarchical nature of blood cell

development has been retained among the malignant cells in AML and that the usual target for

transformation in these diseases is at or upstream of the progenitor cell compartment that

includes normal LTC-IC.

In most cases FISH analysis of CFC plated directly into methylcellulose assays from

patient bone marrow or peripheral blood samples, or CFC from 5-week-old LTC showed a

mixture of cytogenetically normal and abnormal cells. In some cases the apparently high

"normal" CFC number may have been related to the probe that was chosen for FISH analysis,

eg. the Y probe was selected for detection of cytogenetically abnormal CFC from patients 1 and 2 at a time when reliable FISH probes for the inv(16) and t(8;21) were not available. It is possible that malignant CFC with the inversion or translocation but retaining the Y chromosome were present but scored as normal by FISH. Similarly, the absence of the +8+8 abnormality from all colonies analyzed from both direct methylcellulose assays and LTCs of cells from patient 9 was surprising given the high peripheral blood blast cell count and the morphology of the colonies analyzed, which was typical of AML-CFC. It seems likely that at least some of these colonies were in fact malignant, although cytogenetically normal by FISH. This possibility is supported by Van der Lely et al (1995), who found that colonies grown from AML samples with aneuploidies which had abnormal growth patterns did not always carry the aneuploidy that was identified in the leukemic clone at diagnosis. This phenomenon can be explained by the presence of a cytogenetically normal but leukemic precursor cell from which the aneuploid cells arose as the result of secondary genetic changes in the leukemic clone (Van Der Lely et al. 1995). In cases where a translocation resulting in the production of an abnormal fusion protein occurs, the cytogenetic abnormality is more likely to have been the transforming event, and therefore can be more reliably used as a marker for leukemic cells. Thus, the percentages of cytogenetically abnormal CFC detected by FISH shown in Tables 3.3 to 3.6 are minimal estimates of the proportion of AML progenitors present in these samples. Nevertheless, it seems likely that residual normal hematopoietic cells coexist with AML progenitors in many of these samples. The fact that all erythroid colonies examined from 5 of 7 patients were normal by FISH supports this possiblity. Normally, LTC-IC circulate in the peripheral blood at easily detectable frequencies, so it is not surprising that they could be found in cultures from patients 8 and 10, because in these the peripheral blood white blood cell count and the proportion of blasts were relatively low, and the FISH probe for inv(16) would be

expected to discriminate reliably between normal and malignant CFC.

Because the AML LTC assay is linear (Figure 3.3), it can be used to quantitate the numbers of LTC-IC in different clinical samples. Although the expected heterogeneity among AML patient samples was observed in both the frequency and proliferative capacity of these cells, they could be shown to be a relatively rare cell population as compared with AML-CFC (10 to 260-fold less frequent) and, in most cases, to give rise to more than one CFC after 5 weeks in LTC (Table 3.4). This is further evidence for a hierarchical cell population in AML. The overall frequency of LTC-IC in the peripheral blood of AML patients is significantly higher than the frequency in normal peripheral blood, and is in fact in the same range as the frequency of LTC-IC in normal bone marrow ($55/10^6$; Udomsakdi et al. 1992c), and in the peripheral blood of normal individuals after induction of mobilization by G-CSF administration ($60/10^6$; Hogge et al. 1996), as determined under similar culture conditions. A high proportion of these LTC-IC in the peripheral blood of AML patients are cytogenetically normal, with 10 to 100-fold higher frequencies of normal LTC-IC in the peripheral blood of AML patients than in normal individuals. Similar observations have been made in CML, where in some patients the number of normal LTC-IC is more than 3 times the number of leukemic progenitors, and is up to 50 times higher than the corresponding value in normal steady state peripheral blood (Podesta et al. 1997). This indicates that high numbers of normal LTC-IC are mobilized to the peripheral blood as part of the leukemic process in both CML and AML.

Upon comparison of cytogenetically normal and abnormal LTC-IC plated at limiting dilution, the number of CFC produced per LTC-IC did not differ significantly (see Table 3.5). The growth advantage of the leukemic cells, as ultimately manifested by the massive expansion of these cells and eventual population of the bone marrow and peripheral blood of these patients with very high numbers of blast cells, is thus not due to an increased proliferative capacity of

AML-LTC-IC relative to coexisting normal LTC-IC. It may instead be the result of a change in the cycling status of the leukemic LTC-IC and CFC. The majority of progenitors and LTC-IC in the blood of normal individuals are quiescent, as determined by a modified ^{3}H-thymidine suicide assay for cycling cells (Ponchio et al. 1995). Contrastingly, in the case of CML, most of the circulating clonogenic cells and LTC-IC show features of proliferating or activated cells (Udomsakdi et al. 1992a; Udomsakdi et al. 1992b), suggesting "a defective, unregulated mechanism for inevitable expansion of the Ph$^+$ clone" (Udomsakdi et al. 1992b). This has recently been confirmed in studies showing that the growth deregulation of CML LTC-IC is, at least in part, due to a lack of responsiveness to inhibitors of normal LTC-IC cycling (Cashman et al. 1998). This would give a growth advantage to the leukemic clone, resulting in the specific expansion of leukemic cells seen in the patient. The cycling status of both cytogenetically normal and abnormal LTC-IC from AML peripheral blood samples are currently under investigation in our laboratory to determine if a similar growth deregulation has occurred in these primitive AML progenitors.

In contrast to the results presented here, early observations showed a disappearance of AML-CFC from standard LTC (Coulombel et al. 1985). There are a number of possible explanations for the apparent discrepancy between that report and the results reported here. Firstly, the detection of LTC-IC depends on both the sensitivity of the assay conditions and, in cultures containing a mixture of normal and malignant cells, their relative frequency. Thus, under conditions where AML-LTC-IC are relatively rare in the input population as compared with normal LTC-IC, their recognition may be difficult unless culture conditions are optimal. The use of preestablished feeders in LTC and recombinant cytokines in methylcellulose assay has improved the LTC-IC assay since the original AML LTCs were performed, allowing malignant LTC-IC to be observed even in the presence of residual normal hematopoiesis. The

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sensitivity of malignant LTC-IC detection is further improved by the use of FISH. This allows the identification of malignant CFC which may otherwise be classified as normal, since colonies derived from leukemic progenitors are not always distinguishable from those derived from normal progenitors by morphology alone. However, if the detection of normal LTC-IC is substantially favored, as it appears to be in some cases, AML-LTC-IC can still be difficult to detect. The detection of AML-LTC-IC is also made more difficult by their low output of CFC progeny as compared with normal LTC-IC under current assay conditions. A final possibility that would explain the reduced production of AML-CFC in previous LTC experiments, which is suggested by the results of studies done with CML (Udomsakdi et al. 1992b), is that the standard LTC conditions previously tested may favor the self-maintenance of normal LTC-IC, whereas at least some of the conditions tested in the current experiments may provide more equivalent support for primitive normal and malignant progenitors.

In conclusion, these experiments have shown the existence of a rare progenitor among the leukemic cells of patients with AML, which will initiate longterm hematopoiesis in cocultures containing fibroblast feeder layers that support normal LTC-IC. These conditions allow such AML-LTC-IC to be quantitated and characterized. Furthermore, demonstration of leukemic LTC-IC in all samples tested supports the origin of AML in a primitive cell compartment. Manipulation of the cytokine conditions present in LTC was shown to affect AML-LTC-IC or their immediate progeny differently from coexisting normal LTC-IC in many cases. Further exploitation of this system should allow other factors important to the regulation of primitive AML progenitors to be elucidated.

CHAPTER FOUR : GROWTH CHARACTERISTICS OF AML PROGENITORS WHICH INITIATE MALIGNANT HEMATOPOIESIS IN NOD/SCID MICE

The data presented in this chapter have been drafted into a manuscript:

4.1 Introduction

Transplantation models in mice have been critical to the development of our understanding of the most primitive hematopoietic progenitors, which are capable of reconstituting multilineage hematopoiesis when transplanted into lethally-irradiated recipients. It has been possible to use some of the principles that were used to develop these assays for murine "stem cells" to study normal human cells that exhibit similar potential when injected into sublethally irradiated immunodeficient mice. Recent studies have shown that multilineage human hematopoiesis can be successfully established in nonobese diabetic/LtSz-scid/scid (NOD/SCID) mice (Shultz et al. 1995) after intravenous injection of normal human marrow or cord blood cells (Pflumio et al. 1996; Bhatia et al. 1997; Cashman et al. 1997a; Cashman et al. 1997b; Wang et al. 1997).

NOD/SCID mice will also support the growth of malignant progenitors from patients with AML (Blair et al. 1997; Bonnet and Dick 1997). The engraftment of NOD/SCID with both normal and leukemic hematopoietic samples has been reviewed in Chapter 1. Although the ability of some AML samples to grow in NOD/SCID mice had already been demonstrated, the growth potential of different samples was variable. Therefore a large series of AML samples was surveyed, representative of the clinical, phenotypic, and genetic heterogeneity seen in this disease, for their ability to grow in NOD/SCID mice. The human cytokine requirements of NOD/SL-IC were also studied and compared to the growth factor responsiveness observed in in vitro assays of leukemic progenitors from the same patient specimens, and the frequency and proliferative capacity of NOD/SL-IC were investigated and compared to the same parameters obtained for AML-LTC-IC. These data demonstrate the ability of NOD/SCID mice to allow the detection and quantitation of these very primitive leukemic progenitors in most AML samples.

Furthermore, clinical features of the disease with prognostic significance in patients, as well as the growth characteristics of leukemic progenitors in culture, appear to predict the growth potential of NOD/SL-IC from different patient samples in mice.

4.2   Results

The patient samples used for experiments described in sections 4.2.2 to 4.2.6 are listed in Table 4.1.

Table 4.1. AML Patient Samples – Clinical Characteristics

Patient	Age (yr)	Sex	FAB Type	WBC(%blasts)* (X 10⁹/L)	Bone Marrow Cytogenetics [% abnormal]	FISH Probe
3	64	F	M5A	129 (95)	47,XX,+8,rea(11)(q23) [100]	8
4	69	F	M4	65 (83)	47,XX,+8 [100]	8
7	77	M	M2	57 (51)	47,XY,+8,t(12;22)(p13q12)del(20) (q1.2q1.3) [100]	8
11	59	M	M2	75 (53)	46,XY,t(9;22); 46,XY,t(9;22),del(20q) [100]	
12	63	F	M1	295 (>90)	ND	
13	29	F	M4	87 (6)	46,XX; 46,XX,del(16)(q22) [32]	
14	44	M	M1	148 (95)	46,XY	
15	58	F	M4	370 (46)	46,XX; 47,XX,+13 [37]	13
16	60	F	M4	56 (76)	46,XX,del(7q),del(16q); 46,XX,del(16q),+22 [100]	
17	63	F	M1	27 (80)	46,XX,t(8;20)(p12q32) [100]	
18	48	M	M5a	46	46,XY [0]	

*WBC, peripheral blood total white blood cell count at diagnosis.

4.2.1   Variability of Engraftment of AML Samples in NOD/SCID Mice

Light density peripheral blood cells from 61 different AML patients were tested for their

ability to engraft NOD/SCID mice. Mice were injected intravenously with 10^7 patient cells, and

8 weeks later femoral bone marrow was removed and analyzed for the presence of human cells

by flow cytometry for CD45, a human-specific pan-leukocyte marker (Cobbold et al. 1987). An

average of 3 (range 1 to 10) mice were analyzed per patient sample, and mean %CD45$^+$ bone

marrow cells in the group of mice calculated to derive the graphs shown in Figures 4.1 and 4.2.

Human CD45$^+$ cells were easily detectable by flow cytometry in mouse bone marrow for

43 (70%) of the 61 samples (reliable detection of CD45$^+$ cells above background fluorescence

was 0.5%). Among the 61 samples the mean $\pm$ SEM level of engraftment was 13.3% $\pm$ 31

(Figure 4.1). 32 of the 61 patient samples had cytogenetic abnormalities detected in their

diagnostic bone marrow samples. Thirteen samples had cytogenetic changes associated with a

good clinical prognosis, i.e. inv(16) or variants (n=11) or t(15;17) (n=2) (Mrozek et al. 1997).

The mean engraftment level in this group was 5.7% (range <0.1% to 35%). The remaining 19

samples had chromosomal rearrangements associated with a poor prognosis. These

abnormalities included inv(3), +8, 11q23 rearrangements, t(9;22), del(20q), del(7q), +13,

t(12;22) and a variety of complex abnormalities. The mean level of engraftment in mouse bone

marrow in this group was 20.5% (range <0.1% to 100%). Thus it appears that AML samples

with poor risk cytogenetic features often engraft in mice to higher levels than those with

changes associated with a good prognosis. However, there is considerable heterogeneity and

overlap between the two groups and the difference in levels of engraftment obtained did not

reach statistical significance (p=0.06 by Student t-test).

57 of the group of 61 AML samples could be classified into different AML subtypes

according to FAB criteria (Bennett et al. 1985). When the levels of engraftment of these different cell types in mice were compared among the subgroups the lowest mean percent human cells in mouse marrow was seen among the AML M0 (n=1), M2 (n=4) and M3 (n=5) samples, (0.24, 4.5 and 1.8%, respectively), while the highest were seen among those with M1 (n=8), M4 (n=24) and M5 (n=11) subtypes, 13.7, 14.6, and 18.9%, respectively. The difference in levels of engraftment between M3 samples and all others was highly statistically significant (p=0.002) and approached significance (p=0.06) for M2 samples (Figure 4.2).
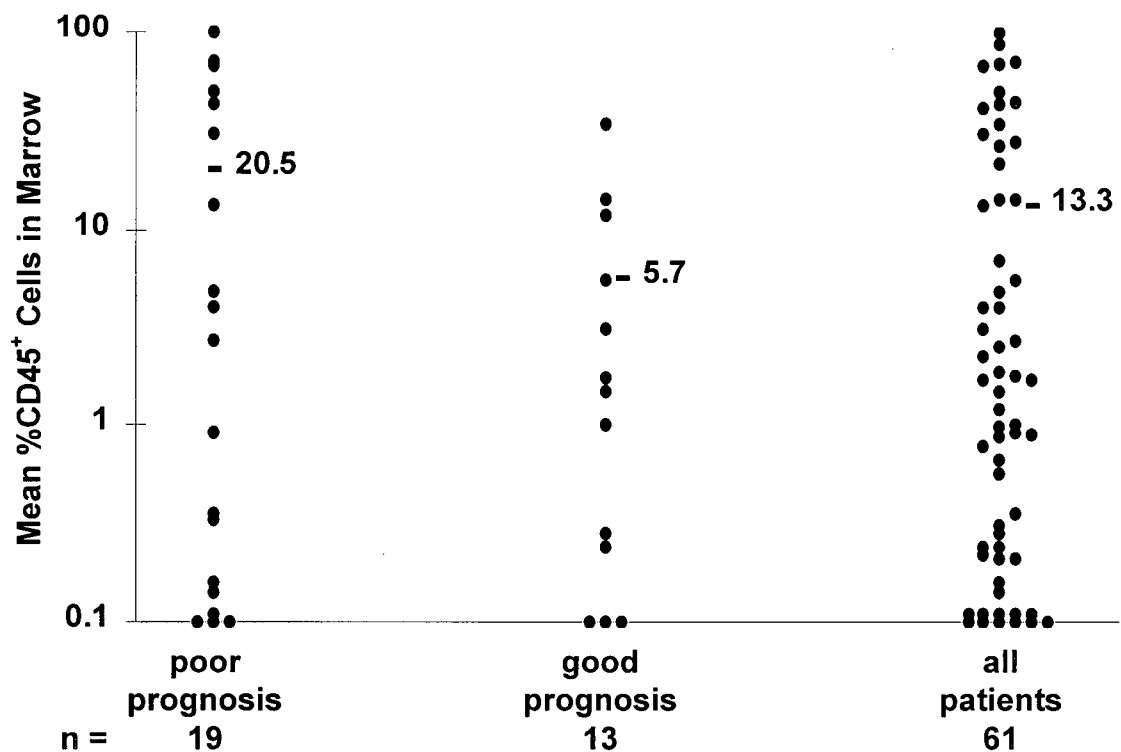
Figure 4.1. Engraftment of AML samples in NOD/SCID mice: Influence of cytogenetic abnormalities. The %CD45$^+$ cells in mouse marrow was determined for each sample 8 weeks after i.v. injection of 10^7 cells. Each dot represents the mean % engraftment in the bone marrow of all mice injected with cells from an individual patient sample (mean, 3 mice per sample; range, 1-10 mice per sample). The numerical values shown are the means for each group of samples. Changes associated with a poor prognosis included inv(3), +8, 11q23 rearrangements, t(9;22), del(20q), del(7q), +13, t(12;22), and a variety of complex abnormalities. Changes associated with a good prognosis included inv(16) or variants, or t(15;17). There was a trend toward higher levels of engraftment with samples with poor risk cytogenetic features (p=0.06).
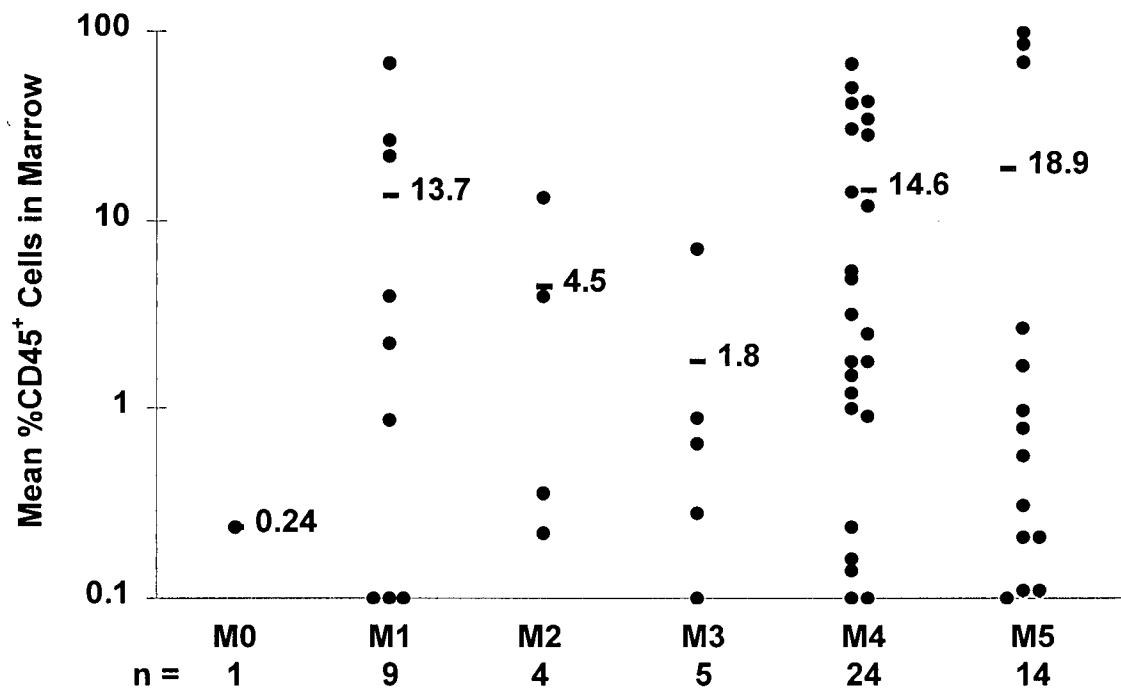
Figure 4.2. Engraftment of AML samples in NOD/SCID mice: Influence of FAB subtype. 57 of the 61 samples could be classified into AML subtypes according to FAB criteria. The mean engraftment levels obtained from each subtype are shown. Levels of engraftment were lower with samples with FAB subtypes M3 (p=0.002) and M2 (p=0.06) than with other subtypes.

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In order to confirm that the human cells growing in the mice were leukemic, May-Grunwald-Giemsa stained bone marrow touch preparations or cytospins of marrow cells from mice 8 weeks post-transplant were examined for 21 patient samples. The proportion of blast cells identified in the differential cell counts on these slides were in most cases comparable to the %CD45$^+$ cells as assessed by flow cytometry (r=0.74, p<0.001). In some cases characteristic morphological features such as dysplastic eosinophils from AML M4eo samples were clearly distinguishable in mouse marrow samples (Figure 4.3). Four AML samples which engrafted well in mice had cytogenetic markers which could be detected easily by FISH on interphase cells. 3 samples with the +8 abnormality (pts 3, 4 and 7) showed levels of engraftment of CD45$^+$ cells at week 8 post-injection of 5%, 3% and 15%. FACS-sorted CD45$^+$ cells from these mice showed the +8 abnormality in 95%, 15% and 100% of analyzed cells, respectively (see Figure 4.4). For a fourth sample (pt 15), in which 37% of the metaphases in the patient's diagnostic bone marrow contained trisomy 13, 50% of mouse marrow cells were CD45$^+$ at week 8 and 27% of these were +13 by FISH. Thus, the human cells engrafting in these animals are characteristic of the malignant clone initially identified in patients both morphologically and cytogenetically.
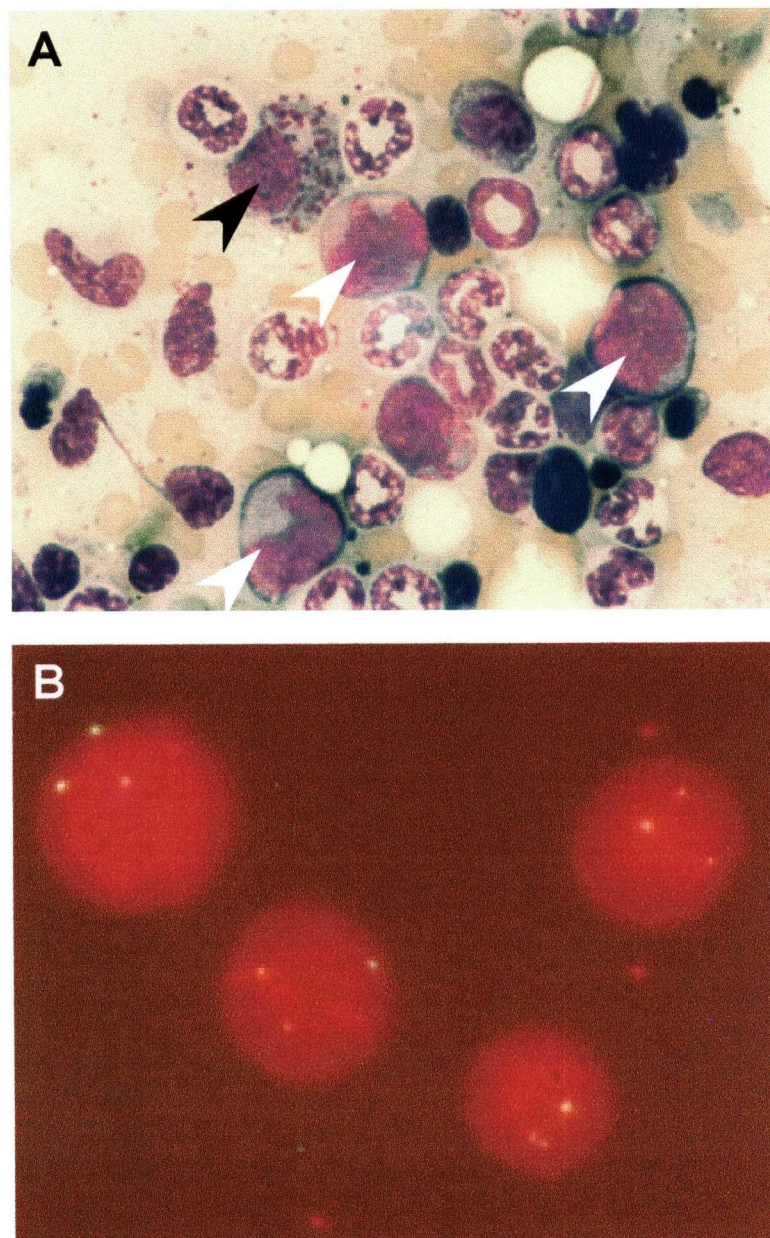
Figure 4.1. Histology and FISH on cells from cngrafted mice. (A)Bone marrow smear from a mouse 8 weeks after injection with cells with AML FAB type M4Eo, and the engrafted cells show the characteristic morphology of this subtype, including dysplastic eosinophils (black arrowhead) and leukemic blasts (white arrowheads). (B)FACS-sorted CD45+ cells from the bone marrow of a mouse 8 weeks after injection with cells from patient 7. The probe was D8Z2. The +8 abnormality previously identified in the leukemic clone of this patient is clearly present.

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The spleen and peripheral blood of animals injected with 59 of the AML samples were also examined for the presence of human leukemic cells. Although in every case the proportion of human cells detected was lower in spleen and blood than in the marrow (mean % human cells 2.7%, 3% and 12%, respectively), there was a direct correlation between the % AML cells in each of these tissues which was highly significant ($p<0.001$, Student t-test) (Table 4.2). All of the 16 patient samples where the proportion of human cells in marrow was >10% had AML cells easily detected in spleen and 15 also had human leukemic cells detected in blood.

Table 4.2. Engraftment of AML Samples in Different Mouse Hematopoietic Tissues

Mouse Tissue	Mean % Human Cells $\pm$ SEM (n=59)	Correlation r=*
bone marrow	12.0 ± 2.8	
		0.63
spleen	2.7 ± 1.1	0.76
		0.85
peripheral blood	3.0 ± 1.0	

* The correlation between levels of engraftment in different tissues was statistically significant, $p<0.001$.

4.2.2 Treatment of Mice With Human Cytokines Does Not Improve the Overall Ability of AML Samples to Engraft NOD/SCID Mice

The original description of growth of human AML cells in immunodeficient (SCID) mice had suggested that repeated injections of human cytokines were necessary to achieve high levels of engraftment. Although this suggestion is consistent with the known cytokine-responsiveness of human AML cells in vitro, in the current study we discovered that 13 of the 61 samples showed $\geq 25\%$ human leukemic cells in mouse marrow 8 weeks post-injection without treatment of mice with any human growth factors. Nevertheless, because we were

unable to demonstrate engraftment with all samples tested, and the level of engraftment of human cells was low with a number of others, we studied the ability of cytokine injections to enhance the levels of human AML cells detected in NOD/SCID mice. Since >90% of AML blast samples will proliferate in response to the combination of IL-3 and SF as measured in short-term ^{3}H-thymidine incorporation assays (Appendix A, Table A-1), these growth factors were tested for their effect on AML cell growth in mice. 10^7 cells per mouse from different AML samples were injected into groups of 4 mice each. Four weeks after injection of AML cells, one animal in each cohort was sacrificed. For 36 AML samples the bone marrow of this mouse contained <25% CD45$^+$ cells. After this had been determined one of the 3 remaining mice in these cohorts began to receive 6 $^\mu$g of human IL-3 and 10 $^\mu$g of human SF intraperitoneally on Monday, Wednesday and Friday while the second mouse received IL-3 only according to the same dose and schedule, and the third mouse continued not to receive any cytokines. Cytokine levels in the serum of a treated mouse 1.5 hours post injection were 8000 pg/mL of IL-3 and >100000 pg/mL of SF, while levels at 48 hours post-injection and immediately prior to the next scheduled dose of cytokine(s) had declined to undetectable levels for IL-3 and 55 pg/mL of SF. When these groups of 3 mice were analyzed at week 8 following initial injection of AML cells the mean $\pm$ SEM proportions of human cells in mouse bone marrow were not significantly different for animals receiving or not receiving human cytokines (2.6%$\pm$1.0 for animals which never received cytokines (n=36), 3.5%$\pm$1.7 for animals treated with IL-3 only (n=19), and 2.6%$\pm$0.9 for animals treated with IL-3 and SF (n=36)).

Although these results suggested that cytokines of the type and dose injected were ineffective in enhancing AML cell growth in mice, there were four samples in which comparison of the individual cytokine-treated and untreated mice suggested a possible

enhancement of engraftment with the combination of IL-3 and SF. Thus, the above experiment was repeated with these cells using larger numbers of mice in the treated and untreated groups. Table 4.3 shows the results from these four experiments. In no case was the difference between mice treated or not treated with cytokines statistically significant.

Table 4.3. Human Cytokines Do Not Enhance the Growth of AML Cells in NOD/SCID Mice

| Patient | Mean %CD45$^+$ Cells in Mouse BM ± SEM (n) | | p= |
	No Cytokines	IL-3 + SF*	
11	5.8 ± 2.2 (4)	2.2 ± 1.4 (4)	0.22
12	33.0 ± 15.8 (3)	10.9 ± 4.1 (3)	0.29
13	13.0 ± 6.1 (6)	16.2 ± 9.3 (4)	0.79
14	0.25 ± 0.18 (2)	6.5 ± 4.2 (3)	0.27

*Human IL-3 and SF were injected i.p. at a dose of 6 μg and 10 μg, respectively, Monday, Wednesday, and Friday of each of the 4 weeks prior to sacrifice. Mice were analyzed a total of 8 weeks following injection of AML cells.

4.2.3 Factor Independent Growth of AML-CFC Predicts AML Cell Growth in Mice

A subset of AML cells from most patient samples will form colonies of blast cells when placed in semisolid medium with a source of cytokines (Buick et al. 1977). However, the growth of these AML-CFC from some patient samples appears to be relatively factor-independent (Appendix A, Table A-3; Young et al. 1988; Lowenberg and Touw 1993). When 45 of the AML samples which were tested in mice were placed in methylcellulose assay containing serum but no other source of cytokines, 17 developed $\geq$5 colonies per 10^5 cultured cells. One additional patient showed <5 colonies but equivalent numbers of small clusters both

with and without cytokines. These 18 samples were considered to show factor independent AML-CFC growth. In 25 cases ≤ 1 colony formed per 10^5 cells without growth factors, but when cytokines were added to cultures colonies formed in every case. In the remaining 2 cases 2 or 3 colonies per 10^5 cells formed without cytokines but there was a >10-fold increase in colony numbers when cytokines were added. The latter 27 cases were considered to be factor dependent. When the ability of AML samples which were either factor dependent or independent in colony assays were compared for their ability to grow in mice, there was a significant difference (p=0.03), with factor independent samples showing higher proportions of human cells in mouse bone marrow than factor dependent samples (Figure 4.4).

100

Mean %CD45⁺ Cells in Marrow

_ 26.8

10

— 7.3

1

0.1

factor
independent
n=   18

factor
dependent
27
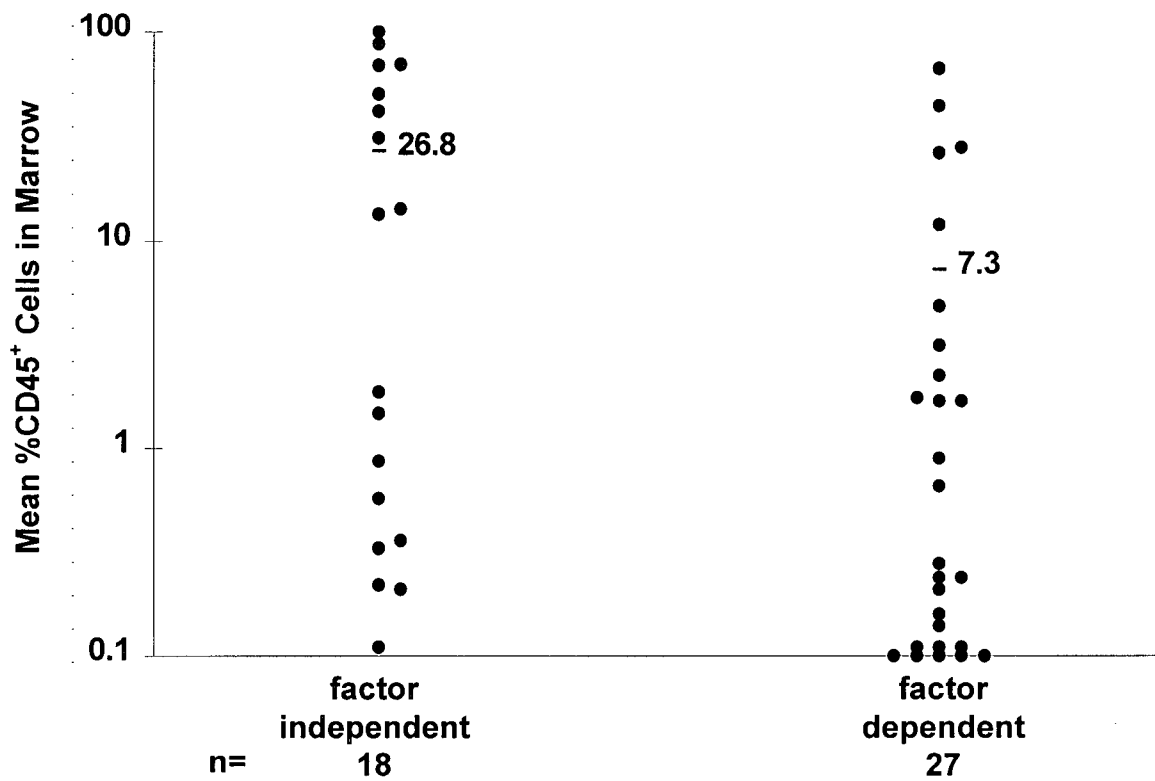
Figure 4.4. AML-CFC factor independence is associated with increased growth of AML cells in mice. Engraftment levels obtained from samples which could be classified as either growth factor dependent or independent in methylcellulose colony assays are compared. Samples which were factor independent showed a significantly higher mean level of engraftment than those which were factor dependent (p=0.03).

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4.2.4 Kinetics of Engraftment

The kinetics with which human leukemic cells engraft and proliferate in NOD/SCID mice were examined in 3 experiments, each performed with a different patient sample. Animals injected with 10^7 cells were sacrificed at various times post-injection, the marrow from 4 long bones was analyzed for $CD45^+$ cells, and the calculated total number of human cells present in the mice was plotted over time as shown in Figure 4.6. Human cells were detectable in 10 to 100-fold lower numbers than the number initially injected 2 days post-injection. Thereafter a rapid expansion of $CD45^+$ cell numbers was seen in the bone marrow which continued throughout the 8 week period of assessment. The fold-increase in human cells between 2 days and 8 weeks post-injection ranged from 20 to almost 5000-fold. These data suggested that a relatively small number of cells with high proliferative capacity in the initial inoculum was responsible for the growth and maintenance of leukemic cells in the animals.
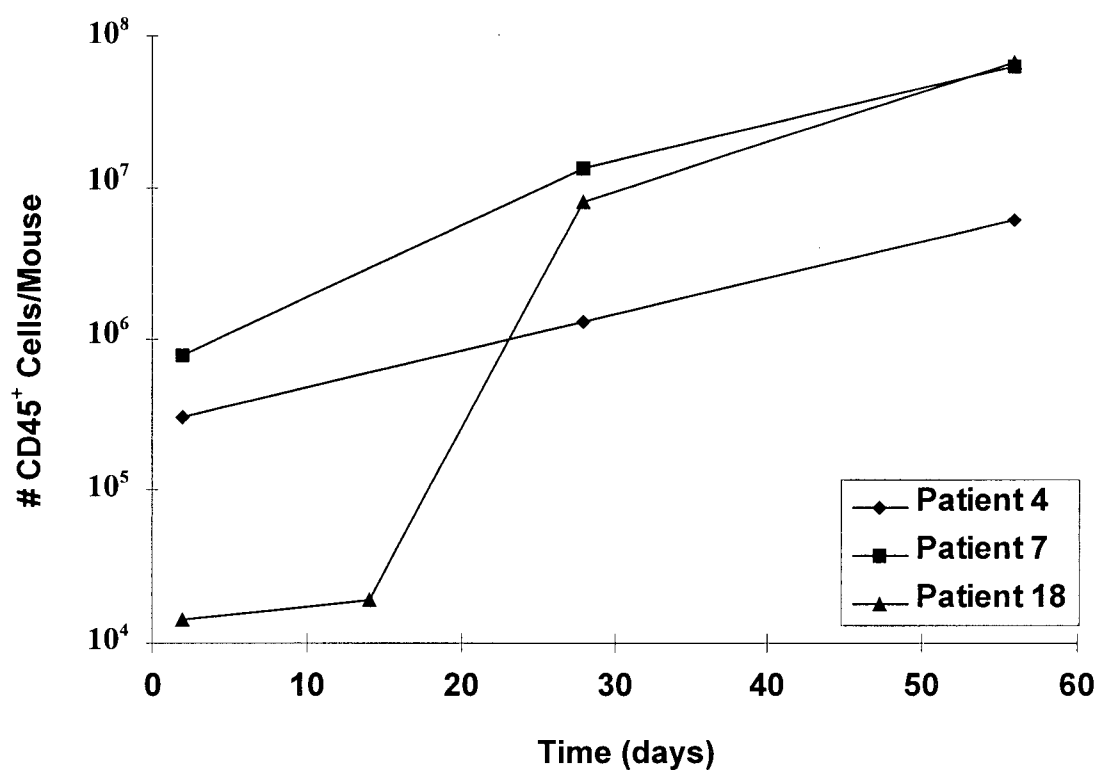
Figure 4.5. Kinetics of AML cell growth in NOD/SCID mice. Animals injected with 10^7 cells from patients 4, 7 and 18 were sacrificed at various time points post-injection and the total number of $CD45^+$ cells per mouse was determined by FACS and plotted over time. A continuous expansion in cell numbers throughout the 8 week period of assessment was seen.

4.2.5   Limiting Dilution Analysis of AML Progenitors Engrafting in NOD/SCID Mice

In the previous chapter it was shown by limiting dilution analysis that the frequency of AML progenitors which initiate longterm culture (AML-LTC-IC) is 10 to >100-fold lower than the frequency of AML-CFC in the same patient sample, and that each LTC-IC will generate more than 1 clonogenic cell progeny over 5 weeks in culture. Using the same principles, the frequency of NOD/SL-IC was determined for several patient samples. Five different doses of the same AML sample ranging from 10^5 to 10^7 cells were injected into cohorts of up to 8 mice each. Using Poisson statistics the frequency of NOD/SL-IC in a given sample was calculated based on the frequency of mice in which $CD45^+$ cells could not be detected in bone marrow 8 weeks after injection of each cell dose. Since the limit of sensitivity of FACS analysis for $CD45^+$ cells was approximately 0.5%, apparently negative FACS results were confirmed by hybridizing Southern blots of genomic DNA from mouse tissues with a human-specific probe for endogenous retroviral sequences (HERV-H; Mager and Freeman 1987). This method allows detection of <0.1% human cells in mouse tissues. The results of limiting dilution experiments with 6 AML samples are shown in Table 4.4. The frequencies of NOD/SL-IC ranged from 0.7 to 45 per 10^7 cells. This number was 214- to 800-fold lower than the frequency of LTC-IC determined previously for 3 of the 6 patient samples.

After determining the frequency of NOD/SL-IC in these samples it was possible to use these values to quantitate the numbers of cell progeny of different types produced per NOD/SL-IC (Table 4.5). The average number of AML blast cell progeny produced by each NOD/SL-IC at week 8 post injection ranged from 1.1×10^6 to 7.5×10^6 cells per NOD/SL-IC (mean $\pm$ SEM = $3.7 \times 10^6 \pm 1.0 \times 10^6$). Assays for AML-CFC were performed on bone marrow cells from mice engrafted with cells from patients 4 and 7. The number of CFC produced at week 8 post-

injection per NOD/SL-IC was 497 and 4877 for cells from patients 4 and 7, respectively. FISH

analysis of cells from colonies grown from such samples showed that 100% of AML-CFC

carried the +8 abnormality characteristic of the leukemic clone (Table 4.5). These same samples

were also analyzed for the presence of AML-LTC-IC. For samples 4 and 7 the average number

of LTC-IC produced per NOD/SL-IC was 299 and 36, respectively, with FISH analysis

confirming the presence of the +8 abnormality in AML LTC-IC from patient 7 (Table 4.5).

Table 4.4. Frequency of NOD/SL-IC in AML Samples

Patient	NOD/SL-IC/10^7 (95% confidence interval)	LTC-IC/10^7 (95% confidence interval)
3	0.7 (0.2-2.6)	150 (102-227)
4	1.8 (0.8-3.8)	500 (367-707)
7	1.5 (0.7-3.0)	1200 (820-1792)
15	21 (11-38)	ND
16	5.0 (2.7-9.9)	ND
17	45 (21-100)	ND

Table 4.5. Cell Production per NOD/SL-IC 8 Weeks Post-Injection into Mice

Patient	Blast Cells Number Produced (x10^6)	Blast Cells FISH*	AML-CFC Number Produced	AML-CFC FISH*	AML-LTC-IC Number Produced	AML-LTC-IC FISH*
3	4.0	13/85				
4	3.4	40/42	497	7/7	299	ND
7	5.1	31/31	4877	30/30	36	3/3
15	1.2	21/78				
16	7.5	ND				
17	1.1	ND				

*FISH – number abnormal/total number of colonies analyzed

4.2.6 Secondary Transplants of NOD/SL-IC

To determine if AML progenitors capable of engrafting in mice persisted for significant time periods post-transplantation and to quantitate their numbers, NOD/SCID animals injected with AML cells 3 to 4 weeks previously were sacrificed and their bone marrow cells transplanted into secondary recipients. When bone marrow from the secondary recipients was analyzed 8 to 12 weeks later, 2 of 3 mice injected with cells from primary recipients of AML cells from patients 15 and 17 and 1 of 3 mice injected with marrow from a recipient of patient 16 cells showed engraftment with human cells (Table 4.6). Using Poisson statistics it can be estimated from this data that the marrow of the primary mice contained approximately 10 to 20 NOD/SL-IC at the time of secondary transfer. Since the primary mice were injected with AML cells from 3 samples for which the frequency of NOD/SL-IC had previously been determined at limiting dilution (Table 4.4), it was possible to compare the number of NOD/SL-IC injected into primary mice initially with the number recovered 3 to 4 weeks later. As shown in Table 4.5, at least in the case of cells from patients 15 and 16, the number of NOD/SL-IC was apparently well-preserved over that time period. Furthermore, FISH carried out on CFC grown from the bone marrow of a secondary recipient of cells from patient 15 harvested 12 weeks post-transplant showed the +13 cytogenetic abnormality characteristic of the leukemic clone in 8 of 9 colonies, confirming the malignant nature of these progenitors.

Table 4.6. Maintenance of NOD/SL-IC Numbers In Vivo

Patient	# NOD/SL-IC per 1° mouse day 0	% BM from 1° mouse injected per 2° mouse *	# positive 2° mice	% CD45$^+$ cells in +'ve 2° mice	Estimated # NOD-SL-IC in 1° mouse at wk 3-4 $^{\psi}$
15	21	5	2/3	1.9, 2.6	20
16	5	5	1/3	1.2	10
17	45	10	2/3	1.8, 3.0	10

* 1° mice were sacrificed 3 to 4 weeks post-injection of AML cells. Marrow harvested from these animals was injected into secondary recipients which were sacrificed for analysis 8 weeks later.

$^{\psi}$ Using Poisson statistics it was estimated that when 2/3 of 2° mice showed engraftment with human cells, on average 1 NOD/SL-IC was present in the fraction of the 1° mouse marrow initially injected into the 2° recipients.

4.3 Discussion

The first description of an in vivo assay for human AML progenitors in immunodeficient mice used sublethally irradiated SCID animals injected with human cytokines (Lapidot et al. 1994). Although this assay for SL-IC was a clear demonstration of the feasibility of this approach, the somewhat low and inconsistent levels of engraftment obtained limited the usefulness of the technique. It has since been shown that the use of NOD/SCID mice as hosts for leukemic progenitors directly isolated from AML patients has greatly enhanced the sensitivity and reproducibility of the NOD/SL-IC assay over that which can be obtained with SCID mice and a variety of other immunodeficient strains (previous work in our lab; Bonnet and Dick 1997). A survey of a large number of AML samples for their ability to grow in NOD/SCID mice showed that 8 weeks after intravenous injection of 10^7 light density peripheral blood cells from newly-diagnosed AML patients, human CD45$^+$ cells were usually easily detected in mouse bone marrow. The leukemic origin of these cells was obvious from their characteristic morphology and could be confirmed by demonstration of chromosomal

abnormalities that had previously been identified in the patient's diagnostic bone marrow cytogenetic analysis. Thus, the use of these animals allows the routine detection and quantitation of primitive leukemic progenitors from most patient samples.

In preliminary experiments it had been observed that the proportion of human cells detected in mouse marrow increased with the number of AML cells initially injected (data not shown). Thus in order to compare the ability of various patient samples to grow in mice a standardized cell dose was selected. The dose of 10^7 cells was chosen because of limitations in cell numbers from some samples and because this number of cells appeared to be adequate to generate reproducible engraftment from many samples. However, as indicated in Table 4.4, the frequency of NOD/SL-IC in some patient samples is less than 1 in 10^7 cells. Thus, the low or undetectable numbers of human cells observed in some cases may reflect the injection of inadequate cell numbers. The 8 week endpoint for most of these experiments was chosen after initial studies had indicated that the level of engraftment continued to increase steadily until that time point and to provide a standard endpoint for comparison of the growth characteristics of different samples. However, it is likely that the choice of a different timepoint would have yielded somewhat different overall results. In a limited number of experiments it was possible to show continued expansion of AML cell numbers up to 12 to 14 weeks post-injection. Unfortunately, due to the inherent tendency of NOD/SCID mice to spontaneously develop malignant thymomas as they age (Shultz et al. 1995), it is difficult to continue these experiments much beyond that time point.

Since the original description of the ability of AML cells to form colonies in semisolid media it has been recognized that AML progenitors share at least some of the cytokine-responsiveness of their normal counterparts (Buick et al. 1977). Although some cytokines which stimulate normal and malignant myelopoiesis show species cross-reactivity between

mice and humans, a number of key molecules, such as IL-3, do not, and others, such as SF, show less potency on cells from a different species of origin (Morstyn and Burgess 1988; Broxmeyer et al. 1991). Thus it seemed reasonable to suppose that provision of relevant human growth factors would enhance the growth of human AML cells in mice. However, it has also been well-documented that progenitors in many AML samples can exhibit varying degrees of factor-independent growth. In many cases this is associated with autocrine growth factor production (Young et al. 1987; Oster et al. 1988; Oster et al. 1989; Rogers et al. 1994). It was therefore not certain whether or not human cytokines would be necessary for the growth of leukemic samples in mice. In fact, using the protocol established in this study, 17 of the 61 samples tested showed >10% replacement of NOD/SCID marrow with leukemic cells in the absence of any human cytokine supplements. In comparing the growth factor requirements for 45 of these 61 AML samples in colony assays with their ability to grow in mice, we discovered that most (9 of 14) samples which showed >10% replacement of mouse marrow with AML cells demonstrated factor independent growth in AML-CFC assays. Although we did not test these samples for autocrine cytokine production, it seems likely that at least some of them were producing factors which stimulated their own growth both in vitro and in vivo.

In this study we chose to inject IL-3 and SF thrice weekly i.p. into mice beginning 4 weeks after injection of AML cells. In previous experiments testing a variety of growth factors alone and in combination it had been found that this combination stimulated maximal proliferation of AML blasts in short term assays (Appendix A, Table A-1), and, as described in Chapter 3, that AML-LTC-IC from some samples also respond to these cytokines. Initiation of cytokine injections was delayed until 4 weeks post-injection of AML cells to allow detection of factor-independent AML cell growth in an initial test animal and thereby avoid cytokine injections if they were unnecessary. Although the dose of both factors administered was high enough to

achieve substantial serum levels immediately post-injection, their relatively short half-life resulted in little or no detectable cytokine by the time of the next injection. Thus, although it is clear that the dose and schedule of cytokine administration chosen did not improve the growth of human AML cells in NOD/SCID mice, beginning the injections earlier or giving them more frequently or at higher dosages may have produced different results. Other investigators have suggested that there is a relationship between the dose of hIL-3 administered and the level of AML cell engraftment observed in SCID mice (Terpstra et al. 1995). The fact that leukemic cells showing cytokine dependent colony growth often failed to grow well in mice also suggests that an alternative strategy for human growth factor administration may be necessary to optimize the sensitivity of the NOD/SL-IC assay. It would be of particular interest to investigate the use of immunodeficient mice transgenic for human cytokines as possible improved hosts for human AML progenitors, as has already been shown for normal hematopoietic progenitors in SCID mice (Bock et al. 1995).

The proportion of leukemic cells detected in mouse marrow after injection of AML samples known to contain chromosomal rearrangements associated with a good clinical prognosis was generally lower than that observed after injection of cells with rearrangements associated with a poor prognosis (Figure 4.1). Similarly, as shown in Figure 4.2, the levels of engraftment were higher with samples from patients with AML FAB subtypes M1, M4, and M5 than with AML-M3 and M2 samples. Clinically AML-M3 has a relatively favourable prognosis as compared to AML-M5, which is associated with an aggressive course and poor overall survival (Tobelem et al. 1980; Warrell et al. 1993). In NOD/SCID mice human leukemic cells will also disseminate from bone marrow into both the peripheral blood and spleen and this occurs as bone marrow infiltration becomes more extensive. Although most of the animals in this study were sacrificed before advanced tumor growth could cause their death,

in some cases unexpected deaths were associated with extensive infiltration of AML cells in marrow and other hematopoietic tissues. Thus the development and spread of leukemia in NOD/SCID mice resembles in many ways the clinical disease in patients.

NOD/SL-IC are rare progenitors as demonstrated at limiting dilution in Table 4.4. These frequencies are consistent with those reported by others using similar techniques and are not greatly different from the frequencies determined for NOD/SCID competitive repopulating units (CRU) in normal human bone marrow (Cashman et al. 1997b). These frequencies are, however, several hundred-fold lower than the frequencies determined for AML-LTC-IC, described in Chapter 3. These relative frequencies are similar to those previously determined for normal CRU and LTC-IC (Hogge et al. 1996; Conneally et al. 1997; Wang et al. 1997). The proliferative capacity of NOD/SL-IC is high. Each of these progenitors is capable of producing more than 10^6 leukemic blasts and hundreds of AML-CFC (Table 4.5). In 2 cases it was possible to show that AML-LTC-IC were also produced in large numbers. These data contrast with the average proliferative capacity of AML LTC-IC, which was shown to be 1.8 and 1.2 AML-CFC per LTC-IC for patient samples 4 and 7, respectively. One interpretation of these differences in frequency and proliferative capacity is that the NOD/SL-IC and AML LTC-IC assays detect different populations of progenitor cells. Studies of retroviral-mediated gene transfer efficiencies and cell surface phenotype have suggested to some investigators that this may be the case for normal LTC-IC and progenitors which are detected in NOD/SCID mice (Larochelle et al. 1996). However, using similar comparisons others have obtained data consistent with a close relationship between these two cell types (Conneally et al. 1997; Conneally et al. 1998). Recent studies have also shown that the majority of AML progenitors which initiate longterm malignant hemopoiesis in culture share the cell surface phenotype of NOD/SL-IC and are predominantly CD71⁻ and HLA-DR⁻ as well as CD38⁻ and CD34⁺

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(Kawagoe et al. 1997; Blair et al. in press). Thus, it appears likely that the LTC-IC and NOD/SL-IC assays detect overlapping if not identical populations of progenitors. The apparent differences in the frequency and proliferative capacity of the cells detected is likely to be at least partly explained by differences in the sensitivity of the two assays and their relative ability to support the proliferative potential of the cells detected.

Under the conditions used in these experiments NOD/SL-IC numbers are maintained at relatively constant levels for at least 3 to 4 weeks post-transplantation into mice (Table 4.6), while other investigators have suggested that an absolute expansion of their numbers may be possible in vivo (Bonnet and Dick 1997). This apparent ability to self-renew their own numbers combined with the potential to differentiate to produce large numbers of more differentiated progenitors and mature leukemic blasts suggests NOD/SL-IC as candidate leukemic "stem cells". These primitive characteristics and the ability to sensitively detect and quantitate NOD/SL-IC from the majority of AML samples suggests the use of this assay for preclinical evaluation of novel therapeutic strategies and to study the relative importance of specific molecular abnormalities in the in vivo behavior of the leukemic clone.

CHAPTER FIVE: GENETIC MODIFICATION OF AML STEM CELLS

5.1 Introduction

The development of optimized protocols for gene transfer into leukemic stem cells in AML would be valuable in several ways. First, the engraftment of mice with retrovirally marked cells would allow clonal analysis of the engrafted cell populations, and provide further information regarding the proliferative capacity and engraftment capability of individual NOD/SL-IC. Second, it would provide a means of developing gene therapy strategies such as those described in Chapter 1. Third, it would provide a means of introducing into leukemic stem cells gene products, such as cytokines, growth factor receptors, or oncogenes, which might affect properties in functional assays, and thereby provide clues to the regulatory processes involved. The effect of introducing such genes could also be examined at the molecular level, for example by looking for changes in gene expression, or at phosphorylation/dephosphorylation and/or changes in binding partners of particular proteins which are known or suspected to be involved in control of cell proliferation, differentiation or apoptosis (eg. tyrosine kinases, transcription factors, proteins involved in cell cycle control). Thus the ability to transfer genes into leukemic stem cells could provide the means for discovering the molecular mechanisms behind leukemogenesis.

One of the goals of the work presented in this chapter was to retrovirally transduce NOD/SL-IC, and to assess the proliferative capacity of these cells by clonal analysis of the engrafted cell population. This was undertaken using the MSCV-Neo retroviral vector described in Chapter 2, using a protocol involving the cocultivation of target AML cells with the retroviral packaging cell line. After transduction, gene transfer efficiencies were assessed by DNA analysis for the neo gene, and cells were injected into mice. At several time points post-injection, mice were assessed for engraftment of leukemic cells, presence of the transgene in the

engrafted cells, and clonality of the engrafted cell population. When it was found that a 5-day transduction protocol led to a loss of the NOD/SCID-repopulating ability in most samples, the effect of placing AML cells in vitro in the presence or absence of various cytokines for various periods of time on subsequent engraftment ability was assessed. This was compared to the effect of the same culture conditions on CFC and LTC-IC from the same samples.

In order to obtain purified populations of transduced cells, primary AML cells were infected with a vector carrying both the neo gene and the murine HAS gene. Very large numbers of AML cells are needed to set up LTC-IC and NOD/SL-IC assays, making the use of FACS to separate transduced from untransduced cells impractical. Instead, large numbers of AML cells infected with MSCV-HSA-Neo virus were purified using an immunomagnetic column to isolate the HSA$^+$ cells, as described in the Materials and Methods, section 2.21.

As already mentioned, it appeared that the 5 day transduction protocol initially used for the MSCV-Neo experiments resulted in a loss of stem cell function. Since it was possible that decreasing the time required in culture would avoid this impairment, a time course study was performed to determine the minimum length of time required for reasonable gene transfer levels to be achieved. If it was found that such a decrease in culture time resulted in an improvement in stem cell maintenance, the use of column separation should allow a highly enriched population of transduced cells to be isolated even if gene transfer efficiencies were reduced.

Similar experiments were performed using filtered retroviral supernatant rather than co-cultivation with viral producers as a transduction protocol. The use of cocultivation has the associated risk of contamination of target cells with retroviral producer cells. This protocol is therefore unusable in a clinical setting, where there would be a risk of transplanting the patient with producer cells, which are derived from immortalized cell lines, and which have a slight possibility of eventually producing replication-competent retroviruses. The use of filtered

supernatant led to significantly lower initial transduction efficiencies than were achieved by co-cultivation. However, after immunomagnetic purification of HSA$^+$ cells, some enrichment was achieved, and easily detectable neo$^+$ CFC and HSA$^+$ LTC-IC were present in the enriched populations.

Different retroviruses utilize different cell surface molecules for cell entry, and expression levels of such receptors in target cells has been shown to have an effect on the transduction efficiency by the corresponding retrovirus (Miller 1996; Kiem et al. 1997). Retroviral packaging cell lines are available which package retrovirus particles in different envelope proteins, the most notable ones being the murine leukemia virus amphotropic envelope, which binds to the Ram-1 receptor on target cells, and the gibbon-ape leukemia virus envelope, which binds to the Glvr-1 molecule on target cells (Miller 1996). It has been shown that Glvr-1 is expressed at higher levels in primitive hematopoietic cells than Ram-1, and that correspondingly, hematopoeitic cells are more efficiently transduced with GALV-pseudotyped retroviral vectors than with amphotropic retroviral vectors (Orlic et al. 1996; Kiem et al. 1997). It therefore seemed possible that gene transfer efficiencies into AML cells could also be improved by using a GALV-pseudotyped vector. In order to investigate this, the retroviral vector MSCV-EGFP-Neo was packaged in either the GP+envAM12 (amphotropic envelope) or the PG13 (GALV envelope) packaging cell lines, and transduction efficiencies of AML cells by these two virus particles were compared. GFP was originally isolated from the jelly fish *Aequorea victoria* (Chalfie et al. 1994), and has since been modified for optimal expression in mammalian cells (enhanced GFP, or EGFP; Yang et al. 1996). It has the advantage that its fluorescence is intrinsic to the primary structure of the protein, and does not require substrates or cofactors to fluoresce (Cubitt et al. 1995). It therefore allows very rapid and efficient assessment of gene transfer by simple flow cytometry and/or fluorescent microscopy. 3 AML

samples were selected and their cells sorted by flow cytometry into CD34$^+$CD38$^-$,

CD34$^+$CD38$^+$, and CD34$^-$ fractions. Each fraction was then transduced with either amphotropic

or GALV-pseudotyped MSCV-EGFP-Neo, and transduction efficiencies into total cells and

CFC were assessed.

5.2   Results

The patient samples used for experiments described in this chapter are listed in Table 5.1.

Table 5.1. AML Patient Samples – Clinical Characteristics

Patient	Age (yr)	Sex	FAB Type	WBC(%blasts)* (X 10^9/L)	Bone Marrow Cytogenetics [% abnormal]	FISH Probe
4	69	F	M4	65 (83)	47,XX,+8 [100]	8
6	69	F	M5B	155 (95)	46,XX,t(9;11)(p22q23)	11q23
15	58	F	M4	370 (46)	46,XX; 47,XX,+13 [37]	13
18	48	M	M5a	46	46,XY [0]	
19	58	M	M4	103 (90)	46,XY; 46,XY,del(16)(q22)	
20	37	M	M4	80 (80)	46,XY,+7(q32),inv(16)(p13q22); 47, idem,+22	
21	65	F	M4	88 (70)	46,XX,inv(16)(p13q22)	
22	64	F	M4	151 (79)	46,XX	
23	74	F	M4	215	46,XX,del(11)(q14q25)	
24	78	F	M1	434 (93)	46,XX	
25	68	M	M2	13.4 (90)	48,X,+I(X)(q10),-Y,-5,+6,+8,+11,-12, -13,+14,-16,-17,+I(21)(q10), +add(22)(q13),+mar1,+mar2,0-43dmin	

*WBC, peripheral blood total white blood cell count at diagnosis.

5.2.1 Gene Transfer of the Neomycin-Resistance Gene to NOD/SL-IC

The MSCV vector carrying the neo gene under the control of the murine phosphoglycerate kinase promoter was used to tranduce primary AML cells. The method of transduction involved prestimulating the cells with a combination of IL-3 and SF, a choice based on previous studies which showed that >90% of AML samples will proliferate in response to this combination of factors as measured in short-term ^{3}H-thymidine incorporation assays (Appendix A). Following 48 hours of prestimulation, the cells were co-cultivated with the AM12-MSCV-Neo producer cells for a further 48 hours, still in the presence of IL-3 and SF, plus polybrene. After a final 24 hours of culture, the cells were removed and injected into NOD/SCID mice at a dose of 10^7 each. An aliquot of cells was removed prior to injection, and genomic DNA purified and analyzed by Southern blotting for the presence of the transgene. A dilution series of DNA from MO7e-J-IL-3 cells, which carry a single copy of the neo transgene, diluted into mouse DNA was used to compare band intensities to determine the gene transfer efficiency. Gene transfer efficiencies to AML cells from patients 4, 6, 19, and 21 were 30%, 80%, 30% and 50%, respectively, as shown in Figure 5.1.
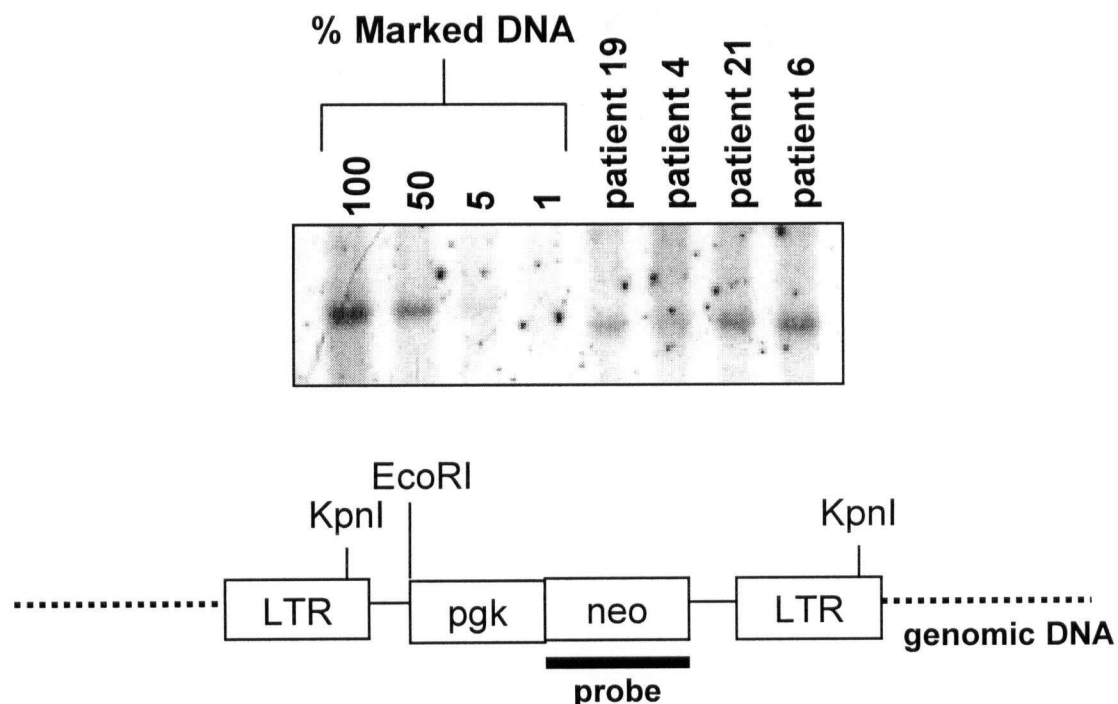
Figure 5.1. Gene transfer efficiency to bulk AML cells. Genomic DNA was purified from cells immediately following the retroviral transduction protocol. DNA was digested with KpnI, which excises a 2.5 kb internal segment of the retroviral sequence containing the neo gene. A dilution series of genomic DNA from the cell line MO7e-J-IL-3 diluted into mouse genomic DNA, digested with KpnI to yield a 3 kb fragment containing the neo gene, was included in the same Southern. The blot was then probed with the neo sequence. Gene transfer efficiencies to cells from patients 4, 6, 19, and 21 were estimated to be 30%, 80%, 30% and 50%, respectively.

The engraftment levels attained in the hind bone marrow, front bone marrow, spleen and blood of these animals are shown in Table 5.2. Engraftment levels were determined by hybridizing Southern blots of genomic DNA from mouse tissues with a HERV-H probe, which is specific for human DNA. By comparison with a dilution series of human DNA into mouse DNA on the same blot, the proportion of human DNA present in each sample could be estimated. The limit of sensitivity of this technique is approximately 0.1%, as illustrated in Figure 5.2.

With patient 4 and 20 cells, week 8 engraftment levels of bone marrow were low upon injection of freshly thawed cells directly into mice (5% and 1%, respectively), so it is not surprising that high engraftment was not achieved in mice that received these cells post-gene transfer. Patient 19 cells had been previously shown to engraft SCID mice to a high level. With pt 6 and 21 cells, injection of 10^7 cells directly into mice led to 70% and 35% engraftment of week 8 hind bone marrow, respectively. These latter 3 samples thus appear to have lost their ability to engraft mice at some point during the gene transfer protocol. Only with patient 15 cells were engraftment levels at least equivalent to those obtained with freshly thawed cells.

In order to assay for the presence of the transgene in mouse tissues, either Southern blots or semiquantitative PCR for the neo gene were performed. The limit of sensitivity of the Southern blotting technique was approximately 5% of neo^+ DNA diluted into mouse genomic DNA, whereas the PCR technique could detect concentrations as low as 0.01% (Figure 5.3). The presence of the neo gene was demonstrated in at least some of the hematopoietic tissues of every single mouse tested except one (pt 20, wk 9), even in cases where engraftment was undetectable (i.e. <0.1%; Table 5.2). An unusual result was obtained in the patient 4, week 12 mouse, where PCR for neo indicated the presence of the transgene at levels >0.1% even though engraftment was undetectable in this mouse. This was most likely due to contamination of the

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DNA samples from this animal with neo sequences. In the case of patient 15, with which high

levels of engraftment occurred, significant levels of the engrafted cell populations were

retrovirally transduced (Table 5.2). In the week 9 mouse, 10% of the hind bone marrow and

80% of the front bone marrow were engrafted, and 5% of each of these bone marrow samples

contained neo$^+$ cells, meaning that 50% and 6% of the engrafted AML cells were transduced in

the hind and front bone marrow of this mouse, respectively. The leukemic nature of the

engrafted cells was confirmed by morphological analysis of cells in bone marrow smears. In the

week 13 mouse, 80% of the hind and 100% of the front BM were engrafted (Figure 5.4A), and

10% and 50% of these samples were neo$^+$ (Figure 5.4B), meaning that 12% and 50% of the

engrafted cells were transduced. In the latter case, sufficient transduced cells were present to

perform clonal analysis. This was done by digesting genomic DNA with EcoRI, which has only

a single site within the provirus, so that the neo probe will hybridize to a uniquely-sized

fragment for each individual retroviral integration site. In this way, the number of individual

retrovirally-marked stem cells from which the transduced cells in the mouse's bone marrow are

derived can be determined. It was shown that the transduced population of cells in the front

marrow sample of the sample 15-engrafted mouse was made up of predominantly 2 clones of

cells (Figure 5.4C), indicating the high proliferative capacity of the cells which initiated

engraftment of the bone marrow in this mouse.

Table 5.2. Engraftment of NOD/SCID Mice with Retrovirally Marked AML Cells

Patient	Mouse Tissue	Week 8 % Eng (Uninfected Cells)	Week 8		Week 15	
			% Eng	% Neo[+]*	% Eng	% Neo[+]*
19	Hind BM	100[φ]	<0.1	0	0.1	0
	Front BM		<0.1	0.01	0.5	0.1
	Spleen		<0.1	0	<0.1	0
	Blood		<0.1	0	<0.1	0
			Week 9		**Week 13**	
15	Hind BM	50	10	5	80	10
	Front BM		80	5	100	50
	Spleen		<0.1	0	0.5	0
	Blood		<0.1	0.01	0.1	nd
			Week 9		**Week 12**	
20	Hind BM	1	<0.1	0	<0.1	0
	Front BM		<0.1	0	<0.1	0.01
	Spleen		nd	0	<0.1	0.01
	Blood		nd	0	<0.1	0.01
			Week 9		**Week 12**	
4	Hind BM	5	<0.1	0.1	<0.1	0.5
	Front BM		<0.1	0	<0.1	5
	Spleen		<0.1	0.01	<0.1	1
	Blood		<0.1	nd	<0.1	1
			Week 9		**Week 12**	
21	Hind BM	35	<0.1	0.01	<0.1	0.02
	Front BM		<0.1	0	<0.1	0.02
	Spleen		<0.1	0.01	<0.1	0.05
	Blood		<0.1	0.01	<0.1	0.05
			Week 8		**Week 14**	
6	Hind BM	70	5	0.1	<0.1	0.01
	Front BM		2	0.2	<0.1	0.01
	Spleen		<0.1	0	<0.1	nd
	Blood		<0.1	0.01	<0.1	nd

* Proportion of cells in tissue which contain the neo gene, as determined by Southern blotting or by semiquantitative PCR. Each time point for each patient sample represents a single mouse.
φ This value was obtained in a SCID mouse, 3 weeks after injection of cells.
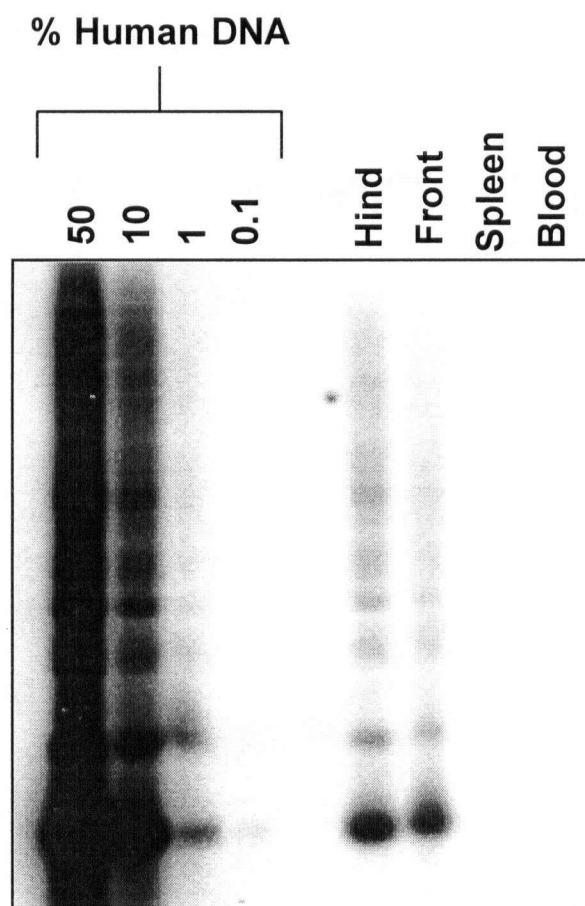
Figure 5.2. Southern blot method to determine levels of human cell engraftment in NOD/SCID mouse tissues. Genomic DNA was purified from mouse tissues and digested with EcoRI. A dilution series of genomic DNA from the human cell line MO7e into mouse genomic DNA, also digested with EcoRI, was included in the same Southern. The blot was then probed with the HERV-H sequence, which is present in the human genome in approximately 1000 copies. This technique is sensitive down to 0.1% of human DNA in mouse DNA. The hind bone marrow, front bone marrow, spleen and blood samples illustrated here are from a mouse that was injected with retrovirally transduced patient 6 cells, 8 weeks post-injection. The engraftment levels in these tissues were estimated to be 5%, 2%, <0.1% and <0.1%, respectively.

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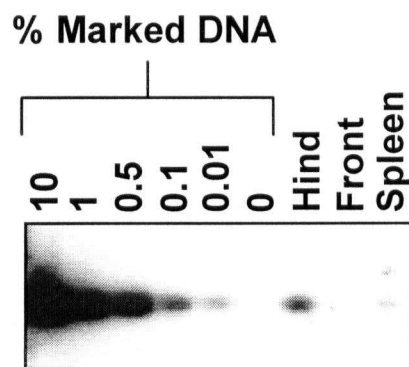
Figure 5.3. Semiquantitative PCR for neo to determine levels of neo$^+$ DNA present in tissues with low or undetectable levels of engraftment. 200 ng of genomic DNA purified from mouse tissues was subjected to 21 cycles of PCR using neo-specific primers, as described in Chapter 2. A dilution series of MO7e-J-IL-3 DNA was included for each PCR run. PCR products were then electrophoresed, Southern blotted and probed with the neo sequence. This blot illustrates that this technique is sensitive down to 0.01% neo$^+$ DNA in mouse DNA. The hind bone marrow, front bone marrow and spleen samples shown here are from a mouse that was injected with retrovirally transduced patient 4 cells, 9 weeks post-injection. The % neo$^+$ DNA in these tissues was estimated to be 0.1%, 0% and 0.01%, respectively.
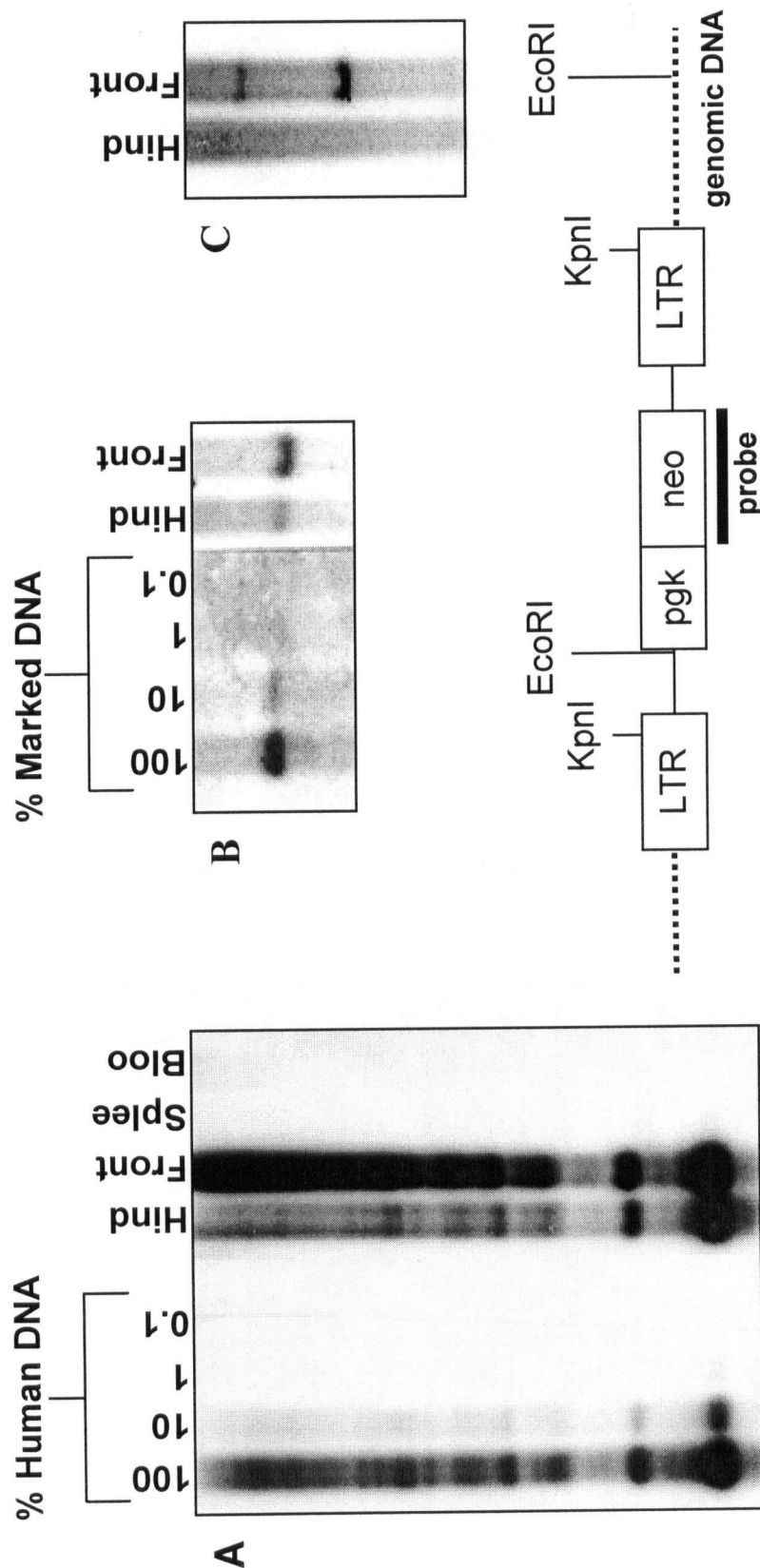
Figure 5.4. Analysis of DNA from tissues of a mouse 13 weeks after injection with retrovirally transduced cells from patient 15. (A) Southern blot of genomic DNA digested with EcoRI and probed with HERV-H. Engraftment levels in hind bone marrow, front bone marrow, spleen and blood were estimated to be 80%, 100%, 0.5%, and 0.1%, respectively (the latter 2 signals became visible upon longer exposure of the same blot to film). (B) Southern blot of genomic DNA digested with KpnI and probed with the neo sequence. Levels of neo$^+$ DNA in hind bone marrow and front bone marrow were estimated to be 10% and 50%, respectively. (C) Southern blot of genomic DNA digested with EcoRI and probed with the neo sequence, yielding a unique fragment size for each individual clone of transduced cells. The front bone marrow contains predominantly two clones, illustrating that the marked population of cells in the front bone marrow of this mouse was initiated by 2 cells that were present in the inoculum.

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5.2.2 Engraftment of AML samples in NOD/SCID mice is impaired within 24 hours of tissue culture

Cells from patients 6, 19 and 21, which could achieve high levels of engraftment in SCID or NOD/SCID mouse bone marrow when injected directly following thawing of cells, failed to do so when subjected to the gene transfer protocol. In order to determine if placing these cells in tissue culture for several days was causing this impairment, a series of time course studies was performed in which 3 AML samples which were known to engraft well when injected directly into mice were placed into suspension cultures for 4, 12 or 24 hours prior to i.v. injection, and bone marrow engraftment levels were determined by FACS for $CD45^+$ cells 8 weeks later. In addition, various combinations of growth factors were tested in an attempt to find conditions which would improve the maintenance of NOD/SL-IC in vitro. These conditions included the addition of FL, TPO, and/or hyper-IL-6 (a fusion of the soluble IL-6 receptor and IL-6; Chebath et al. 1997), as it has been previously shown that these factors facilitate in vitro maintenance of primitive cells present in normal hematopoietic samples (Petzer et al. 1996; Young et al. 1996; Borge et al. 1997; Chebath et al. 1997; Haylock et al. 1997; Ramsfjell et al. 1997). Samples from pts 6, 18 and 21 achieved mean engraftment levels of 70%, 87%, and 35%, respectively when 10^7 cells were injected directly into mice. After 24 hours of culture in the same basic conditions as used for gene transfer (i.e. 20% FCS in IMDM), plus various combinations of cytokines, in all conditions tested a significant reduction in engraftment levels was seen after 24 hours of culture (Figure 5.5). No cytokine combination tested was significantly better than any other in maintaining NOD/SL-IC. In patient 21-injected mice, the levels of engraftment were lower following only 4 hours of culture, whereas in patient 6-injected mice, engraftment levels were still relatively high after 12 hours of culture, and

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decreased sometime between 12 and 24 hours. In patient 18-injected mice, engraftment levels were somewhat reduced after 12 hours, and further reduced after 24 hours of culture. Thus the engraftment ability was impaired in 3 patient samples which are normally capable of achieving high levels of engraftment in NOD/SCID mice. This suggests the absence of appropriate conditions for the maintenance of NOD/SL-IC during the culture period, both in these time course experiments and in the gene transfer experiments described previously. It is therefore necessary to determine appropriate conditions for the maintenance of NOD/SL-IC in vitro before any further gene transfer studies are performed.
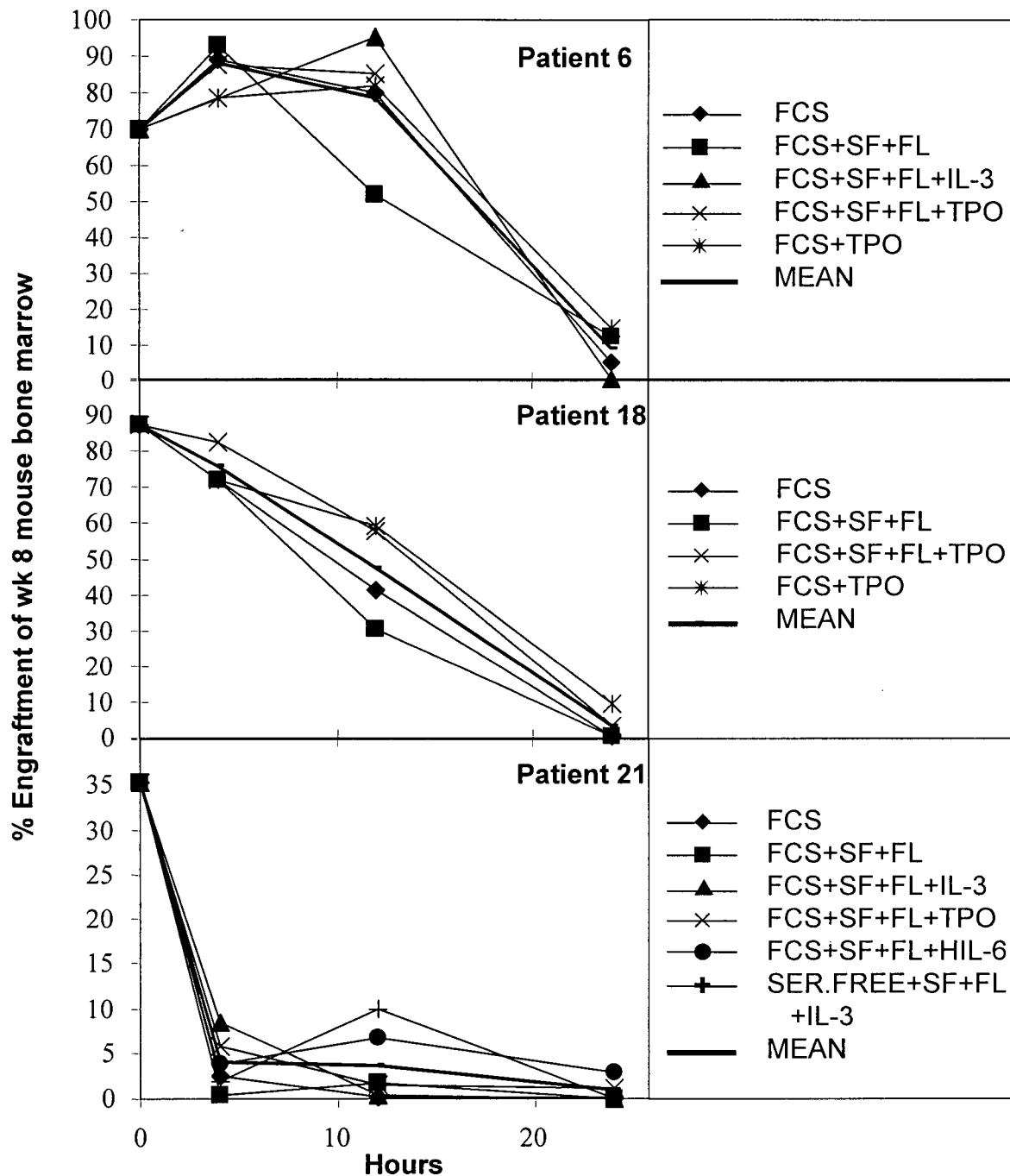
Figure 5.5. Suspension culture of AML cells in a variety of culture conditions leads to a loss of mouse-repopulating ability within 24 hours. 3 samples which are capable of achieving high levels of engraftment when injected directly into NOD/SCID mice (70%, 87%, and 35% of hind bone marrow, respectively, for patients 6, 18 and 21) were placed in suspension cultures with various combinations of cytokines. Cells were harvested after 4, 12 and 24 hours of culture and injected into NOD/SCID mice. In all conditions tested, engraftment levels of hind bone marrow 8 weeks following injection were drastically reduced relative to levels achieved at time 0. FCS=20% fetal calf serum; SF=steel factor; FL=flt-3 ligand; IL-3=interleukin-3; TPO=thrombopoietin; hIL-6=hyper-interleukin-6; ser. free = serum free.

Cells from some of the culture conditions described above were also assayed for CFC and LTC-IC, to see if these cell types were maintained during the 24 hour culture period. The results of these assays are shown in Table 5.3. 24 hours in any of the culture conditions tested did not significantly reduce the number of CFC present in any of the samples; in all 3 cases the numbers are either maintained or expanded. Similarly, AML-LTC-IC numbers were not significantly reduced with patient 21 cells for two conditions tested, or with patient 6 cells for 4 conditions tested. With patient 18 cells, a reduction in LTC-IC was seen after 24 hours with serum and TPO, but in cultures containing serum plus SF and FL or serum plus SF, FL and TPO, LTC-IC numbers were maintained, suggesting that these factor combinations allowed the maintenance of AML-LTC-IC in this sample. However, based on the impaired engraftment ability of these same cells, maintenance of AML-LTC-IC in vitro does not correlate with maintenance of NOD/SL-IC in the same cultures.

Table 5.3. Maintenance of CFC and LTC-IC after 24 Hours in Culture

Patient	Assay	Time 0	FCS	FCS+ SF+FL	FCS+ SF+FL +IL-3	FCS+ SF+FL +TPO	FCS+ TPO	FCS+ SF+FL +hIL-6
6	CFC*	1060	950	1290	1010	1250	950	
	LTC-IC**	>5000		2195	4914	2204	3006	
18	CFC	100	180	290		320	170	
	LTC-IC	160		167		105	43	
21	CFC	10				160		140
	LTC-IC	38				45		56

*number of colonies per 10^6 cells
**number of colonies per 10^6 cells plated into LTC

5.2.3   Gene Transfer of Murine HSA into AML samples and Immunomagnetic Purification of

Transduced Cells

5.2.3.1 Transduction by Cocultivation – A Time Course Study

To enable purification of transduced cells, the murine HSA gene was included in the

retroviral vector together with the neo gene (MSCV-HSA-Neo). Initial experiments were done

by cocultivation of patient 18 cells with retroviral producers. To shorten the culture period as

much as possible, the prestimulation step was eliminated, and a time course study was done to

determine the minimum time required to achieve significant levels of gene transfer. Times in

coculture were 10, 24 and 36 hours. Cells were then removed and immediately assessed for

gene transfer, or purified with the immunomagnetic system described in Chapter 2. The

proportions of HSA$^+$ cells, both before and after immunomagnetic purification, are shown in

Table 5.4. After 10 hours of coculture, cells were 5 to 10% HSA$^+$ by FACS, and this was

increased to 42% and 67% in the enriched fraction by magnetic purification. By 24 hours of

coculture, the initial transduction efficiency was 20 to 25%, with enrichment to 75% after

magnetic purification. Extending cocultivation to 36 hours did not improve on this, with initial

transduction efficiencies of 22% to 28%, and enrichment to 42% to 49%. The 36 hour coculture

period may have had a detrimental effect on the immunomagnetic purification due to the

presence of a greater number of dead viral producer cells mixed with the transduced cells to be

purified. An example of flow cytometry on transduced cells before and after immunomagnetic

enrichment is shown in Figure 5.6.

Cells from the different fractions were assayed for colony forming ability, and the results

of this are shown in Table 5.5. The frequency of CFC in this patient sample at time 0 (i.e.

before retroviral transduction) was 110 per 10^6 cells. This frequency was not reduced in the

precolumn fraction or the enriched fraction at any of the three time points. The proportion of CFC from the enriched fraction that were transduced was determined by PCR for the neo gene on individual colonies. The proportion of neo$^+$ CFC in the enriched fraction, was high, 100% after 10 hours of coculture and 70% after 24 hours of coculture.

Cells from the enriched fraction were also assessed for gene transfer to LTC-IC, as shown in Table 5.6. Time 0 cells produced 246 CFC per 10^6 cells plated. After 10, 24 and 36 hours in coculture with viral producers, this number was somewhat reduced (to 6, 42 and 65 CFC per 10^6 cells plated, respectively), but significant numbers were still produced, indicating no significant loss of LTC-IC during the gene transfer procedure. The proportion of these CFC that were positive for the transgene was again determined by neo-PCR, and it was found that after 10 hours, 0% of CFC arising from LTC were transduced, whereas after 24 hours 56% were transduced and after 36 hours 29% were transduced. It thus appears that, although 10 hours was sufficient to transduce CFC, it was not sufficient to transduce LTC-IC, for which at least 24 hours of cocultivation was required.

Table 5.4. Gene Transfer to AML Cells by Cocultivation with AM12-MSCV-Neo Producers, and Enrichment by Immunomagnetic Purification of HSA$^+$ Cells

Experiment[#]	Hours in Coculture	% Cells HSA$^+$*	
		Precolumn Fraction	Enriched Fraction
1	36	22%	42%
2	10	5%	42%
	24	25%	75%
	36	28%	49%
3	10	10%	67%
	24	20%	75%

*Determined by flow cytometry
[#]All 3 experiments were performed on cells from patient 18

Table 5.5. Recovery of CFC from Cocultures and Proportion Transduced in the Enriched Fraction

	CFC per 10^6 Cells		%CFC in Enriched Fraction	%CFC neo$^+$ (#+'ve/#analyzed)
Hours	Precolumn	Enriched		
0	110			
10	135	75	20%	100% (9/9)
24	110	140	55%	70% (7/10)
36	280	85	40%	ND

Table 5.6. Recovery of LTC-IC from Cocultures and Proportion Transduced in the Enriched Fraction

Hours	CFC/10^6 Cells Plated in LTC	% CFC from LTC Neo$^+$ (#+'ve/#analyzed)
0	246	
10	6	0% (0/7)
24	42	56% (9/16)
36	65	29% (9/31)
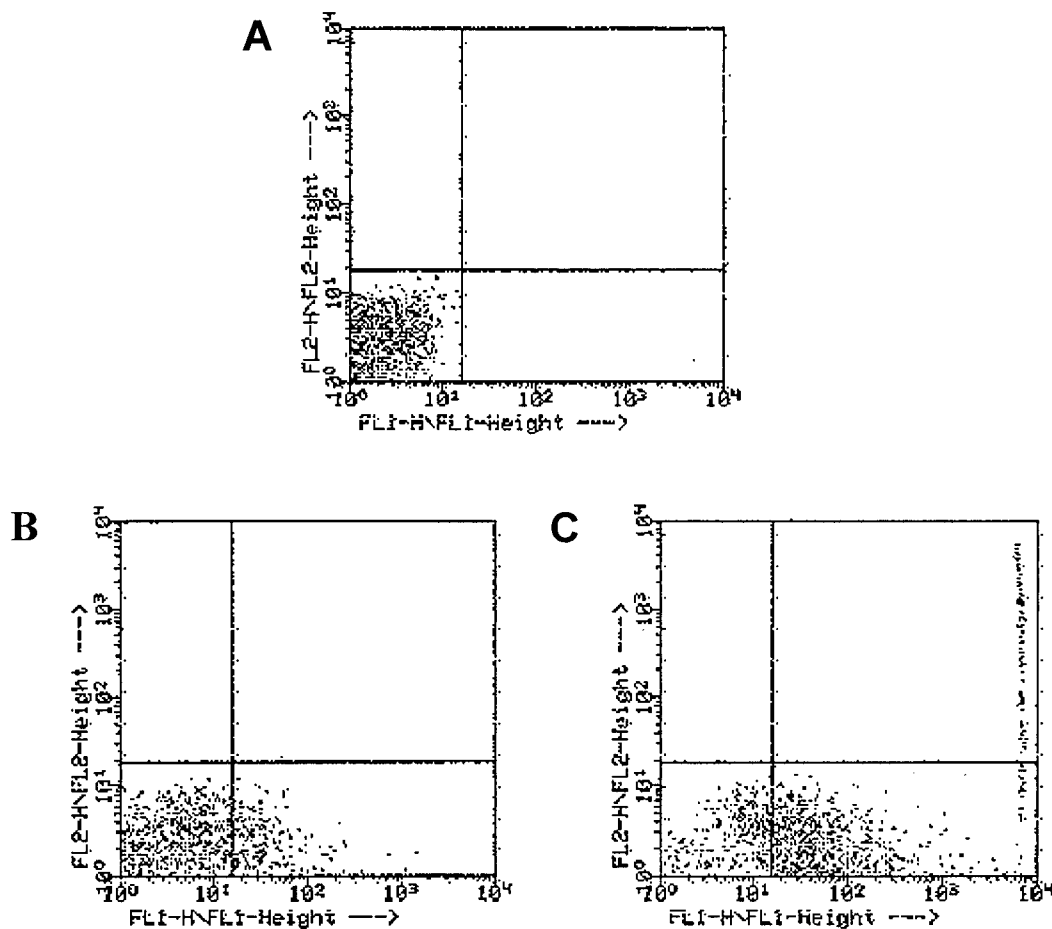
Figure 5.6. Flow cytometry on MSCV-HSA-Neo-transduced cells (coculture method) before and after immunomagnetic separation. (A) Precolumn cells labeled with isotype control (IgG1-FITC). (B) Precolumn cells labeled with M1/69-FITC, which is specific for murine HSA; positive fraction is 25%. (C) Enriched cell fraction, labeled with sheep-anti-mouse-FITC, to detect M1/69-biotin-labeled cells after their removal from the column; positive fraction is 75%. All profiles shown are from the 24 hour time point in experiment 2.

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5.2.3.2 Transduction by Retroviral Supernatant – A Clinically Relevant Protocol

To avoid direct contact of target cells with viral producer cells, experiments were performed using filtered medium conditioned by the producer cells as a source of retrovirus. In all cases, cells were incubated with retroviral supernatant containing MSCV-HSA-Neo on fibronectin-coated dishes for 24 hours prior to immunomagnetic purification and assessment of gene transfer efficiencies in the precolumn and enriched fractions. The use of fibronectin has been shown to improve gene transfer efficiencies to hematopoeitic cells by retroviral supernatant, possibly as a result of colocalization due to binding of both viral particles and hematopoietic cells to the fibronectin molecules. Alternatively, binding of fibronectin to its receptors on the target cell surfaces (VLA-4) may mediate cell signalling events which control viral entry and/or integration (Moritz et al. 1994). Again cells were assayed for CFC and LTC-IC, and PCR was performed on individual colonies to determine gene transfer efficiencies to these subpopulations. The results of these experiments are summarized in Table 5.7. For these experiments, to be more accurate with regard to transduction of CFC as determined by PCR, two changes were made to the protocol. Instead of simply using primers specific for the neo gene, two primer sets were included in each reaction, one for the transgene and one for GAPDH, to serve as a positive control for the presence of DNA. Since the colonies being assayed were frequently very small (<50 cells each), it was desirable to be sure that DNA was indeed present in cases where the colony was negative for the transgene. The second change was that for colonies arising from LTC, the transgene assayed for by PCR was changed from the neo gene to the HSA gene. This was because the LTCs contained the feeder layer Sl/Sl-J-IL-3, which contains the neo gene. It is therefore possible for plucked colonies to be contaminated by neo$^+$ cells from the adherent layer of the LTC, resulting in false positives.

Examples of neo-PCR and HSA-PCR on individual colonies are illustrated in Figure 5.7. The

data in Table 5.7 illustrate that initial gene transfer efficiencies, as shown by the % HSA^+ cells

in the precolumn fraction, were lower after 24 hours of gene transfer, ranging from about 2 to

5% in most cases (with the exception of patient 7). Experiments were done in parallel on

normal bone marrow, and initial transduction efficiencies were 4 to 15%. After purification,

proportions of transduced cells were increased, but very high purities were not obtained, except

with patient 7 cells, where the transduction efficiency was initially high (the high transduction

level with this sample was confirmed by semiquantitative PCR on genomic DNA, which

indicated >50% neo^+ DNA in the enriched fraction). The immunomagnetic column system as

used here does not appear to be efficient for the purification of cells which make up only a

small proportion of the starting population, and conditions to improve this remain to be found.

Again, CFC and LTC-IC were assayed for transduction by PCR for neo and HSA, respectively,

and colonies positive for the transgene could be found in significant numbers in both cases.

Before enrichment the transduction efficiency of AML-CFC was 5 to 22%, and after

enrichment this was increased to 12.5 to 75%. The transduction efficiency of leukemic LTC-IC

before enrichment was 10%, and after enrichment this was increased to 25%. In the case of

normal bone marrow, the CFC transduction efficiency was increased from 6-12 % to 10-38%,

and the LTC-IC transduction efficiency was increased from 47% to 60%. Thus significant

frequencies of transduced CFC and LTC-IC could be obtained using a clinically relevant

retrovirus-containing supernatant method combined with immunomagnetic purification based

on HSA expression.

Table 5.7. Supernatant Gene Transfer Efficiencies

Sample	% Cells HSA$^+$ (FACS)		% CFC neo$^+$ (PCR) (#+'ve/#analyzed)		% LTC-IC HSA$^+$ (PCR) (#+'ve/#analyzed)	
	Precolumn	Enriched	Precolumn	Enriched	Precolumn	Enriched
7	57%	87%	5% (1/20)	12% (2/16)		
11	3%	14%	25% (4/16)	75% (3/4)		
22	5%	27%				
23	4%	18%	22% (4/18)	73% (8/11)	10% (1/10)	25% (3/12)
24	2%	17%				
NBM-1*	4%	10%				
NBM-2	16%	34%	6% (1/17)	10% (2/20)	47% (8/17)	60% (6/10)
NBM-3	7%	20%	12% (2/17)	37% (6/16)		

*NBM=normal bone marrow

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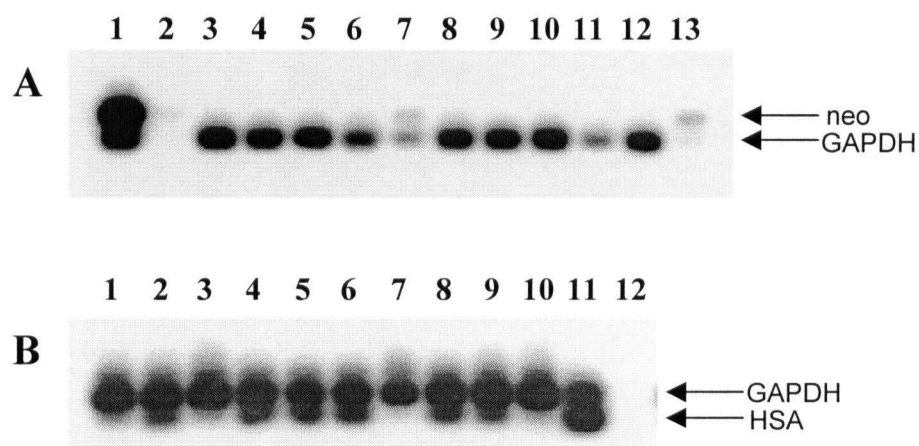
Figure 5.7. Analysis of colonies for the presence of transgenes after transduction with MSCV-HSA-Neo. (A) Lane 1: Positive control; DNA from MO7e-J-IL-3 cells. Lane 2: Negative control; H_2O. Lanes 3-13: Individual colonies from the precolumn fraction of cells from patient 11. Two primer sets were included in each reaction, one specific for the neo gene and one for the GAPDH gene, yielding fragment sizes of 514 bp and 300 bp, respectively. The GAPDH primers are a positive control to confirm the presence of DNA in the reaction. All 11 colonies were positive for GAPDH, and colonies 7 and 13 were positive for the neo gene. (B)Lanes 1-10: Individual colonies from LTCs of the enriched fraction of NBM-2 cells. Lane11: Positive control; genomic DNA from patient 22 enriched fraction, which was shown to be 26.6% HSA^+ by FACS. Lane 12: Negative control; H_2O. The two primer sets in this case were for HSA and GAPDH, yielding fragment sizes of 148 bp and 300 bp, respectively. All 10 colonies were positive for GAPDH, and colonies 2, 4, 5, 6, 8, and 9 were positive for the HSA gene.

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5.2.3.3 Gene Transfer Efficiency to AML Cells by Amphotropic Vs GALV-Pseudotyped Retroviruses

In order to determine whether gene transfer efficiencies into AML cells with the MSCV vector could be improved by using a different pseudotype, as had been suggested for normal hematopoietic cells in the literature (Orlic et al. 1996; Kiem et al. 1997), the MSCV-GFP-Neo vector was packaged in either GP+envAM12 cells, which contain the amphotropic envelope gene, or in PG13 cells, which contain the GALV envelope gene. EGFP was used as a reporter gene for these experiments allowing gene transduction efficiencies to be determined directly by FACS analysis or fluorescent microscopy, with no intermediate steps, such as antibody labeling or PCR, being required. 3 AML samples were separated into CD34$^+$38$^-$, CD34$^+$38$^+$ and CD34$^-$ subpopulations by flow cytometry. Patient samples were selected which, based on previous studies, were known to have a fairly even distribution of cells among the 3 fractions so that sufficient numbers of cells from each would be available for gene transfer (18-32% were CD34$^+$CD38$^-$; 30-62% were CD34$^+$CD38$^+$; 19-47% were CD34$^-$). Each fraction was divided in half, and one half was transduced with amphotropic MSCV-EGFP-Neo and the other half was transduced with GALV-pseudotyped MSCV-EGFP-Neo (also a small number of cells was mock transduced as a negative control). For all 3 samples the efficiency of gene transfer into CD34$^+$CD38$^-$, and CD34$^+$CD38$^+$ cells were consistently higher with the GALV-pseudotyped virus, as determined by FACS for EGFP (see Table 5.8; sample FACS profiles are shown in Figure 5.8). The efficiency of transfer into CD34$^-$ cells was significantly lower than in the other two cell populations, most likely due to a lack of proliferation of these cells.

Table 5.8. Gene Transfer Efficiencies by Amphotropic vs GALV-Pseudotyped MSCV-EGFP-Neo in Different Sorted Cell Fractions of AML Cells.

	% Cells GFP+ in Each Cell Fraction, as Determined by FACS					
	$CD34^+CD38^-$		$CD34^+CD38^+$		$CD34^-$	
Sample	Ampho	GALV	Ampho	GALV	Ampho	GALV
7	27%	40%	16%	32%	3%	11%
23	3%	6%	4%	14%	1%	2%
25	7%	22%	12%	34%	3%	2%

The transduced cells were also assessed for CFC. For 2 samples, gene transfer to CFC was higher with the GALV pseudotype, and in fact, gene transfer with this vector into CFC was quite high (30 to 60% in some cases), without any purification of cells following transduction (Table 5.9). Thus good gene transfer efficiencies can be obtained by combining sorting of cell populations to remove those cells which are largely non-dividing (i.e. CD34⁻), and selection of the appropriate pseudotype to improve virus binding to the cell surface and cell entry.

Table 5.9. Gene Transfer Efficiencies by Amphotropic vs GALV-Pseudotyped MSCV-EGFP-Neo in CFC from Different Sorted Cell Fractions of AML Cells.

Sample	Experimental Group	$CD34^+CD38^-$		$CD34^+CD38^+$		$CD34^-$	
		$CFC/10^5$	$\%EGFP^+$	$CFC/10^5$	$\%EGFP^+$	$CFC/10^5$	$\%EGFP^+$
7	Time 0*	7820	-	5950	-	530	-
	Ampho[#]	ND	-	ND	-	ND	-
	GALV[@]	11260	59%	8170	27%	950	6%
23	Time 0	37	-	7	-	0	-
	Ampho	116	5%	0	-	0	-
	GALV	91	15%	0	-	0	-
25	Time 0	4775	-	1090	-	0	-
	Ampho	1960	3%	370	11%	0	-
	GALV	1945	6%	460	39%	0	-

*Cells from each fraction were plated into colony assays immediately after sorting
[#]Cells from each fraction which were transduced with amphotropic MSCV-GFP-Neo and then placed in colony assays
[@]Cells from each fraction which were transduced with GALV-pseudotyped MSCV-GFP-Neo and then placed in colony assays
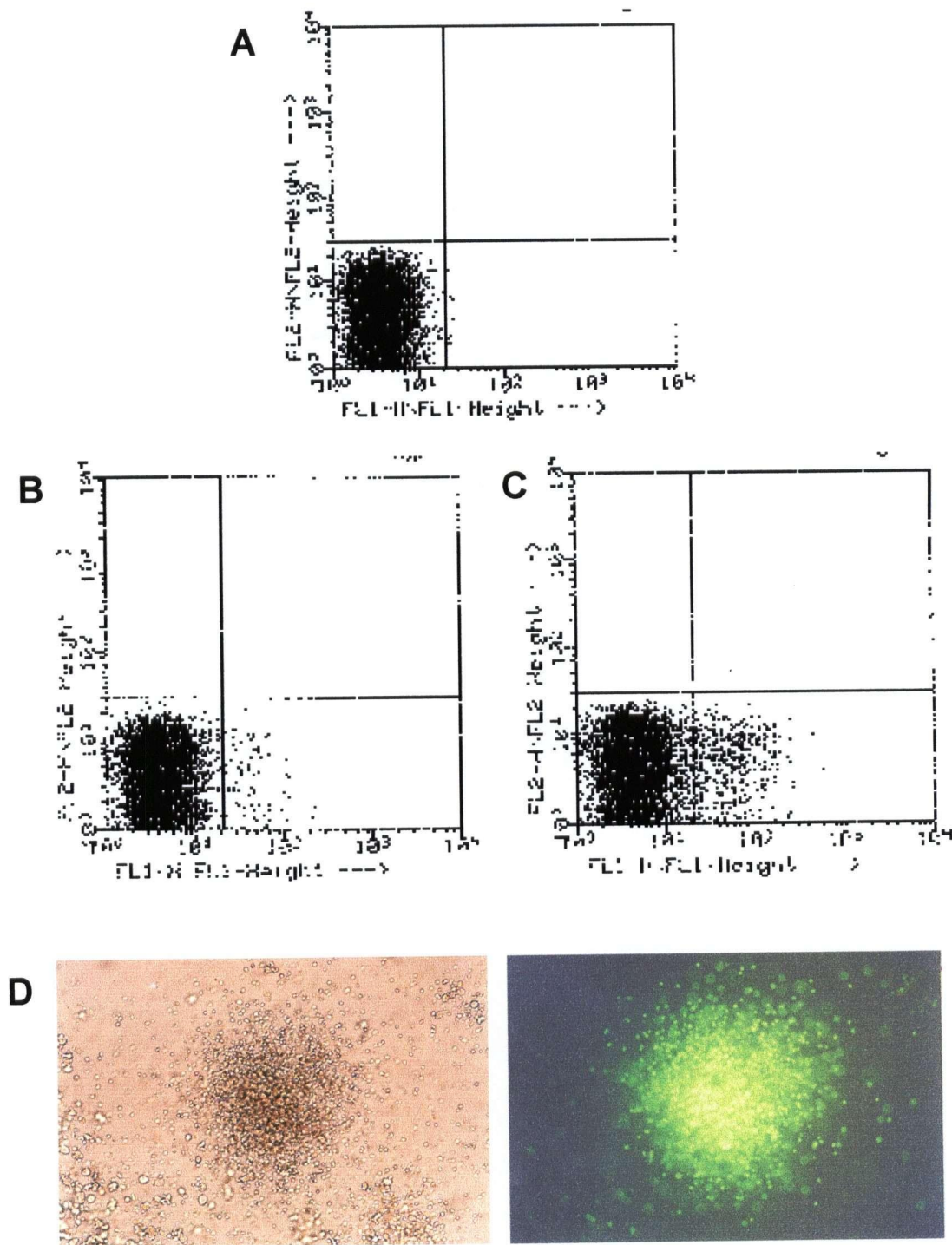
Figure 5.8. Flow cytometry on MSCV-EGFP-Neo-transduced cells: Amphotropic vs GALV-pseudotyped vectors. (A) Mock-transduced $CD34^+CD38^+$ cells from patient 23 (0.2% $EGFP^+$) (B) Amphotropic MSCV-EGFP-Neo-transduced $CD34^+CD38^+$ cells from patient 23 (3.8% $EGFP^+$) (C) GALV-pseudotyped MSCV-EGFP-Neo-transduced $CD34^+CD38^+$ cells from patient 23 (14% $EGFP^+$). (D) Colony derived from GALV-pseudotyped MSCV-EGFP-Neo-transduced $CD34^+CD38^+$ cells of patient 7, shown under regular light and UV light.

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5.3   Discussion

5.3.1   Gene Transfer of the Neomycin-Resistance Gene to NOD/SL-IC

The efficiency of gene transfer into bulk AML cells with MSCV-Neo using a 5-day protocol was high, ranging from 30 to 80%, as determined by Southern blot analysis, indicating that induction of cells into cycle by SF and IL-3 was successful. However, for 3 samples that were known to achieve high engraftment levels when cells were injected directly into mice, the engraftment ability was lost. This suggested that the culture conditions used were either unable to support the maintenance of NOD/SL-IC for the duration of the transduction protocol, or they were stimulating the NOD/SL-IC to differentiate into a more mature cell type which was then no longer capable of initiating engraftment. Similar difficulties have been encountered by others attempting to expand and/or retrovirally transduce normal SRC in vitro. Larochelle et al (1996) reported a time-dependent loss of human SRC during their retroviral transduction period, and Gan et al (1997) showed that the frequency of SRC in BM declined 6-fold after 1 week of coculture with allogeneic human stroma.

A variety of studies have been done to assess the effects of various cytokines and cytokine combinations on in vitro stem cell expansion. A number of conditions were reported which allowed significant expansion of cells with primitive cell surface phenotypes, as well as LTC-IC (Young et al. 1996; Ramsfjell et al. 1997; Zandstra et al. 1997). FL, SF and TPO appear to be of particular importance in stimulating these cells to proliferate, while maintaining their primitive phenotype (Petzer et al. 1996; Young et al. 1996; Borge et al. 1997; Haylock et al. 1997; Ramsfjell et al. 1997). Furthermore, in a single study by Lisovsky et al (1996), it was shown that FL both stimulated proliferation and inhibited apoptosis of AML cells. In addition, it had been shown previously that supplementation of LTCs of AML cells with IL-3, SF and/or

FL resulted in an increase in the output of CFC from these cultures, suggesting a proliferative response, as was also seen with these same cytokines in short term proliferation assays (Chapter 3). On the other hand, it has been suggested that IL-3 may be detrimental to the maintenance of primitive normal murine and human hematopoietic stem cells, probably due to the induction of differentiation in parallel with proliferation, as illustrated by loss of CD34 expression on divided cells in vitro (Yonemura et al, 1997; Luens et al, 1998). I therefore decided to investigate the effect of culturing AML cells under a variety of conditions, selected based on the studies described above, in the hope that cytokines known to promote normal primitive progenitor cell maintenance in vitro may also promote NOD/SL-IC maintenance under the same conditions. The conditions tested included the removal of IL-3, as well as the addition of FL and/or TPO, and in one case hyper-IL-6 (a fusion of IL-6 and its receptor; Chebath et al. 1997) to the cultures. The various cytokine cocktails were tested for their ability to sustain NOD/SL-IC in vitro for 4, 12 and 24 hours. None of the conditions tested could maintain NOD/SL-IC for 24 hours (Figure 5.5).

More recently, expansion and retroviral marking of normal CRU have been reported. Both Conneally et al (1997) and Bhatia et al (1997) report that culture of $CD34^+CD38^-$ cord blood cells in serum-free medium with IL-3, IL-6, G-CSF, SF and FL allows modest expansion (2- to 4-fold) of CRU. Conneally et al (1998) then reported use of the same cocktail for successful retroviral transduction of 30% of CRU from cord blood. Dao et al (1998) achieved retroviral transduction of bnx-mouse repopulating cells in the $CD34^+CD38^-$ fraction of adult BM by 7 days of infection with stroma and FL present. Cheng et al (1998) and Marandin et al (1998) also described conditions under which high levels of in transduction of in vivo repopulating cells from cord blood were achieved. Thus it appears that appropriate culture conditions have recently been discovered which allow the proliferation and retroviral infection

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of CRU to occur, while preserving their ability to initiate hematopoiesis in vivo. The use of cord blood as a source of CRU in these studies may have contributed to their success, due to ontogeny-related differences in functional properties of cells from different stages of development (Lansdorp et al. 1993), which could allow better maintenance of the repopulating potential of these cells in vitro. The culture conditions tested for AML cells in this study are not identical to those used in the above reports, thus further testing of a variety of cytokine cocktails, in combination with serum-free culture conditions, should be undertaken before further gene transfer experiments with AML cells are performed, with the conditions known to maintain normal CRU as a starting point. Whether leukemic in vivo repopulating cells have the same cytokine requirements as normal CRU in vitro, or even whether the cytokine requirements will be the same from one patient sample to the next, remains to be seen.

Interestingly, it was found that when cells from the various culture conditions described above, all of which failed to maintain NOD/SL-IC, were also assayed for CFC and LTC-IC, both of these cell types were maintained during the 24 hour culture period. This suggests that LTC-IC are a distinct population from NOD/SL-IC in the same samples. This has been suggested by other groups for normal hamatopoietic cells. Larochelle et al (1996) found that CRU were rarely transduced, while CFC and LTC-IC in the same samples were efficiently transduced, and Gan et al (1997), who showed a loss of CRU under their culture conditions, had a simultaneous expansion of CFC and LTC-IC. Alternatively, LTC-IC may represent a more heterogeneous population of cells, a small proportion of which can also function as CRU or NOD/SL-IC. This theory was also suggested in the previous chapter as a result of the large differences found in the proliferative capacities of these two cell populations. Either way, the behavior of LTC-IC in biological assays cannot be used to predict the behavior of SRC or NOD/SL-IC in the same assays.

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Even in mice in which engraftment was undetectable (<0.1%), small numbers of marked cells were detectable by PCR for the transgene, and this persisted for as long as 15 weeks post-transplant. This indicates the presence of very long-lived AML cells in the tissues of these mice. Possibly, these marked cells may have been derived from a small number of transduced, more mature cells types, such as CFC or some LTC-IC, that produced small numbers of progeny. Alternatively, the presence of these cells at low frequency in the mice suggests that the loss of NOD/SL-IC activity in vitro under the various culture conditions described above is not due to their death, but rather to induction of a differentiation step which leads to a loss of high proliferative capacity.

Patient 15 cells showed a different behavior from all the others, in that they were still able to engraft mouse marrow to very high levels following 5 days in vitro. The reason for this is unknown. Perhaps this particular AML clone had undergone additional transformation steps, making it no longer responsive to differentiation signals that were present in the cultures. The presence of predominantly 2 clones in the retrovirally marked population of the week 13 mouse indicated that 2 individual cells gave rise to the large number of cells present in the mouse marrow 13 weeks later. This supports the theory that a very rare subpopulation of highly proliferative stem cells exists within leukemic clone that is responsible for the maintenance and expansion of the leukemic population in the patient.

5.3.2   Retroviral Transduction with a Gene for a Cell Surface Molecule and Subsequent Immunomagnetic Purification of Transduced Cells

A time course study showed that 5 to 10% of AML cells could be transduced with MSCV-HSA-Neo after only 10 hours of coculture with retroviral producers, and this population could be enriched to 42-67% by immunomagnetic purification. The CFC in the enriched population

were 100% transduced. By 24 hours in coculture, a maximum transduction efficiency of 25% was achieved, and this could be enriched to 75% by immunomagnetic purification. However, the results of the experiments described in 5.2.2 showed that NOD/SL-IC activity is lost within 24 hours in vitro in all cases tested, but 2 of 3 samples retained significant mouse-engrafting ability after 12 hours in vitro. These two results combined suggest that 10 to 12 hours of coculture may be the optimum procedure for achieving sufficient gene transfer levels while retaining NOD/SL-IC activity. However, when colonies from LTC were assessed for gene transfer after only 10 hours of coculture, 0% of them were transduced, whereas 56% were transduced after 24 hours of coculture. It thus appears that, although 10 hours is sufficient for the transduction of CFC, LTC-IC transduction may require a longer exposure to retroviruses. This may be due to a longer time requirement for the induction of LTC-IC into cycle, whereas CFC may already be cycling at the time of initiating the transduction protocol, or may be more easily induced to cycle by the cytokines provided. It therefore is necessary to find means either to sustain NOD/SL-IC in culture for at least 24 hours, or to achieve efficient transduction of these cells within 12 hours, if gene transfer strategies are to be successful.

Due to the potential contamination of transduced cells with the retroviral producer cell lines, the cocultivation method of retroviral transduction is unlikely to be used in clinical gene therapy. It was therefore of interest to determine what results could be obtained using cell-free retroviral supernatant. AML samples were transduced with viral supernatant on fibronectin-coated dishes, a method which has been described as being optimal for supernatant transduction of normal hematopoietic stem cells (Moritz et al. 1994). The transduction efficiencies with this method were lower than those achieved with cocultivation. This is not surprising given that, in the case of cocultivation, the producer cells are continuously producing fresh virus, to which the target cells are immediately exposed, whereas in the case of supernatant transduction, the

cells are exposed to 2 batches of virus-containing supernatant over a 24 hour period, in which the viruses, being quite labile, immediately begin to decay upon removal from the producer cells. Thus the ratio of viruses to cells, or multiplicity of infection (m.o.i.) will be much higher in the cocultivation protocol, even though the cells are plated at the same concentration.

When cells which had been transduced using the supernatant protocol were immunomagnetically purified, the resulting fold-enrichment was in approximately the same range as it was with the 24 hour cocultivation experiments (1.5-9.1-fold vs 3-fold, respectively). However, since the starting populations had a smaller proportion of positive cells, the enriched population was not as pure. It thus appears that a low proportion of target cells in the precolumn fraction has a negative effect on the purity of the enriched fraction. This phenomenon has been described by others (Thomas et al. 1993). It may be possible to improve the purity of the enriched fraction by altering the flow rate at which the cells and buffer are pumped through the column, or the cell numbers used per given column size, or by changing the magnetic field strength used. A systematic testing of all of these parameters needs to be done to optimize this purification system specifically for HSA-transduced cells.

In spite of the low purities of the enriched fractions of supernatant-transduced AML and NBM cells, the transduction levels of CFC and LTC-IC in these populations were significant (12.5 to 75% of AML-CFC and 25% of AML-LTC-IC). These numbers could be improved in the future by optimization of the immunomagnetic purification system.

5.3.3   A Comparison of Two Pseudotypes of Retroviral Vectors for Efficiency of

Transduction of AML Cells

All of the experiments with retroviral transduction of AML cells up to this point had been done with retroviruses packaged in a cell line containing the amphotropic envelope gene.

Recent reports in the literature had suggested that better transduction efficiencies into hematopoietic cells could be obtained by using vectors that were instead packaged with the GALV envelope, as the receptor for this envelope is more highly expressed in the target cells (Orlic et al. 1996; Kiem et al. 1997). To test whether this would also be true for AML cells, the MSCV-EGFP-Neo vector was packaged in either GP+envAM12 cells, giving it the amphotropic envelope, or in PG13 cells, giving it the GALV envelope, and transduction efficiencies of AML cells by the two vectors were compared. For these experiments, the 3 AML samples were first sorted into $CD34^+CD38^-$, $CD34^+CD38^+$ and $CD34^-$ populations. Each fraction was then divided and transduced with the two different pseudotypes of vector. As described in the literature for normal hematopoietic cells, the GALV-pseudotyped vector yielded higher transduction efficiencies of all cell fractions than did the amphotropic vector. This was evident in CFC as well as in total cells. Unfortunately, transduction efficiencies into LTC-IC with these vectors were not determined due to bacterial contamination of the cultures. With respect to transduction efficiencies of the different cell fractions, the transduction efficiencies of the $CD34^+CD38^-$ and $CD34^+CD38^+$ fractions were not significantly different from each other with either of the pseudotypes, whereas the transduction efficiency of the $CD34^-$ cells was always significantly lower. This is most likely due to the fact that the $CD34^-$ fraction contains a population consisting largely of mature, nondividing cells and therefore cannot be retrovirally transduced. This is further illustrated by the fact that the CD34- populations contained very few or no CFC (Table 5.8). Thus by separating cells based on their CD34 and CD38 expression, a subpopulation which is untransducible is removed from the target cells, and the m.o.i. for the remaining cells is increased.

Based on all of the work described above, an optimized strategy for obtaining populations of transduced leukemic stem cells would involve the following steps:

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1. Identification of culture techniques that maintain NOD/SL-IC for at least 24 hours in vitro.

2. Use of GALV-pseudotyped vectors for higher transduction efficiencies.

3. Purification of CD34$^+$ cells prior to transduction. This would remove non-transducible CD34$^-$ cells, thereby increasing the m.o.i. and thus also, most likely, the transduction efficiency. This would be particularly useful in cases where the majority of cells are CD34$^-$, as cell numbers would be greatly reduced without loss of NOD/SL-IC in most cases (Blair et al. 1997; Bonnet and Dick 1997). For those samples which have a high proportion of CD34$^+$ cells, this step would not be necessary, as it would not provide a significant benefit.

4. For cases where CD34$^+$ selection reduces the target cell population to a small number, EGFP could be used as a reporter gene, and transduced cells could subsequently be purified by FACS. For those cases where a high proportion of cells are CD34$^+$, unsorted cells would be transduced, but the necessity of using larger numbers of cells would make the use of HSA as a transgene and subsequent immunomagnetic purification of transduced cells a more practical approach.

The column purification approach is more likely to be of benefit in the clinical gene therapy setting, where very large numbers of cells will be required to perform transplants to human recipients.

CHAPTER SIX: SUMMARY AND CONCLUSIONS

In order to gain an understanding of the underlying mechanisms of growth deregulation in the hematopoietic hierarchy that lead to AML, experimental models are required which will allow primitive leukemic cells to be detected, characterized, and manipulated. Such models exist for normal primitive hematopoietic progenitors, these being the LTC system and the NOD/SCID mouse model. The first major goal of my thesis was to determine the utility of these model systems for the detection and characterization of analagous cell types from AML samples. In Chapter 3, it was demonstrated that a leukemic LTC-IC could be routinely detected under appropriate conditions, and also that most AML samples contain a mixture of normal and abnormal LTC-IC. The assay was shown to be quantitative, and calculation of the frequencies of both leukemic and normal LTC-IC in AML peripheral blood samples showed that the frequency of both is considerably higher than the frequency of LTC-IC in normal peripheral blood. In fact, the frequency of normal LTC-IC in AML blood was 10 to 100-fold higher than that of normal blood, suggesting that normal LTC-IC are mobilized as a part of the leukemic process. Finally, differences in the responses of normal and leukemic cells to various cytokines active on normal LTC-IC were revealed. This suggests that further studies investigating the manipulation of LTCs by the addition of various cytokines may reveal hitherto unknown factors important to the regulation of primitive AML progenitors, as well as leading to conditions for the selective maintenance of normal stem cells, thus providing therapeutic "purging" strategies for AML patients. The evaluation of the cell cycling status of normal vs leukemic LTC-IC within these samples in response to the various culture conditions could also lead to therapeutic strategies that could specifically target leukemic stem cells while sparing the normal ones. Finally, the LTC system could be used as an assay to assess the effects of various manipulations, such as retroviral transduction with genes involved in growth control, differentiation, or cell death, on leukemic stem cell function, thus aiding in the elucidation of

the molecular mechanisms involved in leukemogenesis, as well as evaluating additional therapeutic strategies.

A second and more reliable assay for stem cells is the animal repopulation assay. The ability of a small subset of cells within AML samples to repopulate immunodeficient mice was demonstrated in Chapter 4, again confirming the existence of a rare stem cell in AML that is responsible for maintenance of the leukemic clone. This cell was quantitated and its high proliferative capacity and self-renewal ability were demonstrated. Like the LTC system, this assay system provides a model in which to assess the manipulation of human AML cells, both for evaluation of therapeutic strategies and for dissecting the mechanisms of leukemogenesis, as described above.

The second major goal of my thesis was to achieve retroviral-mediated gene transfer into AML stem cells. This would allow clonal analysis of populations of cells engrafted in mice, as well as providing a tool for the genetic manipulation of leukemic cells. It was found that CFC and LTC-IC were easily transduced under a variety of conditions, including those that are clinically relevant. However, although transduction of NOD/SL-IC was successful in one case, in all other cases the repopulating ability of these cells was lost when these cells were placed in vitro for as short as 24 hours. It is therefore of utmost importance to focus studies in the immediate future on discovering culture conditions which allow the maintenance of NOD/SL-IC in vitro for periods of time long enough to achieve retroviral transduction. Initial experiments should focus on those conditions described very recently in the literature which allowed the successful maintenance and retroviral transduction of normal CRU (Conneally et al. 1997; Conneally et al. 1998; Marandin et al. 1998).

Strategies for the enrichment of transduced cells within a test cell population were also investigated. Transfer of a murine cell surface molecule into human AML cells enabled the

enrichment of transduced cells using an immunomagnetic separation technique. The protocol used, however, did not yield very pure populations of transduced cells when the initial transduction efficiency was low. The purification protocol should therefore be optimized by varying a number of parameters in the system, including the flow rate of cells through the column, cell numbers and cell densities used, and the magnetic field strength used. If such optimization strategies could reveal conditions in which more pure populations of transduced cells could be obtained, this would provide a very useful tool for situations in which very large numbers of transduced cells are required, such as in the clinical setting, when sorting transduced cells by flow cytometry is not practical.

Another potential method for increasing the efficiency of gene transfer is by packaging viral vectors with an envelope whose receptors are highly expressed on the target cell surface. Reports in the literature have indicated that the receptor for the GALV envelope is more highly expressed in human hematopoietic cells than the receptor for the amphotropic envelope. Therefore a comparison study was done with GALV vs amphotropic vectors carrying the EGFP gene, and it was found that GALV-pseudotyped vectors did indeed have higher transduction efficiencies of AML cells than amphotropic vectors. Using EGFP as a transgene has the added advantage of rapid assessment of gene transfer efficiency and purification of transduced cells by simple flow cytometry.

Once conditions for the optimal maintenance and transduction of AML stem cells have been established, many avenues of investigation would be opened. Strategies for gene therapy could be tested, such as the antisense or tumor vaccine strategies that were described in Chapter 1. The in vitro and in vivo models described here would provide the means for assessing the effects of such strategies in preliminary studies. The effect of introducing genes for proteins which are known or suspected to have roles in cell cycle control, apoptosis, or differentiation

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and development in the hematopoietic system (eg. transcription factors like CBF, MLL or HOX proteins, cell cycle control and apoptosis-related genes like RB, bcl-2 family genes, and p53, as well as cytokines and oncogenes) could be assessed. Analysis of resulting changes in gene expression patterns, phosphorylation or dephosphorylation of various cell signalling molecules, or the formation of protein complexes, combined with changes in behavior of target cells in the various progenitor assays, could all provide valuable information about the underlying mechanisms of leukemogenesis, and thus provide the means by which the disease could be successfully treated. The work described in this thesis has contributed to the achievement of these goals.

APPENDIX A - CYTOKINE RESPONSIVENESS OF AML CELLS IN SHORT-

TERM ASSAYS

Table A-1. Stimulation of AML Blast Proliferation by Various Cytokines

Cytokine(s)*	Proportion of AML Samples Responding % (no. tested)	Fold ^{3}H-Tdr Incorporation$^\Psi$ Median (range)
IL-3	79 (53)	4 (1-126)
GM-CSF	67 (49)	5 (1-51)
G-CSF	50 (50)	1 (1-23)
IL-6	0 (7)	1
SF	63 (48)	2 (1-65)
IL-11	68 (44)	1 (1-19)
IL-1	54 (41)	2 (1-83)
IL-2	43 (35)	1 (1-11)
FL	62 (26)	2 (1-57)
IL-3 + SF	96 (46)	11 (1-253)
IL-3 + FL	96 (26)	10 (1-99)
SF + FL	91 (23)	4 (1-118)
SF + IL-3 + FL	96 (24)	16 (1-122)

*Cytokines tested at final concentrations of 40 ng/mL for IL-3, GM-CSF, G-CSF, G-CSF, IL-6, IL-11, and IL-1 and 100 ng/mL for SF, IL-2, and FL.
$^\Psi$As compared with control cultures without cytokines.

Table A-2. Cytokine-Stimulated ^{3}H-Thymidine Incorporation into AML Blasts from Patient Samples Tested in LTC

Patient$^\Psi$	CPM Without Cytokines*	Fold-Increase in ^{3}H-Tdr Uptake Compared with Control Without Cytokines						
		IL-3	G-CSF	SF	FL	IL-3 + SF	IL-3 + FL	IL-3 + SF + FL
1	88 ± 12	1	2	2	1	4	2	2
2	44 ± 4	126	2	24	ND	253	ND	ND
3	86 ± 5	30	2	5	2	56	65	85
4	97 ± 7	35	8	65	57	114	99	122
5	242 ± 36	5	4	1	39	7	54	52
6	85 ± 3	2	1	1	1	2	2	2
7	415 ± 78	16	3	3	3	20	22	24
8	270 ± 12	4	11	9	8	12	10	13
9	82 ± 19	3	1	2	2	3	5	7
10	339 ± 50	3	6	9	5	11	10	13

*Mean ± SEM.
$^\Psi$Patient numbers match those of Table 3.1.

Table A-3. Blast Colony Formation per 10^6 AML Peripheral Blood Cells with Different Cytokine Combinations.

Patient[Ψ]	Growth Conditions*			
	GM-CSF+IL-3 +SF	GM-CSF+IL-3 +SF+FL	FL	No Added Cytokines
1	70	125	10	0
2	10	30	0	0
3	1980	2350	1750	0
4	320	740	1010	10
5	>10000	16500	>10000	65
6	215	240	180	155
7	24250	>24250	4220	2900
8	15	45	5	10
9	2050	2625	800	250
10	170	215	20	0

* Methylcellulose assay containing 30% FCS and human cytokines at the following concentrations: SF and FL at 50 ng/mL, IL-3 and GM-CSF at 10 ng/mL.
[Ψ] Patient numbers match those of Table 3.1.

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