Development of Gene Therapy for Hematopoietic Stem Cells using Lentiviral Vectors

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Development of Gene Therapy for Hematopoietic Stem Cells using Lentiviral Vectors

by

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PREAMBLE

Definition: gene therapy *n* : the insertion of normal or genetically altered genes into cells usually to replace defective genes especially in the treatment of genetic disorders. Webster's New Complete Medical Dictionary. Smithmark publishers, New York, 1996.

The first scientific article ever published containing the catchphrase "gene therapy" was the article, "DNA and gene therapy: uncoating of polyoma pseudovirus in mouse embryo cells", written by Osterman JV, Waddell A, Aposhian HV and published in September 1970 in the journal Proceedings of the National Academy of Science of the United States of America; 67(1):37-40. It speculated on the use of a pseudovirus, manipulated to contain therapeutic genes for insertion into target cells. Slowly, over the next 10 years to 1980 about 50 other scientific articles would be published containing the phrase in the title or the abstract, and about 600 more in the following ten years to 1990. However the exponential growth seen here was nothing compared to the 1990's, where significant breakthroughs in vector and genetic technologies emerged increasing the feasibility of the modality for the treatment of disease. This led to higher medical research funding for the field, expansion of facilities, and most importantly increased knowledge. Almost 15,000 scientific articles were published during this period. Moreover, in just the last two and a half years since the millennium shift (2000), the arbitrary year separating our humble days of phlebotomy as a form of treatment for many diseases, to the new age of gene and cell therapies, over 7,500 articles have been published. From its inception, to its first successful clinical trial, to today, all the articles ever published are readily available to anyone through Pubmed's medical index WebPages. With such vast and detailed knowledge gained in the field of gene therapy and all that it encompasses, from the genetic basis of diseases, gene transfer technology, stem cell culturing and transplantation, the success of gene therapy in the clinical setting is imminent.

But not only as originally thought…

Advances in cell biology sciences such as the expansion of hematopoietic stem cells and differentiation of ES cells combined with the ability to perform nuclear transfer into ES cells (technology used for cloning of sheep, e.g. Dolly) have opened new and exciting avenues for the treatment of disease. While these new techniques are still in their infancy, together with techniques developed in the field of gene therapy, more diseases are potentially curable than ever before. In this thesis, I hope to show you what the latest advances in gene therapy of the hematopoietic system are while leaving you with a taste of what is to come when cell therapy and gene therapy converge in the near future.

TABLE OF CONTENTS

ABBREVIATIONS

AIDS acquired immune deficiency syndrome
BFU-E burst forming unit, erythroid
BM bone marrow
BRCA1 breast cancer 1 gene
CB umbilical cord blood
CD34 CD38 cluster of differentiation 34, 38
CID chemical inducer of dimerization
CFC colony forming cell
CFU-GM colony forming unit - granulocyte and macrophage
DNA deoxyribonucleic acid
dNTP deoxynucleotide triphosphate
EB embryoid body
ES embryonic stem
EF-1α elongation factor 1α
FL fetal liver tyrosine kinase-3 ligand
GFP green fluorescence protein
G-CSF granulocyte colony stimulating factor
HIV *human immunodeficiency virus*
HOX B4 *homeobox B4*
HSC hematopoietic stem cell
IRES internal ribosomal entry site
IL-3, -6 interleukin-3, -6
LIF leukemia inhibitory factor
Lin lineage
LTC-IC long-term culture, initiating cell
MESV *murine embryonic stem cell virus*
LAM PCR linear amplification mediated polymerase chain reaction
LTR long terminal repeat
MFI mean fluorescence intensity
MFU mean fluorescence units
MOI multiplicity of infection
MMLV *Moloney murine leukemia virus*
MPB mobilized peripheral blood
MSCV *murine stem cell virus*
NOD/SCID non-obese diabetic/severe combined immune deficient
PCR polymerase chain reaction
PGK phophoglycerate kinase
RNA ribonucleic acid
SCID severe combined immune deficient
SIN self-inactivating
SRC SCID repopulating cells
SCF stem cell factor
SDF-1 stroma derived factor 1
TE transduction efficiency
TPO thrombopoietin (bioactively equivalent to MGDF)
VSV-G *vesicular stomatitis virus* glycoprotein
WPRE woodchuck posttranscriptional regulatory element

LIST OF PAPERS

This thesis is based on the following communications that will be referred to in the text by their roman numerals:

I. Isao Hamaguchi, **Niels-Bjarne Woods**, Ioannis Panagopoulos, Elisabet Andersson, Hanna Mikkola, Cecilia Fahlman, Romain Zufferey, Leif Carlsson, Didier Trono, Stefan Karlsson. 2000. Lentivirus Vector Gene Expression during ES Cell-Derived Hematopoietic Development *In Vitro*. **J Virol. 74(22):10778-84**.

II. Hanna Mikkola, **Niels-Bjarne Woods**, Marketa Sjögren, Hildur Helgadottir, Isao Hamaguchi, Sten-Eirik Jacobsen, Didier Trono, Stefan Karlsson. 2000. Lentivirus Gene Transfer In Murine Hematopoietic Progenitor Cells is Compromised by a Delay in Proviral Integration and Results in Transduction Mosaicism and Heterogeneous Gene Expression In Progeny Cells. **J Virol. 74(24):11911-8**.

III. **Niels-Bjarne Woods**, Cecilia Fahlman, Hanna Mikkola, Isao Hamaguchi, Karin Olsson, Romain Zufferey, Sten-Eirik Jacobsen, Didier Trono, Stefan Karlsson. 2000. Lentiviral Gene Transfer into Primary and Secondary NOD/SCID Repopulating Cells. **Blood. 96(12):3725-33.**

IV. **Niels-Bjarne Woods**, Johan Flygare, Arne Muessig, Manfred Schmidt, Karin Olsson, Hanna Mikkola, Eva Nilsson, Thomas Relander, Johan Richter, Patrick Salmon, Didier Trono, Christof von Kalle, Stefan Karlsson. 2002. High-Level Lentiviral Vector Transgene Expression Requires Optimal Vector Design and Multiple Vector Copy Integration into Repopulating Cells: Risk for Insertional Mutagenesis. **(subsequently divided into two manuscripts, one of which is accepted for publication in Blood and the other is in manuscript form)**

INTRODUCTION

Corrective-gene transfer into hematopoietic stem cells is an attractive concept for the treatment of a host of inherited or acquired diseases of the hematopoietic system. Since a genetically modified hematopoietic stem cell would have the potential to give rise to corrected cells of all hematopoietic lineages, and would have the ability to self-renew, it has been theorized that life-long cures for some diseases would be possible. The finite lifetime of more mature hematopoietic cells of most hematopoietic lineages precludes them from their use for life-long treatments and makes the hematopoietic stem cell the appropriate target for genetic correction. The benefits of such a treatment would include normalcy of life for the patient, minimal doctor patient interventions after initial treatment, and little cost to society after the initial treatment. The routine clinical procedure with which hematopoietic stem cells are harvested from a patient or donor and the innate ability of the stem cells to return to their niche in the bone marrow after transplantation (homing) provides for the practical basis of the therapy. This allows the harvested cells to be genetically manipulated *in vitro* prior to their transplantation back into a patient; known as *ex vivo* manipulation.

Since hematopoietic stem cells possess enormous proliferative potential, methods for permanently integrating genes into its genomes are required to maintain the presence of the gene in the progeny cells. The murine oncoretrovirus based vectors have been developed for this purpose and have been successfully used to genetically modify murine and human hematopoietic progenitor cells in both *in vitro* and *in vivo* assays (36, 62, 251, 256, 260). However, the efficiency of transduction by the oncoretroviral based vectors of hematopoietic stem cells capable of reconstitution in patients and large animals has been disappointingly low (68, 132, 221). The low efficiency of transduction into hematopoietic stem cells has been attributed to the fact that standard oncoretroviral vectors require the collapse of the nuclear membrane during mitosis in order to gain access to the target cell's genome prior to integration (160, 208). Attempts at stimulating primitive hematopoietic cells to divide *in vitro* during vector transduction have been complicated by the fact that stimulation reduces the repopulating potential of stem cells (55, 60, 267). Recently, advances in transduction conditions have significantly improved gene marking efficiencies in non-human primates to a respectable 10-15% (129, 136). However, the conflict between stimulation and loss of repopulating ability persists. This conflict was the impetus for the development of a relatively new vector, derived on the human immunodeficiency virus (HIV) type 1, based on HIV's ability to infect non-dividing cells (142, 257). This vector, known as a lentiviral vector, has demonstrated an ability to transduce numerous non-dividing cell types, as well non-dividing cells of the hematopoietic system (4, 34, 199, 248). Therefore, we sought to test the vector's efficiency of transducing human hematopoietic stem cells.

Prior to using lentiviral vectors in clinical protocols, basic preclinical research must first be performed to characterize hematopoietic stem cell's permissiveness to lentiviral vector transduction. Thanks to the efforts over the last 20 years to define the *in vitro* conditions for efficient transduction using oncoretroviral vectors, much of this knowledge can be applied to help determine the optimal conditions for efficient lentiviral vector gene transfer into hematopoietic stem cells. Although lentiviral vectors can transduce numerous types of non-dividing cells, it must be determined if the quiescent cells of the hematopoietic system are also susceptible to infection. If stimulation is required, this must then be balanced with the optimal conditions for transduction and the maintenance of the properties of stem cells; namely their repopulating and self-renewal abilities.

In addition to being required to efficiently transduce hematopoietic stem cells, lentiviral vectors must demonstrate an ability to maintain transgene expression throughout hematopoietic differentiation, and possibly even enable tissue-specific expression of the corrective gene. Therefore, preclinical studies assaying transduction efficiency and transgene expression of vectors must adequately simulate the development of human hematopoietic stem cells into their mature progeny as it would in a patient. While this can be effectively achieved using transplantation studies into murine models of hematopoiesis such as the C57B6 mouse, or the xenotransplant NOD/SCID mouse, the use of embryonic stem cells lines, which can differentiated into hematopoietic cells *in vitro*, is an quick and effective means of studying transgene expression throughout hematopoietic differentiation. Issues such as expression per vector copy and the effects of variegation can be addressed using this system. Intriguingly, should lentiviral vectors prove to be effective a transducing and maintaining expression in ES cells, new genetically modified tissues could potentially be generated from the ES cells, including the generation of modified hematopoietic stem cells themselves, posing new avenues for treatment of disease.

STEM CELLS

Embryonic Stem Cells

The pluripotentiality of embryonic stem (ES) cells makes them an interesting target for genetic manipulation as corrective tissues could potentially be generated for patients with as yet incurable diseases. ES cells are derived from the inner cell mass of a developing preimplantation embryo at the blastocyst stage (64). Given the correct conditions, ES cells are capable of differentiating into a new organism with normal reproductive capability. This pluripotency of the ES cells is the basis for the assumption that any desired tissue could be differentiated *in vitro* from these cells.

Evidence that ES cells could to be maintained (and manipulated) *in vitro* and subsequently reintroduced into a blastocyst to generate a transgenic animal was first presented in 1981 (74, 109). However, ES cells can also be induced to differentiated *in vitro* into cell types such as neurons, glial cells, cardiac myocytes, hematopoietic cells, skeletal myocytes, and insulin secreting islet-like cells, to name a few (24, 29, 148, 209, 264, 266). The *in vitro* differentiation of cells from early embryos has enabled insights to be gained on the complex biological mechanisms regulating differentiation and development. However, now this knowledge is being used to develop ES cells to generate tissues for treatment of disease, as is exemplified in the curing of an immunodeficient mouse by Rideout *et al.* (204). To this end, it could be advantageous to determine the ability of novel vectors to mediated genetic modification of ES cells. Furthermore, as is described in Paper I of this thesis, ES cells can be used to address gene therapy related issues, such as the capability of vectors for transgene expression throughout differentiation. ES cells have been generated from mice, non-human primates, and more recently humans (91, 150, 202, 235, 241, 242). Some features of interest of ES cells are their high telomerase activity, and low level of methylated DNA (64, 176). The great potential of the ES cell to be cultured *in vitro* indefinitely followed by induction of differentiation into a specific tissue upon demand has far-reaching medical applications.

ES Cell Self-Renewal

ES cells, as all stem cells, have the ability to self-renew, however, the genetic mechanisms of this ability are poorly understood. Under the correct conditions, an ES cell is able to divide into 2 daughter cells, where each has the same undifferentiated state as the original and without loosing its differentiation potential. This means that once ES cells are isolated, it may be possible to expand them indefinitely in culture in an undifferentiated state (to generate an ES cell line), and induce their differentiation into specific tissues whenever needed (64). Multiple ES cell lines have been generated and show a maintained karyotype and ability to self-renew if cultured under the correct conditions. In order to maintain most ES cell lines, a stroma cell feeder layer is required, which provides certain mostly unknown stimuli either by direct contact with signal transducing molecules or by their secretion of soluble factors (64, 176). The soluble cytokine leukemia inhibitory factor (LIF) is always required for the preservation of primitivity of the cells in these cultures. The primitivity of ES cells is maintained largely through the signal transduction through the LIF receptor complex, of which the glycoprotein GP130 is a component (282). Downstream in this signaling pathway is the activation of the signal transducer and activation of transcription (STAT)-3 (172). Another molecule that appears to be necessary for the maintenance of ES cell primitivity is the POU transcription factor Oct4 gene product, which in present in high-levels in undifferentiated ES cells and decreases upon differentiation (170, 173).

ES Cell Differentiation to Hematopoietic Cells

During embryogenesis in the mouse, the first sign of blood is concurrent with the appearance of endothelial cells at 7.5 days post coitum (7.5 p.c.) in the yolk sac blood islands (96). The hematopoietic cells arise from the mesoderm germ layer and appear to require contact from the adjacent visceral endoderm for their generation (75). The hematopoietic cells of the yolk sac are mainly of the erythroid lineage and contain fetal hemoglobin, and these cells are capable of forming colonies when grown in semi-solid medium (271). However, they are not capable of lymphomyeloid repopulation in an adult organism if transplanted (167). ES cells differentiated *in vitro* can also generate hematopoietic progenitor cells (176, 265). Upon induction of differentiation of an ES cell, development of a cluster of cells known as EB bodies will occur. The induction of differentiation is performed simply by the removal of LIF or the feeder layer, or by letting the ES cells grow in close proximity to each other, in a hanging drop, or confluent plate. EB bodies are so named for their likeness with embryos generated *in vivo* having progeny cells from the three germ layers present, however, the disorganized manner of this body prevents its development into an organism (176). During the differentiation of the embryoid body, areas of primitive hematopoiesis, similar to blood islands of the yolk sac of a precirculation embryo, can been seen. The cells of an EB body can be dissociated and plated in semi-solid medium containing hematopoietic growth factors for hematopoietic cell proliferation and differentiation into colonies of the erythroid, macrophage, mast cell, and occasionally neutrophil lineages (266).

The development of hematopoietic stem cells is not normally seen at the early stages of hematopoiesis in either the embryo proper or in *in vitro* derived embryoid bodies. The first day that hematopoietic cells from an embryo can engraft in a lethally irradiated adult recipient is not until day 10. These cells capable of adult hematopoiesis are present in the aorto-gonad-mesonephros (AGM) region of the embryo (156). It was later found by Matsuoka *et al.* that day 8 fetal blood cells from the yolk sac or para-aortic splanchnopleures, which are normally unable to engraft in adult recipients, could engraft if cultured (or "educated") *in vitro* for 4 days on an AGM based endothelial cell line prior to transplantation (153). Furthermore, as growth on the cell line continued, the numbers of colony forming cells (CFC) decreased while the numbers of hematopoietic stem cells and colony forming unit-spleen (CFU-S) cells increased. These results suggest that particular cells from the AGM region provide signals required for the development of fetal blood cells into hematopoietic stem cells. Interestingly, these results also suggest that hematopoietic precursors capable of developing into hematopoietic stem cells can originate from two distinct sites of hematopoiesis (intra- and extra embryonic). However, it is unclear which site (if not both) is the source of hematopoietic stem cells *in vivo*. In fact, controversy over the origin of embryonic hematopoietic precursors that mature into hematopoietic stem cells has existed for some time (280, 87). Regardless of their origin, hematopoietic progenitor and stem cells migrate to the fetal liver, which is the primary site of

hematopoiesis in the fetus. From there, they migrate further to the bone marrow of the developing fetus where they contribute to hematopoiesis for the life of the organism (46).

Although the generation of hematopoietic stem cells in *in vitro* culture systems of ES cells has not been possible, it was recently found that the over expression of *HoxB4* during EB differentiation up to day 6 or in yolk sac derived blood cells from embryos, followed by growth on the OP9 feeder layer could generate hematopoietic stem cells (137). These hematopoietic stem cells showed lymphoid and myeloid repopulating ability in mice and demonstrated for the first time that hematopoietic stem cells could be generated *in vitro* from ES cell lines. These results may prove to have clinical relevance as genetically modified hematopoietic stem cells could be generated on demand.

Hematopoietic Stem Cells

The defining features of hematopoietic stem cells are their ability to self-renew, and to differentiate into all hematopoietic lineages of the hematopoietic system (Figure 1). The mechanisms for hematopoietic cell differentiation into the mature cells of the varying lineages are not fully understood, as they are complex and unique for each lineage. However, considerable progress to understanding these mechanisms as they relate to cytokines and transcription factors have been achieved, as has been reviewed in part by Orkin and by Wiessman (177, 263). A functional feature of hematopoietic stem cells is their ability to repopulate the hematopoietic system of a myeloablated organism. This is achieved by the inherent ability of the stem cells to home to their niche in the bone marrow microenvironment, to self-renew, and to differentiate into all hematopoietic lineages of the hematopoietic system.

Adult human hematopoietic stem cells, which have the ability to home to the bone marrow and reconstitute the hematopoietic system of a myeloablated patient, can currently be obtained from three sources. Hematopoietic stem cells can be directly aspirated from the bone marrow (BM) microenvironment where they normally reside, or can be mobilized using cytokines or adhesion molecules into the peripheral blood (MPB) (135). MBP is the most common source hematopoietic stem cells for transplantation into patients due to the rapid hematological recovery after myeloablation and the relative ease of stem cell extraction from the peripheral blood of the donor (14). The third source of hematopoietic stem cells is from the usually discarded umbilical cord of newborn babies. Unfractionated umbilical cord blood (CB) cells have the highest frequency of cells capable of reconstituting the hematopoietic system of immune compromised mice, with 3 times more repopulating cells than BM,

and 6 times more than MPB (261). However, the low total stem cell number obtained from a single cord blood extraction, and the lack of banking systems, continues to limit their practical use in adult stem cell transplantations.

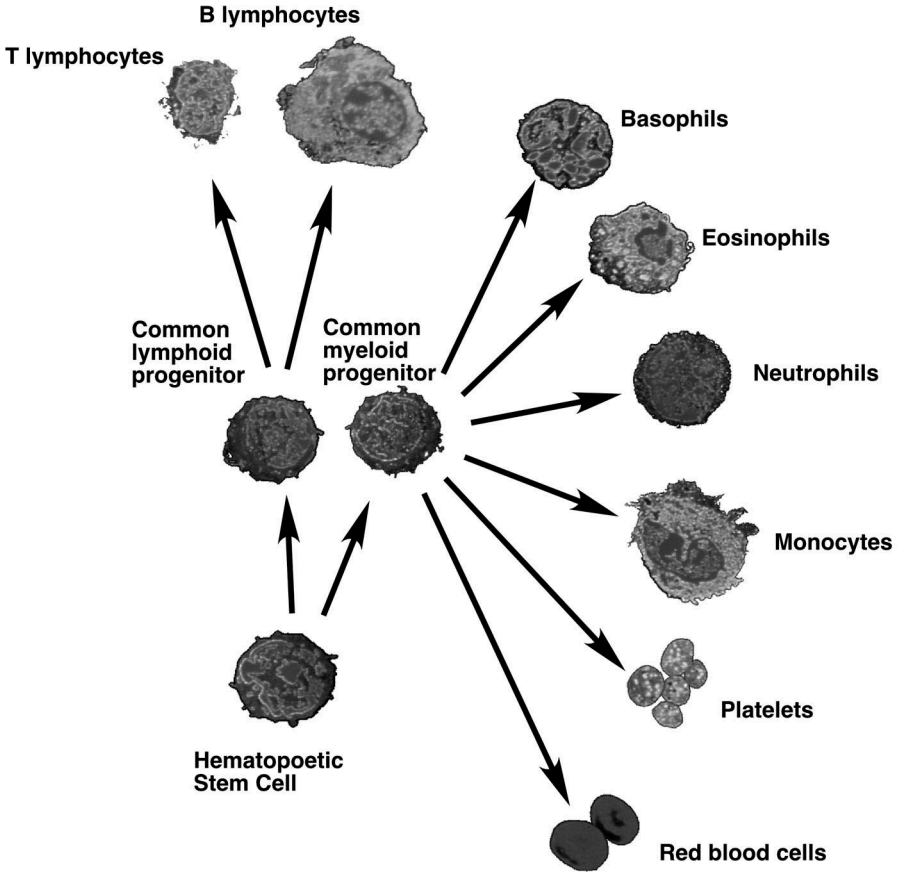

Figure 1. How hematopoietic cells develop from hematopoietic stem cells. This model implies that hematopoietic stem cells can develop into all hematopoietic lineages, both lymphoid and myeloid. Hematopoietic stem cells also possess the ability to self-renew.

Although hematopoietic stem cells are present within the extracts of the stem cell sources mentioned above, it is not as yet possible to isolate individual human hematopoietic stem cells. In contrast to the human setting, hematopoietic cells from murine BM and MPB can be highly enriched for stem cells by selection of key phenotypic cell surface markers. Enrichment of murine hematopoietic stem cells can be achieved by antibody selection for the tyrosine kinase receptors c-kit and Sca-1, while depleting the progenitor and mature cell lineages possessing lymphoid and myeloid markers. These purified cells are known as lineage negative (Lin^-) c-kit^+ Sca-1^+ cells. Further selection of these cells by excluding cells possessing the sialomucin surface adhesion molecule, CD34, from steady state BM cells, enables the isolation of individual hematopoietic stem cells, each capable of reconstituting the hematopoietic

system of a mouse (179). Interestingly, murine stem cells present in MPB show the reciprocal (i.e. $CD34^+$) phenotype, however, the $CD34^-$ phenotype returns to the stem cells after returning to steady state conditions in the BM following transplantation (240). The results of this study suggest that in mice the presence of the CD34 receptor is a marker of activated hematopoietic stem cells (i.e. activated from the stimulation of the mobilization procedure). Closely linked to the surface expression of CD34 is the expression of the glycoprotein CD38, which is expressed in stem cells in a precisely reciprocal manner to the CD34 marker, i.e. stem cells are either $CD34^+$ $CD38^{lo}$ or $CD34^-$ $CD38^+$.

Human hematopoietic stem cell purifications based on the findings of the murine system have been attempted, however, isolation of single stem cells has not been achieved. Selection for $CD34^+$ cells from human BM and MPB and CB mononuclear cells has been shown to enrich human hematopoietic stem cells (16), however, the population of enriched cells predominantly contains cells of less primitive potential. Human hematopoietic stem cell populations can be further purified by selecting for the CD38 lowest expressing cells ($CD38^{lo}$) (45), however, assays in immune compromised mice show that only 1/617 $CD34^+$ $CD38^{lo}$ cells from BM transplanted is capable of reconstituting the hematopoietic system of the mouse (22). Interstingly, it has been recently demonstrated that an extremely low proportion of human $CD34^-$ mononuclear cells have repopulating ability. This suggests that purifying stem cells by $CD34^+$ cell selection may be removing some of the more primitive cells with repopulating ability (21, 53, 94). The ability to isolate single human hematopoietic stem cells would greatly facilitate preclinical studies of the fate and function of hematopoietic stem cells (263).

Models for Assaying Adult Hematopoiesis

In vitro models of hematopoiesis such as the colony forming unit (CFU) assay used to assay clonogenic progenitors such as CFU-GEMM (11), or the long-term culture-initiating cell (LTC-IC) assay (236) and cobble-stone area forming cell (CAFC) assay (191) used to assay more primitive cells in culture for up to 12 weeks, have long been used for assaying both human and murine primitive hematopoietic cell differentiation of the myeloid lineage. By varying the growth conditions, most notably the cytokines used during culturing, the assays could be used to direct hematopoiesis of macrophage, granulocyte, erythrocyte, and megakaryocyte lineages. More recently, *in vitro* culturing of primitive human cells has enabled the differentiation of the lymphoid lineages (natural killer (NK) cells, B-cells and T-cells) as well as the myeloid lineages, enabling studies of multipotent hematopoietic cells with both lymphoid and myeloid differentiation ability (15, 101, 196). However, these *in vitro* assays may not be able to assay the true hematopoietic stem cell compartment adequately. When cells capable of long-term expansion *in vitro* in a an LTC-IC assay were compared to cells capable of reconstituting the hematopoietic system *in vivo* in a murine xenotransplant model of hematopoiesis, it was found that the cells with repopulating ability were different in terms of their frequency, phenotype, multilineage differentiation potential, and their

ability to be maintained in culture, compared to cells capable of expanding *in vitro* (20, 22, 58, 61, 80, 140). *In vivo* models of hematopoiesis are considered superior as they require the assayed cells to home and anchor to the site of hematopoiesis where they differentiate and self-renew to reconstitute the animal's hematopoietic system, as they would be required to do in a patient.

While there are several murine xenotransplant models available, such as Beige-Nude-Xid (BNX) mice (174) or Rag-2 mice (223), the most commonly used is the non-obese diabetic severe combined immunodeficiency (NOD/SCID) mouse, which yields the highest level of engraftment per transplanted human hematopoietic cell of the immune compromised mouse models (35). It was first used to assay human cell reconstitution by Lapidot *et al.* (139), and is used for assays of human hematopoietic stem cell biology, including hematopoietic stem cell homing and self-renewal ability, as well as studies of *in vivo* stem cell differentiation (54). Within the field of gene therapy, the NOD/SCID mouse is an invaluable tool for assaying the efficiency of viral vector mediated transduction of hematopoietic stem cells. Following intravenous transplantation of human hematopoietic stem cells into sub-lethally irradiated recipient mice, repopulation the hematopoietic system with a fraction of human cells occurs. These cells capable of reconstitution are known as SCID repopulating cell (SRC). There is no rejection of the transplanted human cells since the mice possess multiple defects in both adaptive and innate immunological function. They lack both functional B- and T-cells (220). In addition, they have impaired NK cell and macrophage cell function, and lack complement activity (224). The inability of the immune-compromised mouse to mount a host versus graft response, and the inability for the transplanted cells to mount a graft versus host response results in the stable human hematopoietic development within the mouse.

A limitation of the assay, which is based in part on the mouse's immune deficiency, is that it is unable to support human hematopoiesis of all the hematopoietic lineages. The lack of the ability to generate the human T-cells precludes it from many studies of the immune cell function. Moreover, the fact that the assay is a xenotransplant assay and that there are inherent differences between mouse and human hematopoiesis, results in, for example, the inability for human erythroid progenitor cell maturation to mature enucleated erythrocytes even in the presence of the human cytokine erythropoietin (35). Furthermore, there is no clinical evidence to suggest that the cells that are capable of reconstituting a NOD/SCID mouse are the same cells capable of long-term reconstituting in a patient. In fact, the relatively short life span of the mice limits assaying of transplanted hematopoietic cells to a maximum of several months, and hence limits the analyses to human stem cells with short-term repopulating ability. The length of study of hematopoiesis can be prolonged by serially transplanting bone marrow cells into secondary and tertiary recipients, as was performed in Paper III and by Lewis *et al.* (141). However, the poor seeding efficiency in the subsequent recipients is low (5-10%) and prevents the practical evaluation of long-term repopulating cells.

To circumvent the NOD/SCID mouse model's inability to support the full differentiation of all hematopoietic lineages, and to be able to perform long-term studies of hematopoiesis, other models can be used. Immune competent animals, such as the C57B6 mouse serve as excellent models for the study of hematopoiesis of the murine system (238). Here differentiation of all lineages, following transplantation of the murine stem cells, can be studied for up to 2 years (the approximate life span of the mouse). The other advantage is that all hematopoietic lineages can be assayed as they have functional immune systems. Other animal models involving larger animals, such as dogs (169) or non-human primates (69, 258) have very similar hematopoietic systems to humans and hence have been used for assaying aspects of hematopoiesis in autologous transplant settings. A large animal model that allows for the assaying of human hematopoietic progenitors is the xenograft preimmune sheep model, where human stem cells are transplanted in utero, prior to immune development, to permit the recognition of the human cells as self (45, 222, 285).

Hematopoietic Stem Cell Self-Renewal

The ability of transplanted hematopoietic stem cells to self-renew within a patient is a critical feature for transplantation therapies. *In vivo* competitive repopulation studies of bone marrow and fetal liver in mice estimate that there is a 15 to 150 fold expansion of repopulating units following transplantation, respectively (184). However, expanding hematopoietic stem cells *in vitro* has been more problematic. Having the ability to expand human hematopoietic stem cells *in vitro* would be an invaluable means of circumventing risks to the patient/donor of large scale stem cell extractions and the limiting quantities of hematopoietic stem cells obtained from umbilical cord blood harvests. The ability to culture stem cells *in vitro* is also a requirement for oncoretroviral vector mediated transduction of hematopoietic stem cells as will be discussed later. *In vitro* self-renewal divisions of short-term cultured human hematopoietic cells capable of repopulating NOD/SCID mice has been demonstrated (84), however, the majority of short-term culturing conditions result in the maintenance of, or the marginal increase in the total number of repopulating cells.

The cytokine combination used during the culturing of human hematopoietic stem cells and the duration of the culture has major effects on the expansion of stem cells. The cytokine thrombopoietin (TPO) when present in culture alone has been shown to be able to maintain the viability of primitive human bone marrow LTC–IC without inducing cell-division (26, 111). However, when TPO is used in combination with other cytokines, such as stem cell factor (SCF) and fetal liver tyrosine kinase ligand (FL), it acts synergistically to induce a potent proliferative signal to primitive hematopoietic cells (147, 168). In a study by Luens *et al.*, despite the 5-fold expansion of total cells during the 6 days of culture, there is only the maintenance of the number of cells capable of repopulating NOD/SCID mice. Such studies suggest that

hematopoietic stem cell self-renewal *in vitro* is the result of asymmetric divisions where one daughter cell may begin differentiating to a particular lineage while the other daughter cell maintains its primitivity. Other studies have shown the maintenance or marginal expansion (2-4 fold) of human bone marrow and umbilical cord blood NOD/SCID repopulating cells (47, 131, 175). Bhatia *et al*. found that cord blood $CD34^+$ $CD38^{lo}$ cells, although showing an initial 4 fold increase in SRC number after 4 days in culture, end up the total loss of repopulating ability if culture is continued past 9 days (20). While these studies contain various combinations of the cytokine TPO, SCF, FL, interleukin-3 (IL-3), IL-6, granulocyte-colony stimulating factor (G-CSF), there is mounting evidence that the use of the cytokine IL-3 has negative effects on the maintenance of repopulating ability after culturing(147, 281). However, evidence contesting this has also recently been provided in a large animal model study where a marginal improvement in the level of reconstitution from cells that had divided *in vitro* was seen using TPO, SCF, FL, IL-6 compared to IL-3, 6, SCF in the long-term, but that the combination with TPO, SCF, FL, IL-6 resulted in faster repopulation by short-term repopulating cells (136). These findings are in agreement with studies showing that TPO SCF and FL are capable of rapidly inducing cell-division of primitive cells compared to IL-3, IL-6, and SCF (147, 168). Interestingly, one particular study showed that human umbilical cord blood $CD34^+$ cells could be culture *in vitro* using TPO, SCF, FL, and IL-6 for 4 weeks and longer and significantly increase the total number of SRC by up to 70 fold (190). Unfortunately, long-term culturing of patient cells is not practically feasible within the clinical setting, and the modest repopulating cell expansions of the short-term culture of the other studies, while showing statistically significant increases, is not significant enough to warrant change in the established clinical protocols.

Other attempts at expanding hematopoietic stem cells by constitutively over expressing genes involved in signaling hematopoietic proliferation, such as the *c-mpl* gene has been tested (119, 253). The liquid soluble sonic hedgehog protein has also been tested *ex vivo* on human BM cells (19). However, significant expansion of hematopoietic stem cells has not been achieved. *Notch1* overexpression was shown to result in hematopoietic repopulating cell expansion *in vitro*, from an immortalized blast-like cell (253). Interestingly, the transcription factor homeobox gene (*HOXB4*) has been shown to be able to expand hematopoietic stem cells 1000 fold *in vivo* and 40 fold *in vitro* without affecting hematopoietic lineage differentiation or transforming cells (9, 10, 33, 212, 243). *HOXB4* is one of the member of a group of homeotic selector genes originally found 80 years ago that when mutated were able to affect the anterior and posterior patterning of *Drosophila* (*antennipedia*). Interestingly, *HoxB4* knockout mice show only minor reductions in steady state endogenous hematopoiesis, and transplantable repopulating cells showed a resistance to 5-FU myeloablation probably due to a lower stem cell cycling characteristics of the knockout mice (23). It was only under conditions where rapid proliferation was required or induced that the effects of the deficiency were detectable, i.e. a reduced ability to proliferate *in vivo* following transplantation or *in vitro* under strong cytokine stimulation.

Hematopoietic Stem Cell Trafficking and Engraftment

Although adult hematopoietic stem cells are found predominantly in the bone marrow, it has long been known that a small fraction of these primitive cells is regularly found in the peripheral blood. It is therefore believed that stem cells periodically migrate from their niche in the bone marrow only later to return to a new site in the bone marrow (65, 272). Presumably some of the mechanisms that regulate stem cell trafficking in this event are the same that regulate homing and lodging of hematopoietic stem cells during stem cell transplantations. The key structures found on hematopoietic progenitor cells that have been proposed to be involved in stem cell homing and lodging to the bone marrow micro-environment are known as adhesion molecules. Based on their structure and function they can be divided into integrins, selectins, and chemokine receptors, among others (195). Recently, some adhesion molecules have revealed that in addition to aiding in homing and lodgment, they play a significant role in hematopoietic stem cell proliferation and survival.

The concept of homing was first described in leukocyte migration to a site of infection, where the process of leukocyte extravasation is initiated by selectin mediated capture and rolling along activated endothelia. Following rolling, the leukocytes establish firm adhesion through interactions between β1 or β2 integrins and corresponding adhesion molecules on the endothelial cells to enable extravasation (123, 228). It has been hypothesized that hematopoietic stem cell homing to the bone marrow could mirror this process (229). Direct involvement of adhesion molecules in the homing of hematopoietic progenitors has been demonstrated. The α4β1 integrin (also known as very late antigen 4 (VLA-4)) was shown to bind to its receptor, fibronectin (an extra cellular matrix protein in the bone marrow), to elicit homing and engraftment of adult bone marrow cells in transplanted fetal sheep (284). The same study revealed that migration of transplanted cells to the bone marrow could be modulated using anti β1 integrin antibodies. Interestingly, in addition to integrin being a requirement for efficient engraftment *in vivo*, it was also found that *in vitro* culturing of $CD34^+$ cells on the carboxyl (COOH) terminal domain of the fibronectin (FN) (CH-296), could better maintain the *in vivo* repopulating potential of the cells in NOD/SCID mice (52). Furthermore, that fibronectin binding to hematopoietic stem cells during short-term culturing could mediate a β1-integrin induced prevention of apoptosis (63), and increased production of a cell-cycle inhibitor (117). The β1-integrins and their ligands (VCAM-1 and fibronectin) have also been implicated in the mobilization of hematopoietic progenitors from the bone marrow into the peripheral blood (181, 182). Moreover, the FN fragment has been shown to increase oncoretroviral vector transduction efficiency into hematopoietic progenitors, through the colocalization of the viral particle to the target cell (63, 100, 164).

Hematopoietic progenitor cell migration to the bone marrow has also been suggested to be modulated by chemoattractants, such as stroma cell derived factor-1 (SDF-1), produced by stroma cells (3). The chemokine receptor for SDF-1, CXCR4, is found on a subset of primitive hematopoietic cells, and in a study using antibodies which inhibited CXCR4 receptor binding to SDF-1, an inhibition of engraftment in NOD/SCID mice was noted (187). However, another study found that CXCR4 receptor binding was not required for stem cell engraftment in the same mice (210). While the precise role of CXCR4 and SDF in stem cell homing and engraftment remains to be elucidated, CXCR4 signaling was recently found to block entry of the S phase of the cell-cycle in short-term incubated $CD34^+$ cells. Thus, CXCR4 signaling may contribute to the quiescent nature of the stem cell compartment (86).

Cell-cycle Status of Hematopoietic Stem Cells and Effects on Repopulating Potential

Intimately related to the engrafting ability of hematopoietic stem cells is their cell-cycle status. Despite the high proliferative potential of the hematopoietic stem cell, these cells usually lie quiescent in the bone marrow until they are either summoned to proliferate based on a need for more mature cells, or alternatively periodically proliferate only to have their progeny regulated by signals for continued proliferation or to undergo apoptosis. Primitive human hematopoietic cell isolates such as $CD34^+$ cells from the three main sources of adult hematopoietic cells, BM, MPB, and CB, have distinct cell-cycle characteristics. The percentage of actively cycling $CD34^+$ cells ($S/G_2/M$) is low from MPB and CB (< 3 %) and may be up to 15 % in $CD34^+$ cells derived from the BM (92, 247, 270). The percentage of $CD34^+$ cells residing in G_1 also varies between the cell sources with the lowest percentage in BM and MPB at around 50-60% and CB cells having approximately 60-70% (108, 270). Interestingly, although the proportion of G_1 cells from BM and MPB are similar for the $CD34^+$ fraction of cells, the more primitive fraction ($CD34^+$ $CD38^{lo}$) show significant differences, with MPB having 2.5 fold more cells residing in G_1 than BM (108), suggesting an activated state of the more primitive cells of MPB (108).

The most primitive hematopoietic cells capable of reconstituting the hematopoietic system are non-dividing. Both human BM and MPB derived SRC reside almost exclusively in the G_0 stage of the cell-cycle (22, 92). Similarly, *in vitro* culturing of the MPB $CD34^+$ cells under cytokine conditions that stimulate cell-cycle progression of G_0 cells into G_1, such as in the presence of interleukin-3 (IL-3) and IL-6 and stem cell factor or TPO, SCF, and FL also show that cells in the G_1 stage of the cell-cycle have a virtually no repopulating potential (92). In contrast to these findings in BM and MPB, human umbilical cord blood $CD34^+$ cells have a similar engraftment potential in the G_0 and G_1 subfractions (270) which likely has to do with its ontogeny and requirement for extensive self-renewal divisions in the developing organism (269).

Repopulating cells, while being able to self-renew *in vitro* (84), are required to be in a non-dividing state in order to repopulate a transplanted animal. Therefore, mechanisms must exist that diminish the repopulating ability of dividing stem cells. In the murine setting, it has been proposed that since *in vitro* induced cell-cycling lowers the expression of the integrin VLA-4 on the hematopoietic progenitor cell surfaces, that may be the mechanism of reduced engrafting ability of dividing cells (13, 18). However, this is not the case in human hematopoietic progenitor cells. CB cells incubated *in vitro* for 5 days and then sorted for the $S/G_2/M$ phase of the cell-cycle showed an inability to engraft despite there being no differences in VLA-4, -5, or CXCRA expression compared to G_1 cells (85). Moreover, bone marrow $CD34^+$ cells in $S/G_2/M$ were found to have a higher VLA-4 expression than G_1/G_0 cells (276). An increased expression of several adhesion molecules has also been seen on the primitive $CD34^+$ $Thy\text{-}1^+$ cells from MPB after 5 days of culturing (283). Findings by Jetmore *et al*. may provide clues as to why these cycling stem cells do not repopulate mice. They suggest that, although homing of cycling and non-cycling cells to the bone marrow is equal within the first 40 hours after transplantation, the cycling cells are more prone to undergo bone marrow micro-environment mediated apoptosis (115). Recently, it was found that by inhibiting hematopoietic stem cell-cycle progression following *ex vivo* expansion and prior to transplantation, that it was possible to refurbish the repopulating potential of the activated cells. In this study, stroma derived growth factor-1 (SDF-1), which has been shown to block entry to the S phase of the cell-cycle, resulted in the doubling of SRC numbers with its use (86).

RETROVIRUS BASED VECTORS

Oncoretroviral Based Vectors

Retrovirus particles contain two copies of their single stranded RNA genome (7-10 kb long) consisting of the 3 genes, *gag*, *pol*, and *env* (70). The genome pair is packaged around a nucleocapsid made of viral proteins, from the *gag* gene, which in turn is surrounded by an envelope derived from the cytoplasmic membrane of the cell from which the virus was produced (57). The envelope contains viral glycoproteins, produced from the *env* gene, which act as receptor binding domains for the virus to adhere to the target cell. Within the viral nucleocapsid are proteins required for reverse transcription of the RNA genome into double stranded DNA, and for integration of the newly formed DNA into the target cell's genome (89). These proteins are produced from the *pol* gene. Evolution has enabled the retrovirus to be efficient at integrating its DNA into a target cell's genome for the purpose of producing new viral particles. Retroviruses can be modified to maintain their ability to transfer genes into target cells without being able to replicate and produce new virus in the target cell (151). The modified viruses are known as vectors, of which the most commonly used today are based on the *Moloney murine leukemia virus* (MMLV) (38).

Separation of *Cis* and *Trans* Elements

It is the ability to separate the *cis* and *trans* elements for transduction that has enabled the development of vectors for clinical purposes, i.e. to be able to insert therapeutic genes into patients' cells without generating replication competent retrovirus. Numerous oncoretroviral based vectors, have been successfully developed and contain only the sequences required in *cis* for function of the vector. Examples of permanent cell lines used to produce such vectors are the Φ-2, Φ-am, PA12, PA317, PE 501, Φ-cre, Φ-crip cell lines (38, 50, 106). The sequences required on the transfer vector itself for the efficient transduction of the target cell are, the long terminal repeats (LTR), the packaging signal, the polypurine tract, and the primer binding site (194). All genes and additional sequences can be removed from the vector and are only needed to be expressed in *trans* to generate the structural and functional proteins of the virus. Thus, the vector itself has none of the genes required for its replication. These genes only need be expressed in a producer cell line. The newer packaging cell lines are "split" packaging cells, i.e. the *gag-pol* and the *env* genes are coded by separate plasmids which are integrated in separate locations in the genome (for example the GP+Am12 cells). The removal of all genes in the vector itself ensures that no new viral particles can be produced, but also that no potentially immunogenic viral proteins can be expressed in the target cell. This should prevent an immunological rejection of the transduced cells in a patient. The ultimate separation of *cis* and *trans* is illustrated in the third-generation lentiviral vector 4-plasmid system shown in Figure 2, where the transfer vector is present on one plasmid and the genes required for assembly and function are separately located on three additional plasmids (67).

Receptor Binding and the Envelope

The first step in transduction for a virus particle involves the binding of its envelope proteins to a receptor on the cell membrane of the target cell. Therefore, the receptor binding protein present on the virus envelope must be encoded in the producer cell for efficient transduction. As the isolation of individual hematopoietic stem cells is still, as yet, impossible, it is difficult to be sure that the receptor is in fact present. The receptor binding protein is usually a surface glycoprotein and can be changed to enable the vector to specifically infect a particular cell type. The process of substituting the endogenous envelope glycoprotein of the virus for that of another virus is called pseudotyping. Oncoretroviral vectors have been successfully pseudotyped with several envelopes. The most commonly used are the wild-type amphotropic and the *gibbon ape leukemia virus* envelopes, which bind to the sodium dependent phosphate transporters Pit-1 and Pit-2 respectively (124, 186). Pseudotyping can also confer new characteristics to the virion particle. For example, the pseudotyping retroviruses with the *vesicular stomatitis virus*-glycoprotein (VSV-G), or the envelope protein from a feline endogenous retrovirus, RD114, increases the mechanical stability of the vector

particles, enabling ultra-centrifugation for concentration of the virus, and repeated freeze thaw cycles without loss of viral titer (32, 88, 125, 178).

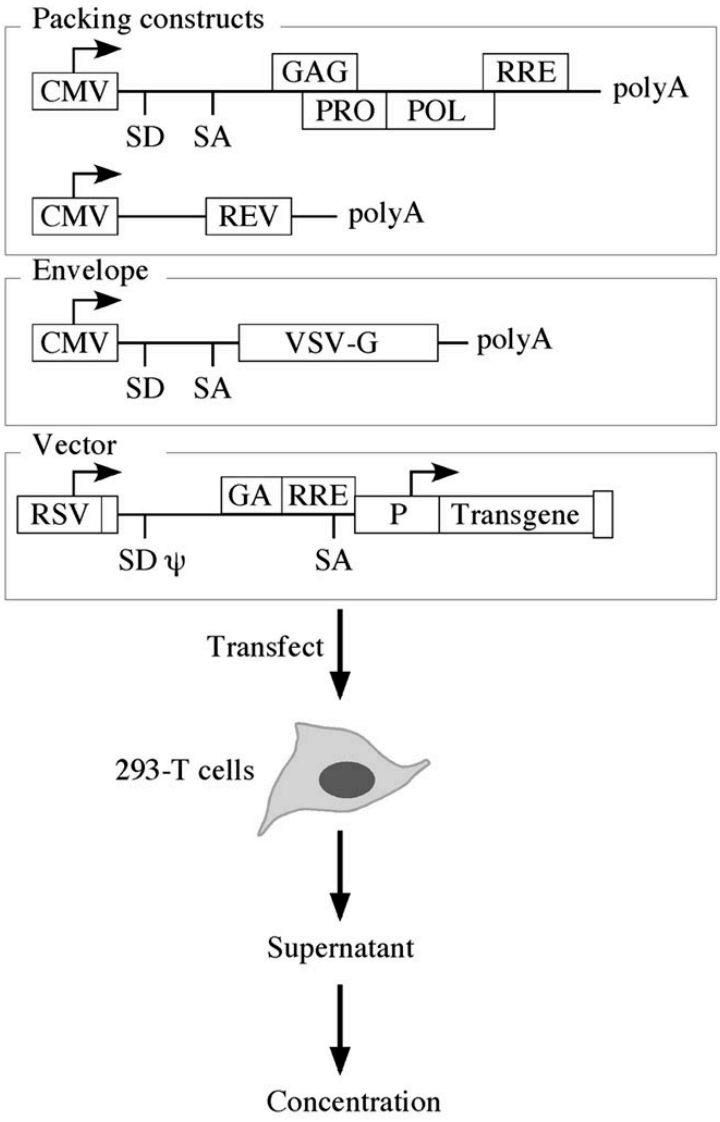

Figure 2. A retroviral based vector production system with separation of *cis* and *trans* elements. The figure shows four constructs used to produce third-generation lentiviral vectors. There are two packaging constructs, one expressing the *gag* and *pol* genes and another expressing the *rev* gene. A third plasmid expresses the VSV-G envelope protein. The vector plasmid contains a chimeric LTR, consisting mostly of the *Rous sarcoma virus* (RSV) LTR fused to the U5 region of the HIV LTR. The vector contains the packaging signal that is absent in the packaging constructs (to prevent packaging the RNA of the packaging constructs into vector particles), and the *rev* responsive element, a promoter (P) and the transgene. These four constructs are transiently transfected into 293-T cells and the supernatant is harvested after approximately 48 hours. The supernatant can be concentrated 100-1000 fold by spinning in an ultracentrifuge. SD indicates splice donor, SA splice acceptor and CMV indicates the promoter/enhancer from *cytomegalovirus*.

Requirement for Cell-Division for Oncoretroviral Vector Integration

Following receptor binding, endocytosis or fusion occurs depending on which glycoprotein is encoded by the *env* gene, which leads to the eventual release of the contents of the capsid into the cytoplasm. It is in the cytoplasm that the reverse transcription and conversion of the RNA genome into double stranded DNA occurs. Once the vector has undergone reverse transcription, the preintegration complex (PIC) awaits for the access to the target cell's genome. Oncoretroviral vectors require the collapse of the nuclear membrane during mitosis in order to gain access to the target cell's genome and integrate (160, 208). Integration is mediated by the Integrase protein and is believed to result in integration in random sites within the genome (107). The requirement for cell-division to occur for successful integration has particular significance since, as previously stated, most hematopoietic stem cells at any given point in time are non-dividing.

Transgene Expression

Oncoretroviral vectors have been designed to transfer genes into the target cells of interest, namely primitive hematopoietic cells. The deletion of *gag*, *pol*, and *env* genes from the vector leaves space for the insert of therapeutic genes up to about 7-8 kb. However, in preclinical research often it is useful to express a marker gene which enables the screening for infected cells, e.g. the gene for the green fluorescence protein (GFP) from the jelly fish *Aequorea Victoria* (39, 43, 188). Other selectable marker genes include truncated receptors whose intracellular signaling domains have been removed and the extracellular domain is able to bind to antibodies conjugated to fluorochromes which can be detected by fluorescence microscopy or by flow cytometry, e.g. the clinically used LNGFR neurotropic growth factor receptor (25). Genes which confer resistance to cytotoxic substances have also been used as selectable markers. If the desired outcome is the expression of more than one gene, for example, a therapeutic gene and a marker gene, then dual gene expression vectors must be used. One method of obtaining expression from of two genes within the retroviral vector containing a single promoter is to use the internal ribosomal entry site (IRES) sequence from a virus such as the *encephalomyocarditis virus* (113, 114). This allows the translation of two separate proteins from a single mRNA, as in the MGIN vector, which drives expression of the GFP and the neomycin resistance gene as described by Cheng *et al.* (42).

The level of transgene expression is dependent on the efficiency of the promoter to drive transcription of the transgene in the target cell. In MMLV based vectors, transgene expression is driven from the U3 promoter and enhancer region of the LTR. However, MMLV LTR expression has been shown to be sensitive to transcriptional inactivation shortly after transduction in both murine ES cells and primitive hematopoietic progenitors (40, 233). The transcriptional inactivation has been attributed to *de novo* methylation and trans-acting factors (180, 227, 279). In order to

overcome this limitation, Ostertag's group developed a recombinant oncoretroviral vector based on a mutated *myeloproliferative sarcoma virus* (MPSV). This new virus showed an ability to maintain expression of the transgene in ES cells, and hence was named the *murine embryonic stem cell virus* (MESV) (77, 93). The MESV based vector was then further modified by Hawley *et al*. to increase titers, and renamed it as the *murine stem cell virus* (MSCV) (103). Most of the oncoretroviral vector used today are derived from MSCV vectors, such as the MGIN and the MGirL22Y vectors used in Papers I, II, and III.

Lentivirus Based Vectors

The most common lentiviral vector used in preclinical research for gene therapy applications has its origins from the *human immunodeficiency virus-type 1* (HIV-1). HIV like the MMLV is a member of the retrovirus family of viruses. The subclass of the retrovirus family, "Lenti" virus (Latin for slow, referring to slow and persistent rate of infection). Other members of the subclass of lentiviruses include the *simian immunodeficiency virus* (SIV), the *bovine immunodeficiency virus* (BIV), the *feline immunodeficiency virus* (FIV), and the *equine infectious anemia virus* (EIAV). HIV-1 from which the lentiviral vector discussed in this thesis is based, is considered to have a complex genome since in addition to the gag, pol, and env genes of standard retroviruses, it contains 6 accessory genes; *tat*, *rev*, *nef*, *vpr*, *vpu*, and *vif*. These accessory genes have been implicated in the virus' pathogenicity (72, 244). However, it is the ability of lentiviral vectors to integrate into the genomes of non-dividing cells that has brought it to the attention of the field of gene therapy of the hematopoietic system.

Lentiviral Vector Packaging

The lentiviral vectors used today, although having their origins in HIV, have few HIV genomic sequences remaining. As in the MMLV based vectors, the LTR, the truncated *gag* gene containing the packaging signal, and the primer binding site are required for function of the vector. The packaging genes are separated from vector sequences as in the MMLV based vectors. They contain the HIV sequences necessary for the packaging of the transfer vector into the functional virion particle, namely the *gag* and *pol* genes. Four of the six accessory genes can be removed from the packaging construct (*nef*, *vpr*, *vpu*, *vif*) as their deletion has little effect on the virus' ability to infect cells, leaving only the *rev* and *tat* accessory genes. The *rev* protein is necessary to prevent splicing of the transfer vector RNA in the producer cell by efficiently exporting it out of the nucleus during viral production. This enables the full-length

vector RNA to be package into a virion particle. The Tat protein is a transcription factor, which binds to the *tar* element in the HIV LTR, necessary for the activation of the LTR promoter (244). The envelope protein used for most lentiviral vectors is the VSV-G, first shown to be successful in pseudotyping oncoretroviral vectors (4, 32). The VSV-G binds to an as yet uncharacterized receptor in the phospholipid bilayer of the cytoplasmic membrane in both vertebrates and invertebrates, and thus has a very broad host range (152). As wild-type HIV-1 enters a $CD4^+$ cell by fusion (231), and the VSV enters its target cell through endocytosis (207), a VSV-G pseudotyped lentiviral vector particle undergoes a slight change in the normal mechanism of entry, but to seemingly no serious consequence. Since the majority of accessory genes have been removed, and the envelope replaced to that of another viruses, there is no chance that the lentiviral vector will ever be able to form the wild-type HIV from which is was derived. That and other safety features of the vector (described in the safety issues section ahead) make lentiviral vectors a theoretically safe means of transducing cells.

The Ability to Infect Non-Dividing Cells

After viral entry and reverse transcription in the cytoplasm of the target cell, the preintegration complex (PIC) localizes to the nucleus, where by active transport through a nuclear pore, the viral genome enters the nucleus of a non-dividing cell (31, 72, 79). The Vpr accessory protein, the Integrase protein, and the *gag* encoded Matrix protein play both additive and redundant roles in this by connecting the PIC with the cellular nuclear import machinery (30, 78, 104). Once in the nucleus, the viral genome can integrate into target cell's genome via the viral Integrase protein. Despite the removal of the accessory gene *vpr* in the lentiviral vector, the lentiviral vector is still capable of entering the nucleus of the non-dividing cell with only slight disadvantage compared to its wild-type counterpart (171). The maintenance of the Matrix and Integrase proteins is the presumable reason for the maintained capability for infection of non-dividing cells (244). This can be achieved independently of mitosis, giving it an advantage over MMLV based vectors in the transduction of non-dividing cells. The non-dividing (or rarely dividing) cell types that have been shown to be pervious to infection by lentiviral vectors are neurons, myocytes, hepatocytes, retinal cells, corneal, cochlear, and pancreatic islet cells (83, 99, 121, 163, 252, 262, 290). Of particular interest is the ability to transduce the non-dividing cells of the hematopoietic system (as will be discussed in length in the section for Transduction of Hematopoietic Stem Cells.)

An interesting feature of wild-type HIV-1 is that following the release of the contents of the HIV-1 into cytoplasm of a quiescent T-cell, the unintegrated PIC is surprisingly stable. A PIC can remain unintegrated for as long as 3 weeks in a quiescent T-cell before integrating upon cell activation (232). In contrast, a PIC from an oncoretroviral vector is stable only for a matter of hours (8), suggesting an advantage for transduction of quiescent cells using lentiviral vector as the PIC could potentially wait for the

conditions for transduction to arise. Another interesting feature of HIV-1 is that the concentration of dNTP in the cytoplasm appears to play a role in the efficiency of the virus replication and likely increasing the efficiency of reverse transcription (133, 159). Therefore, by modulating the dNTP during lentiviral vector transduction may increase gene transfer efficiency in some cell types.

Lentiviral Vector Design and Safety

The great potential of lentiviral vectors as a tool for gene therapy applications may be hampered by certain issues of safety which need addressing prior to their use in clinical protocols. Most lentiviral vectors used to date for the transduction of hematopoietic stem cells have been produced by transiently transfecting packaging constructs and the transfer vector into a producer cell line. This technique for production has an increased likelihood for recombination that could potentially cause the production of replication competent vectors, compared to production from cell lines where the constructs are stably integrated into the producer cell's genome. Stably integrated vector systems have long been used in the generation of oncoretroviral vectors (38, 151). Although the formation of replication competent vectors has rarely been reported for modern vectors produced, by either transiently generated or stable integrated producer cells, it is the general opinion that methods of reducing the risk are warranted. In order to reduce the chances of recombination potentially capable of forming replication competent lentiviral vector, second- and third-generation vectors have been generated, which include a self-inactivating (SIN) deletion of the LTR (67, 161, 289). In these vector systems the envelope gene, the packaging genes, and the transfer vector are on separate plasmids. Only tens of base pairs of overlapping sequences with the other plasmids are present. The third-generation vector in addition to the above features, separates the accessory gene *rev* onto a fourth plasmid further reducing the likelihood of generation of replication competent recombinants (67).

A second concern with the lentiviral vectors is that they share sequence homology with wild type HIV-1. While this poses a threat only if the patient contracts HIV-1 after the gene therapy treatment and the HIV recombines with the vector to form a hybrid unknown vector, it may also complicate the patient's life as HIV vectors may cause the positive scoring of HIV in common tests without the person having been infected with HIV-1. It has been shown to be relatively common that the two copy genome in the virus particle recombines, suggesting if two heterogeneous copies were packaged into the virion (i.e. one vector genome and one wild type HIV genome) a new viral species could emerge (116). Oncoretroviral based vectors based on viruses, such as the MMLV, which do not infect human cells, do not pose the same risks and therefore may be theoretically safer than traditional lentiviral vectors. The development of second- and third-generation lentiviral vectors has dramatically improved the safety of the vector as four of the six accessory genes present on the vector have been removed in the second-generation and all but one removed from the packaging construct in the third-generation vector. The third-generation vector also

has the replacement of the Tat dependant 5'LTR promoter U3 region with the *rous sarcoma virus* (RSV) promoter, which further reduces sequence homology with the wild-type virus and removes the requirement for the *tat* gene in the packaging construct (67). The transfer vector only contains the minimum sequences for functionality of the vector namely portions of the LTR (required for reverse transcription, poly-adenylation and integration), and a portion of the *gag* gene which contains the packaging signal. The 180 bp central polypurine tract (cPPT) was returned to the transfer vector in the third-generation vector, after having been removed in the second-generation vector, as it was shown to improve the efficiency of PIC nuclear import (76, 286).

The development of self-inactivating (SIN) lentiviral vectors further reduces sequence homology with the wild-type virus, but more importantly has the feature which renders an integrated vector incapable of mRNA transcription from the LTR. It is a 400 bp deletion in the 3' LTR promoter region, which removes the LTR promoter in the 5' position following reverse transcription, and therefore prevents full length vector transcription when integrated in the target cell's genome (67, 95, 110, 161, 289). This has implications for safety as without the transcription from the LTR there can be no generation of virus genome capable of being packaged into a virus particle. Furthermore, since lentiviral vectors may, by chance, integrate into genes in the target cell's genomes, the removal of LTR promoters ensures they will not activate genes which may be potentially dangerous for the patient, such as proto-oncogenes. Two recently developed lentiviral vector packaging systems capable of being producing virus from a stable integrated packaging cell line (i.e. eliminating the need for transient transfection of packaging and transfer vector constructs) have recently been developed (Figure 3). However, their efficacies have yet to be assessed (127, 273).

From the standpoint of biosafety, since vectors based on HIV are derived from a virus that infects humans, it has been proposed that there may be increased risks associated with their use in patients. In lieu of this, alternative lentiviral vectors derived from viruses that infect other species have been developed. These viruses have been based on SIV (218), BIV (17), FIV (193), and EIAV (275), and have also demonstrated the ability to transduce non-dividing cells. However, as proposed by Trono, there are several factors that make HIV based vectors theoretically the safer than non-human derived lentiviral vectors (245). 1) Since HIV based vectors are derived from a virus which infects human cells, it is conceivable that the HIV based virus will be more efficient at transducing human cells compared to animal derived vectors. 2) The complexity of the HIV genome is far greater than that of most other lentiviruses (nine genes in HIV instead of six in other lentiviruses) and since all lentiviral vectors require at least three genes (*gag*, *pol*, and *rev*), HIV would be the most distally removed vector from its parental virus. 3) Experience has revealed that infection of one species with the pathogen from another is largely unpredictable (158). Moreover, millions of people worldwide have been screened for HIV-related illnesses, and mutations in HIV have been shown to be able to be carried by humans without causing pathogenicity.

Furthermore, some of these non-pathogenic mutant HIV strains share significantly more sequence homology with wild-type HIV than the third-generation lentiviral vectors.

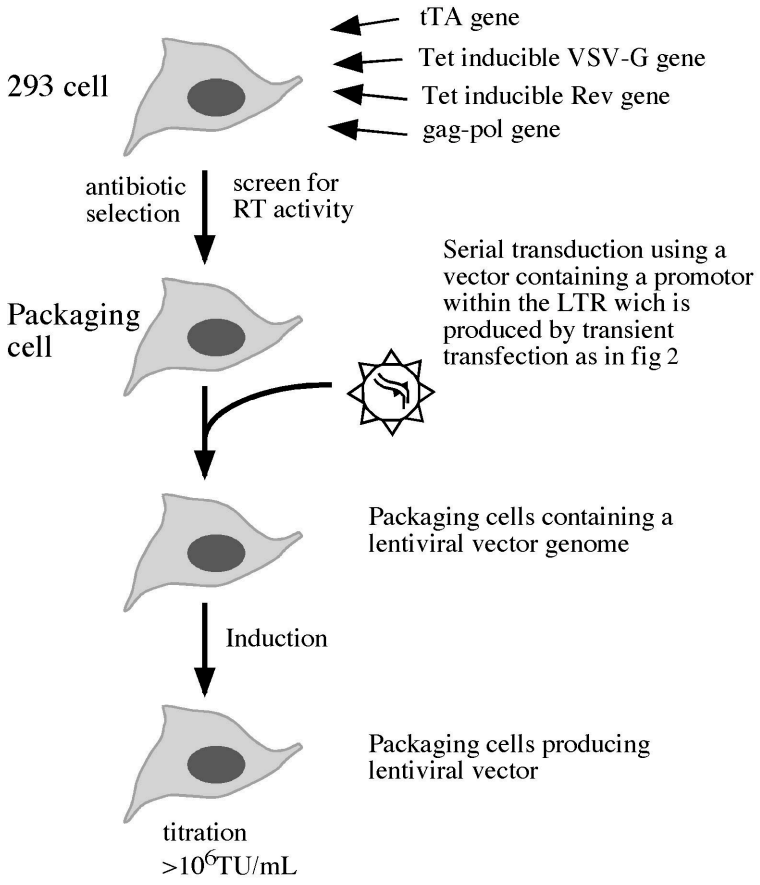

Figure 3. Production of a permanent packaging cell for HIV-1 based vectors. In the first step the vector, packaging constructs and the VSV-G envelope construct are transfected into 293 cells, as explained in Figure 2, to produce a cell line that can express the rev protein and the envelope and gag-pol proteins. The packaging cell line that is created in step 1 contains chromosome integrated constructs that express the *VSV-G* and *rev* genes by a tetracycline regulated promoter and the tTA transactivator, and *gag-pol* genes are expressed by constitutive promoters. The *gag-pol* construct is shown in Figure 2. The *gag-pol* transcripts are quickly degraded in the nucleus without the rev posttranscriptional regulator. Therefore, the *gag-pol* transcripts are stabilized upon rev induction. The expression of the *VSV-G* gene must be regulated since its permanent expression would lead to cell death. This cell line is now repeatedly transduced by a vector from transiently transfected 293-T cells in order to generate multiple vector integration sites and as a consequence, high titer producers that can upon induction produce the viral proteins and the vector RNA (step 2 to 3). In order to suppress expression of the cytotoxic viral proteins the cells are grown in medium containing doxycycline while the cells are maintained. To produce vectors, the viral proteins are induced in the absence of doxycycline. This figure is modified from Klages *et al.* where a description of the generation of the LVG packaging line was made.

PRESENT INVESTIGATION

Specific Aims

- To determine the utility of lentiviral vectors in gene therapy treatment of hematological disorders as assessed by the ability of lentiviral vectors to efficiently transduce human hematopoietic repopulating cells capable of both myeloid and lymphoid reconstitution.

- To determine the ability of lentiviral vectors to transduced repopulating cells under conditions that do not stimulate cell-division, and hence preserve the stem cell repopulating and differentiation potential.

- To determine the ability of lentiviral vectors to transduce primitive murine hematopoietic cells and use this system to define the optimal conditions for transduction of primitive hematopoietic progenitor cells, for the ultimate goal of testing gene therapy based treatments in murine models of human diseases.

- To define the optimal conditions for efficient transduction of primitive hematopoietic progenitors, with respect to cytokine stimulation and cell-cycle status of the cells in hematopoietic progenitors.

- To determine the ability of lentiviral vectors to efficiently transduce embryonic stem cells and use this system to study the lentiviral vector transgene expression throughout differentiation to hematopoietic progenitor cells.

- To optimize lentiviral vector design for efficient transgene expression in the progeny of repopulating cells, using regulatory elements capable of delivering high-level expression with minimal variegation effects.

- To address issues of safety for lentiviral vector transduction of hematopoietic stem cells with regards to vector design and insertional mutagenesis.

TRANSDUCTION OF STEM CELLS

Transduction and Transgene Expression in Embryonic Stem Cells

With the enormous clinical potential of embryonic stem cells, based on its differentiation potential and ability to self-renew in culture, the ES cell is an ideal target for vector mediated genetic modification. Moreover, the ability for ES cells to differentiate *in vitro* to hematopoietic progenitor allows it to be used to assay vector transgene expression throughout hematopoietic differentiation. Oncoretroviral vector transduction of ES cells undergoing self-renewal divisions in culture is efficient. However, the oncoretroviral vector's ability to maintain transgene expression after differentiation to hematopoietic progenitor or other tissues, including the entire organism itself, is hampered by the silencing of the LTR promoter (44, 112, 138). Hence, as part of the study in Paper I of this thesis, the lentiviral vector system was tested in the context of transduction of and expression in ES cells differentiated to embryoid bodies and then further to hematopoietic cells. The results using a lentiviral vector driving GFP expression from an internal murine phosphoglycerate kinase (muPGK) promoter, were compared with results using two oncoretroviral vectors; the MSCV based oncoretroviral vector, and an LTR-independent vector driving expression from an internal muPGK promoter.

We compared the transduction efficiency and transgene expression characteristics of the three vectors described above possessing different envelopes at varying MOI, followed by ES cell differentiation to EB bodies and subsequently to hematopoietic cells. We found that the VSV-G envelope yielded higher transduction efficiencies at similar MOI compared to the same vector pseudotyped with an ecotropic envelope (GP+E-86), suggesting a greater abundance of VSV-G receptor than ecotropic receptor on ES cells. We also found that using the same MOI, lentiviral vectors could transduce the ES cells slightly more efficiently than oncoretroviral vectors with the same envelope, with a near 100% TE at an MOI of 59 TU/cell compared to the MOI of 590 TU/cell required to achieve the same level of transduction by the oncoretroviral vector. The difference in MOI required to achieve the same transduction efficiency is likely due to inherent differences between the vectors in terms of their mechanisms of transduction, and the difference in proliferation kinetics of the ES cells and the cells in which the viral preps were titered.

The ES cell's ability to be clonally expanded enables vector copy number analysis to be performed. It was found that using a high MOI, most of the cells contained multiple

vector copies integrated, as determined by southern blot analysis of digested genomic DNA from individually expanded ES clones. Up to twelve (12) integrations per ES clone were seen using the lentiviral vector, and six (6) using the oncoretroviral vector. The results of our study also demonstrated that the transgene expression correlated with the number of lentiviral vector copies in the ES cell expanded clones, whereas the expression from the oncoretrovirus did not. GFP expression from the lentiviral vector was found to be directly proportional to the vector copy number, with approximately 50 mean fluorescence units (MFU) per vector copy. The GFP expression levels from ES clones transduced with the oncoretroviral vectors was not proportional to copy number with great variation in the expression, suggesting greater position dependent expression from the LTR dependant oncoretroviral vector. Furthermore, when ES cell clones were allowed to proceed with differentiation into EB bodies (day 6) by the removal of LIF, expression was significantly reduced when the transgene was driven by either the LTR promoter or the internal promoter in the oncoretroviral vector, and expression was only slightly reduced for the lentiviral vector driving expression from the internal promoter. Moreover, the expression after differentiation of the EB to hematopoietic colony was only possible with the lentiviral vector where some expression was lost. The study also revealed that the HIV LTR promoter is transcriptionally inactive in ES cells as only mRNA produced from the internal muPGK promoter could be detected. These results suggest that lentiviral vectors are relatively resistant to position-effect variegation and other types of transcriptional downregulation compared to oncoretroviral vectors.

In this study, we demonstrate for the first time the ability of lentiviral vectors to efficiently transduce ES cells, and to maintain their capability for transgene expression throughout differentiation to hematopoietic progenitors. Since these studies, several other studies from other labs have demonstrated that following transduction of ES cells, a transgenic mouse could be generated with virtually all transduced cells of the mouse expressing the transgene (145, 189). The vector transduction of ES cells circumvents the need for the largely inefficient genetic manipulation by homologous recombination for the generation of such mice. The transgenic mice could be generated by two methods 1) by the injection of transduced ES cells into a developing embryo at the blastocyst stage followed by transplantation into a pseudopregnant recipient mouse. 2) by infection of early stage embryos either at the single cell stage or at the morula stage (8-16 cells) having had the zona pelucida removed (or alternatively by injection of the virus directly into the perivitelline space of the embryo) followed by transplantation into pseudopregnant recipients. Mating of the generated offspring with other mice and the ability to visibly score the transgene in the progeny revealed that the transgene was transmitted through the germ line (145, 189). By driving the transgene expression from tissue-specific promoters, Lois *et al.* were able to derive mice with transgene expression in either myocytes or T-cells exclusively.

Transduction of Hematopoietic Progenitors

Transduction of Murine Hematopoietic Progenitors

The extensive knowledge of the murine hematopoietic system and the effectiveness of the assays used to assess hematopoiesis make the murine model an ideal tool for the study of factors involved in efficient lentiviral vector transduction. Moreover to be able to define efficient conditions for transduction of the hematopoietic cells of the murine system would enable the testing of the feasibility of lentiviral vector mediated gene therapy of human disease in murine models. Lentiviral vectors had not previously been used for the transduction of hematopoietic cells from species other than humans adding novelty to lentiviral vector transduction of murine progenitor cells. As described in Paper II, we transduced primitive hematopoietic Lin-, c-kit$^+$, Sca-1$^+$, cells derived from mouse bone marrow under variable conditions using lentiviral vectors and assayed for the transduction efficiency using *in vitro* suspension and CFC assays by flow assisted cytometry (FACS) or by polymerase chain reaction (PCR) and microscopy on individual CFU-GM colonies. Following transduction of the murine hematopoietic progenitors and scoring of individual CFU-GM colonies by microscopy, it was apparent that although a colony may have contained a significant portion of GFP$^+$ cells, there was a significant portion of cells within the colony that were not expressing GFP. In order to ascertain if this was the result of silencing of the vector expression, or a true lack of vector presence in those cells (despite all cells in the colony originating from a single cell) secondary plating of individual colonies was performed. PCR on the secondary colonies revealed that, although all the colonies in the plate originated from a single cell, only a subset of the colonies contained the vector, indicating that the originating primary colony was a genetic mosaic of cells containing and not containing the integrated vector. Since only part of the primary colony contained the vector, but all cells from the colony originated from a single cell, suggested that the vector integrated after the originating colony forming cell had divided. We therefore proposed that there was a delay in the lentiviral vector integration in murine progenitor cells. This was supported by the finding that increased cytokine stimulation after the 20-hour transduction period resulted in a decreased percentage of total cells expressing GFP. The highest form of stimulation resulted in the lowest percentage of transduced cells compared to if the cells had been culture in low stimulation conditions.

These results demonstrated that the high stimulatory conditions used during or immediately after the transduction, resulted in rapid cell-division, which diluted the vector and reduced its integration into the target cell. They also revealed that lentiviral vector integration into murine hematopoietic progenitors was not immediate; that at some stage in the life-cycle after receptor binding, a minimum amount of time was required to complete the integration. These results were further supported when the number of cells expressing GFP per colony was increased by delaying the length of time after transduction prior to plating in methylcellulose by up to three days. A

second viral hit also increased the percentage of GFP^+ cells, presumably because more viral particles were able to infect the target cell, but also because of the additional 20-hour incubation required for the double-hit prior to plating. We also assessed the effect of adding dNTP to the medium during the transduction as it had been previously shown that wild-type HIV-1 was limited in its ability to reverse transcribe its genome in quiescent lymphocytes due to a shortage of dNTP in those cells (133). Interestingly, the addition of dNTP had a positive effect on the transduction efficiency. Although the percentage of colonies that contained GFP cells did not change, the percentage of cells that were GFP^+ within each colony increased, indicating that a major rate limiting step in the transduction of murine hematopoietic progenitors lies in the reverse transcription step of the virus life-cycle.

A second component of the study was to perform the transductions using variable cytokine stimulation. It was found that increasing the cytokine stimulation (from no cytokines to SCF alone, or further to SCF, IL-3 and IL-6) during the 20-hour transduction period increased the percentage of colonies expressing GFP cells. These results are in keeping with the previous findings where lentiviral vectors were shown to be more efficient at transducing human hematopoietic progenitors that resided in the G_1 or $S/G_2/M$ stage of the cell-cycle compared to quiescent cells (237). It was also observed that only the highest form of stimulation yielded any significant level of transduction using the control oncoretroviral vector MGIN, as expected (248).

Since our studies defining the optimal conditions for lentiviral vector transduction of murine hematopoietic progenitors, several additional studies have been performed on murine hematopoietic stem cells demonstrating long-term reconstitution of the irradiated recipient mice (41, 239). Furthermore, lentiviral vector transduction of hematopoietic stem cells from murine models of human diseases have demonstrated the ability of the lentiviral vector to correct genetic deficiencies in the murine hematopoietic systems. These will be discussed later in more detail.

Transduction of Human Hematopoietic Stem Cells

Although oncoretroviral vector transduction of murine hematopoietic stem cells is efficient, only recently have the advances in transduction protocols allowed for efficient oncoretroviral vector transduction of stem cells capable of reconstitution in the NOD/SCID xenotransplant model or the non-human primate autologous transplant model (105, 129, 136, 215). These optimized transduction conditions usually contain combinations of cytokines, such as FL, TPO, and SCF, capable of rapidly inducing cell-division of primitive cells, compared to the older methods usually containing IL-3, IL-6, and SCF (147, 168). However, these conditions continue to push cells to enter the $S/G_2/M$ stage of the cell-cycle, which can be detrimental to the engraftment of hematopoietic stem cells as described earlier (20, 51, 267), and may cause the loss of

potential of the true hematopoietic stem cells required for effective engraftment in patients.

Several *in vitro* transduction studies using lentiviral vectors on freshly isolated human hematopoietic progenitors demonstrated efficient transduction of cells that had previously been resistant to transduction by oncoretroviral vectors (34, 237, 248). These studies revealed that populations of largely non-dividing cells could be efficiently transduced without prior stimulation and without cytokines. From these findings it became apparent that there was potential for gene therapy based treatment of disease using lentiviral vectors as they allowed for the transduction of primitive non-dividing hematopoietic cells to be achieved. This ability is based on the lentiviral vector's karyophillic pre-integration complex, which allows for active vector transport into the nucleus independently of mitosis. However, the precise requirements for transduction of these primitive cells remained to be defined. Although stable transduction of primitive human hematopoietic cells has been achieved *in vitro*, the maximum transduction efficiencies achieved rarely exceed 70% (66, 73, 237, 248). Moreover, the most primitive mitotically inactive portion of these cells remained largely untransduced (< 15 % GFP^+) (34), suggesting that stimulation of the cells during transduction may play an important role in achieving higher transduction efficiencies.

With the efficient *in vitro* transduction of both human and murine hematopoietic progenitors using lentiviral vectors being achieved, we then wanted to address the efficiency of lentiviral vector transduction of more primitive and more clinically relevant human hematopoietic repopulating cell. Thus the efficiency of transduction of primitive human hematopoietic repopulating cells was determined using the NOD/SCID mouse xenotransplant assay, as described in Paper III. A study performed just prior to our study by Miyoshi *et al*. demonstrated that lentiviral vectors could transduce human NOD/SCID repopulating cells from umbilical cord blood using a 5-hour transduction protocol with no cytokine stimulation (162). They achieved a respectable transduction efficiency into repopulating cells of about 35%. However, other studies performed *in vitro* have shown that wild-type HIV-1 infection requires the traverse of cell to the G_{1b} stage of the cell-cycle stage (G_{1b} is defined by an increased level of RNA within the cell compared to G_{1a} and G_0 cells) (134). Furthermore, another study revealed that lentiviral vectors, while being capable of transducing quiescent cells, preferentially transduced cells that resided in the G_1 stage of the cell-cycle as opposed to G_0 (237). This suggests that it might be possible to further increase lentiviral transduction efficiency of human hematopoietic stem cells by some limited cytokine stimulation from G_0 to G_1, but not further to $S/G_2/M$, during transduction, with minimal loss of repopulating capacity or self-renewal capacity of hematopoietic stem cells (85, 270). It had also been documented that while the cytokine TPO alone does not stimulate cell-division, it does contribute to the maintenance of cell viability (26, 111). Therefore, we transduced human umbilical cord blood $CD34^+$ cells using weak stimulatory conditions (TPO in serum free medium), in order to maximize transduction efficiency while minimizing the loss of repopulating ability.

Using a high resolution cell-cycle analysis technique previously described by Jordan *et al*. (120), we saw that the overnight transduction protocol using the single cytokine TPO in serum free medium on a fibronectin coated well did not stimulate the cells to enter into the $S/G_2/M$ phases of the cell-cycle (<3%), and slightly increased the population of G_1 cells from G_0 compared to fresh control cells (62% to 77%). The transduced $CD34^+$ cells were subsequently transplanted into NOD/SCID mice to assess the transduction efficiency into primitive human repopulating cells.

Six weeks after the transplantation of the transduced cells into the NOD/SCID mice, the bone marrow was harvested and analyzed by FACS or by methyl cellulose CFC assay. The average transduction efficiency of the repopulating cells as determined by PCR for the vector on individually picked CFC was 48±23%, demonstrating efficient transduction of NOD/SCID repopulating cells. When the bone marrow of the mouse was then transplanted into a secondary transplant recipient and bone marrow harvested another six weeks later, CFC colonies analysis revealed 64±13% vector positivity. No statistically significant difference was seen between primary and secondary recipients. When FACS analysis was used to assay the bone marrow of these mice GFP expression could be detected in both the lymphoid and myeloid lineages, as well as $CD34^+$ population of progenitors, and $CD38^+$ mature progenitors, in similar levels in all lineages. These results suggest lentiviral vectors are capable of efficient transduction of NOD/SCID repopulating cells with both lymphoid and myeloid lineage reconstituting capabilities, as well as the even more primitive progenitors capable of both myeloid and lymphoid reconstitution in secondary transplant recipient mice.

In order to verify that the transduced repopulating cell was capable of both lymphoid and myeloid repopulation we transplanted mice in cell-dose limiting dilution to generate mice repopulated by single stem cells. The concept of the cell-dose limiting dilution assay was first described by Szilvassy *et al*. as a means of assaying the quantity of stem cells within a population as well as the stem cell lymphoid-myeloid cell repopulating ability (238). Poisson statistics can be used to define the situation where if the majority of animals are negative for human engraftment, the animals that are positive are so by virtue of a single repopulating cell. (In fact, 63% of the animals must be negative in order for the 37% positive mice to have been repopulated by a single cell 95% of the time.) In our study, NOD/SCID mice were transplanted with cell doses of 400 000, 100 000, 35 000, 12 500, and 5 000 of lentiviral vector transduced $CD34^+$ cells, and as expected the engraftment level was found to correlate to the cell dose. One particular mouse, transplanted with 35,000 cells, was found to have a low level of engraftment (3.8%), however, virtually all of the human reconstituting cells expressed GFP, suggesting it may have been reconstituted by a single transduced repopulating cell. In addition, similar high-levels of GFP were found in both the lymphoid and myeloid cells, further confirming lentiviral vectors are capable of transducing primitive human repopulating cells with lymphoid and myeloid potential under minimal stimulatory conditions.

A further component of this study was to develop a vector integration site assay based on inverse PCR protocol. The purpose of the assay was originally intended to aid in the tracking of integration for the assessment of common integrants in both lymphoid and myeloid compartments, as well as common integrants in the primary and secondary mice. Unfortunately, the inverse PCR technique was not sensitive enough for this analysis, but did confirm the ability of the lentiviral vector to integrate into genomes of repopulating cells.

Since these initial studies of lentiviral transduction of CB derived SRC, numerous other studies have been performed, however, the majority of these studies have been performed using lentiviral vectors containing the previously mentioned central polypurine tract (cPPT) (76, 214, 225, 226). In these experiments similar or higher efficiency transduction of SRC have been obtained with up to 70 % of the cells expressing GFP (76, 226). While it has been clearly demonstrated that the cPPT increases the efficiency of lentiviral vector transduction in permissive cells, it has not been addressed whether the element can actually enable the transduction of SRC not normally permissive to transduction using non-cPPT vectors. However, recently Scherr *et al.* demonstrated using a cPPT vector, that the transduction of SRC from MPB were transduced at a high efficiency (40 %) with a 36-hour transduction protocol with cytokines TPO, SCF, FL and IL-6. This is considered efficient since the cells capable of long-term reconstitution are known to be in G_0 (92). Future experiments describing the transduction of primitive repopulating cells from MPB and BM is required as these will likely be the sources of hematopoietic stem cells for transplantation into patients. Comparisons of cell-cycle status of MBP and BM show that the mobilization using the cytokine G-CSF results in the primitive $CD34^+$ $CD38^{lo}$ fraction to enter the G_1 stage of the cell-cycle, and that these cells contribute to the rapid short-term repopulation in (92, 108). However, the long-term repopulating cells are likely to remain in G_0, thus transduction conditions must be designed accordingly (92).

The high efficiencies of transduction of SRC achieved using lentiviral vectors have rarely been achieved using oncoretroviral vectors (94, 105, 201, 215). This is particularly relevant since in addition to transducing at superior efficiencies, lentiviral vectors can do so using lower stimulatory conditions. Although the efficiencies of transduction achieved using oncoretroviral vector is a respectable 10-45%, the transduction conditions to achieve this are a minimum of 3 days with the cytokines TPO SCF, and FL, and this has consequences on the level of engraftment achieved (94, 105, 201, 215). While the total number of repopulating cells during culturing for oncoretroviral vector transduction can be maintain or marginally increased, the results of one study demonstrates that the proliferative potential of the cycled cells is diminished (201).

Transduction of Human $CD34^+$ $CD38^{lo}$ Repopulating Cells

We then wanted to assess lentiviral vector transduction of the more primitive $CD34^+$ $CD38^{lo}$ fraction of CB cells in NOD/SCID mice. Therefore, we transduced our target cell using the same transduction protocol as in Paper III. The average transduction efficiency achieved was approximately 25%, as determined by FACS for GFP in BM from NOD/SCID mice and by PCR on individually picked CFU-GM colonies from the mice. This is comparable to percentages achieved in NOD/SCID mice in another study of transduced $CD34^+$ $CD38^{lo}$ cells where 23±7.2% of human cells was achieved in the NOD/SCID mice (95). However, studies of lentiviral vector transduction of $CD34^+$ SRC demonstrate transduction efficiencies of 30-70% (76, 81, 162, 214), suggesting that repopulating cells present in the $CD34^+$ $CD38^{lo}$ population are slightly less susceptible to transduction. There are two possible explanations for the $CD34^+$ $CD38^{lo}$ cells having lower susceptibility of transduction. One possibility is that the $CD34^+$ $CD38^{lo}$ cells are more easily transduced when surrounded by $CD34^+$ $CD38^+$ cells during the transduction conditions (involving some sort of stimulation of the repopulation cells by the more differentiated $CD34^+$ $CD38^+$ cells). The second and more probable explanation is that the stringent sorting of the $CD34^+$ $CD38^{lo}$ population (lowest 6 %) excludes some of the $CD34^+$ $CD38^{fairly\ low}$ cells also capable of repopulation in NOD/SCID mouse (22). These $CD34^+$ $CD38^{fairly\ lo}$ cells may be slightly more susceptible to lentiviral vector transduction given the short culture conditions. The preferential transduction of $CD34^+$ repopulating cells over $CD34^+$ $CD38^{lo}$ repopulating cells has been seen before in an oncoretroviral vectors study in Beige-Nude-Xid mice but with more dramatic effects. In that study, $CD34^+$ $CD38^{lo}$ cells were impervious to transduction by 4 days of culture, although some repopulating activity was maintained (55). Similarly, in a lentiviral transduction study of repopulating cells, transduction was more efficiently when more stimulatory cytokines were used during the 24 hour transduction protocol than when TPO alone was used (76, 226). However, the use of high stimulatory conditions during transduction may have negative effects on the clinically relevant long-term repopulating ability of stem cells despite the encouraging results in the NOD/SCID mouse.

Multiple Vector Copy Integration into SRC

Optimization studies on lentiviral vector transduction have demonstrated that in order to achieve a maximum transduction efficiency into primitive hematopoietic cells, a high multiplicity of infection (MOI) is required (97, 211). Hence, most lentiviral vector transductions performed on hematopoietic cells, including those described within the context of this thesis, have used viral titers well in excess of the cell number being transduced. However, even at MOI as high as 500-1000 TU/cell and viral concentrations exceeding 10^7 TU/mL, transduction of all primitive hematopoietic cells without stimulation remains an elusive goal (76, 226). Moreover, the consequence of the high concentration of virus on the fraction of cells permissive to transduction has not been addressed, with regards to the integrated vector copy number per transduced

cell. Lentiviral vector transduction and transgene expression analyses in hematopoietic cells have assumed single (or very few) vector copies per cell, based on single oncoretroviral vector copies found in murine hematopoietic clones derived from Beige-Nude-Xid mice (56).

However, as demonstrated in Paper 1, lentiviral vector transduction of murine embryonic stem cells could result in as many as 12 copies per cell by increasing the MOI used during infection. To address whether multiple vector copy integration occurred during transduction of primitive human hematopoietic cells, we decided to measure the transduction efficiency into SRC using semi-quantitative PCR analysis on the total bone marrow from transplanted NOD/SCID mice as described in Paper IV.

Semi-quantitative PCR was used to quantitate the amount of vector DNA normalized to the amount of human genomic DNA. Using the percentage of GFP expressing cells, one could then determine the average vector copy number per human cell. Surprisingly, an average of 5.6±3.3 (n=12) copies per transduced hematopoietic cell were detected in the transduced progeny of the SRC. To confirm this result, individual CFU-GM colonies derived from a transplanted NOD/SCID mouse were analyzed by linear amplification mediate (LAM) PCR, a highly sensitive PCR technique that allows for discrimination of single vector integrants in the complex mixtures of DNA (216, 217). The mouse used for the analysis was the mouse 7.23 from Paper III that was hypothesized to have been transplanted with a single repopulating cell. The results of the analysis reveal that five distinct vector integration sites were detected in the CFU-GM colonies. The fact that all five integration bands could be seen in a single colony confirms that the repopulating cell that gave rise to the colony also had five vector integration sites, confirming the ability of the lentiviral vector for multiple vector copy integration. Furthermore, analysis of more colonies revealed that all contained the same five integration sites and that no additional sites were detected in the in the total bone marrow when analyzed in the same manner. These results confirmed, molecularly, the hypothesis that the mouse 7.23 had been repopulated by a single SRC further demonstrating the ability of the lentiviral vector to transduced human repopulating cells with both lymphoid and myeloid potential. Therefore, we propose that the subset of SRC permissive to lentiviral vector transduction is susceptible to multiple vector copy integration into the genomes of each cell. Furthermore, since this study was performed on many of the same mice as for the transgene expression analysis study mentioned ahead, we hypothesized that the high-level of transgene expression achieved was in part due to the multiple vector copies contributing to the expression.

To further characterize the multiple vector copy events seen we decided to sequence the individual integration sites in mouse 7.23. This enabled the determination of the precise location of the vector within the human genome. Interestingly, one of the integration sites was found to be located in intron 17 of the known tumor suppressor

gene, breast cancer 1 (BRCA1), as depicted in Figure 4. The insertion of the vector into the gene would most probably cause the malfunction of the gene, which mutations therein are associated with the increase likelihood of development of breast and ovarian cancer, but also in impaired double stranded DNA repair. While multiple vector copy integration into human NOD/SCID repopulating cells is efficient in terms of gene transfer and gene expression, multiple copy integrations of lentiviral vectors in hematopoietic cells may increase the risk for insertional mutagenesis (219).

Insertional Mutagenesis

Vector mediated gene therapy for treatment of disease has, since its inception, been associated with its theoretical risk for insertional mutagenesis. This may have implications for the safety of the patient. Although the theoretical chances of activation of a proto-oncogene are small (10^{-7}) (234), the repetitive infections of cells from a wild-type virus infection in an organism eventually lead to the undesirable mutation event. In fact, MMLV infections in the rats have shown some areas of the genome are particularly prone to the development of tumors from insertional mutagenesis. They are the genes *c-erbB*, rat *c- myc*, and a rat locus *dsi-1* (230, 254, 255). Interestingly, only one example of insertional mutagenesis from an oncoretrovirus based vector has ever been reported (143). It was the result of the activation of the Evi-1 proto-oncogene by both the viral LTR upstream of the gene, which in combination of transgene could induce leukemia. The fact that only a single reporting of an insertional mutagenesis event by a vector may demonstrate the complications with determining such an event. As described in by Li *et al.*, a leukemia inducing mutagenesis event may not cause a lethal phenotype in a primary transplant recipient. Therefore, unless serial transplantation into secondary and tertiary recipients was part of the experiment the phenotype might remain invisible. Furthermore, thorough analyses of all animals that have ever died in gene marking studies have not been performed, and nor have there been adequately sensitive techniques available to scientists for the determination of the integration site of the vector, until now (i.e. LAM PCR). The fact that the Evi1 proto-oncogene was only able to induce the leukemia only in combination with the marker gene used in this vector is no evidence of the rarity of the risk, especially since the genomes of patients in a wide population would also contain mutations of unknown genes that together could elicit an undesirable phenotype.

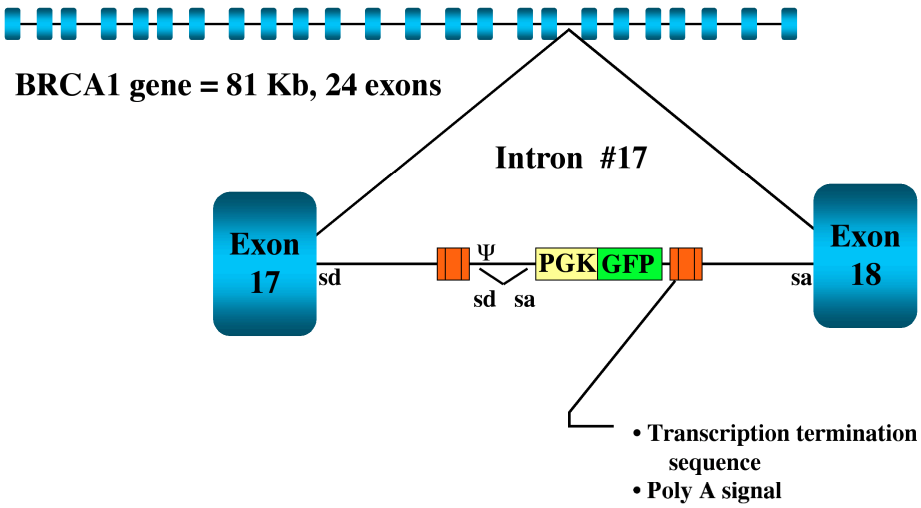

Figure 4. Schematic of lentiviral vector integration into the 17th intron of the known tumor suppressor gene, BRCA1. The vector integration into the intron would likely result in the disturbed function of the BRCA1 protein by premature transcription termination or by aberrant splicing on the RNA level. It is unclear whether the integration by lentiviral vectors into genes is a purely random event, however, wild type HIV-1 has recently been shown to preferentially integrate into active genes.

The BRCA-1 gene, into which the lentiviral vector integrated, as described in Paper IV, is involved in DNA damage repair (166) and associated with hypersensitivity to double stranded DNA damage in Fanconi anemia patient cells (277). Although no unusual phenotype was apparent in the mouse at the time of sacrifice, most leukemias require a minimum time for a leukemic phenotype to develop, well in of excess of the 6 weeks as performed here. It is highly probable that the vector integration into the BRCA-1 gene would disrupt normal function of the gene product as a result of changes incurred in the C-terminus domain. Truncations of the BRCA1 C-terminus domain have been associated with the reduced protein stability and loss of tumor suppressing function (71, 250). However, the role of BRCA-1 and its pro-apoptotic features as defined in other tissues is still unknown within the hematopoietic system. In addition, in order for its oncogenic properties to be manifested, BRCA-1 must also be accompanied by at least one other mutation. It is postulated that a non-functional BRCA-1 gene yielding the inability to repair damaged double stranded DNA would require a mutation, for example, in the p53 signaling pathway rendering the cell unable to evaluate completion of the S to G_2M cell-cycle checkpoint, and hence have abnormal determination if a damaged cell should proceed to the next stage of the cell-cycle or undergo apoptosis (27). The fact that, of the few integration sites sequenced in our laboratory, one of them is located in a known tumor suppressor gene, begs the question of how rare this event actually was. Only recently, Schroder *et al.* demonstrated that wild-type HIV-1 integrates preferentially into active genes (219).

Further analyses to determine whether this ability of HIV-1 is maintained in lentiviral vectors remains to be performed.

Lentiviral Vector Transgene Expression in Hematopoietic Cells

While the ability of viral vectors to stably integrate into a significant number of primitive hematopoietic cells is a requirement for the success of gene therapy of hematopoietic diseases, so too is the vector's ability to express the transgene long-term in the progeny of the stem cells. Although oncoretroviral vectors have demonstrated high-level, ubiquitous transgene expression in all hematopoietic lineages from the MESV LTR promoter, some studies suggest that vector silencing during hematopoietic stem cell differentiation to mature progeny occurs (94, 128, 165). There is a concern that expression may not persist for extended periods in patients due to oncoretroviral vector LTR transcriptional inactivation. As demonstrated in Paper I, oncoretroviral vector expression was severely diminished following transduced ES cell differentiation into EB bodies and further to hematopoietic progenitors. In stark contrast, the lentiviral vector driving expression from the internal muPGK promoter yielded more consistent expression throughout differentiation. Moreover, transgene expression correlated highly with the vector copy number. However, one of the problems of lentiviral vectors has been the low-level of transgene expression in transduced hematopoietic cells. In fact, it is theorized that for many gene therapy applications, the level of expression is too low to elicit a therapeutic response should these vectors be used in clinical protocols. Disease where high-level expression of a transgene is required or desirable in as many cell types as possible, or disease where transgene expression is necessary at an early progenitor cell level will require high-level ubiquitously expressing promoters driving transgene expression.

When the study in Paper II was initiated, other lentiviral vector transduction studies in hematopoietic cells were driving transgene expression driven from the CMV or muPGK internal promoters (7, 12, 162), or HIV LTR based promoters (that are likely to be incompatible with the clinic trials for safety reasons) (4, 82). The expression study performed in Paper II was the first expression study performed in the context of the lentiviral vector system, not driving transgene expression from the wild-type HIV LTR. In order to improve the transgene expression we tested vector containing several additional ubiquitously expressing promoters (Figure 5) in murine Lin^- c-kit^+ $Sca\text{-}1^+$ cells for high-level expression as determined by FACS analysis 3 days following transduction. In addition to the CMV and muPGK, we tested the *elongation-1* (EF-1) and the hybrid CMV/*chicken actin* (CAG) promoters. The intron containing EF-1 promoter drives expression of an elongation factor required for GTP-dependent catalysis of the binding of aminoacyl-tRNA to the ribosome during protein translation. This enzyme is one of the most abundant proteins found in eukaryotic cells. When the

EF-1α promoter was used to drive expression of other genes, high efficiency gene expression was seen in multiple cell types both *in vitro* and *in vivo* (126, 249, 278). The results of this study showed the superiority of the EF-1α intron-containing promoter to express in the lentiviral vector when compared to the vectors containing the muPGK, CMV and CAG promoters by 3-4 fold.

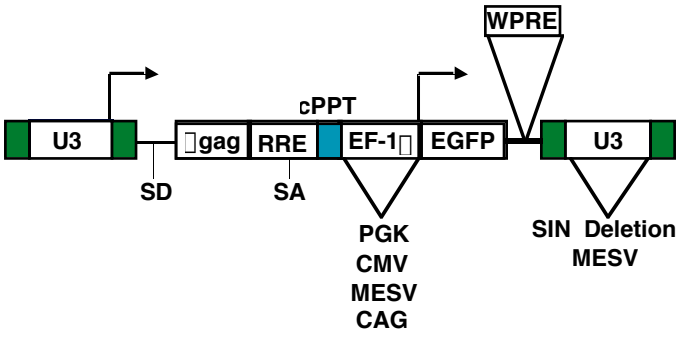

Figure 5. Lentivirus gene transfer vectors tested for high-level transgene expression in hematopoietic progenitor cells. The five internal promoters tested are shown. The *woodchuck posttranscriptional regulatory element* (WPRE) was added to the vectors containing the EF-1α, PGK and CAG promoters. The self-inactivating (SIN) configuration was tested on vectors containing the PGK CMV and EF-1α promoter. The MESV LTR promoter was tested within the context of the lentiviral vector as an internal promoter as well as a promoter from within the HIV LTR. The cPPT, which is required to be located centrally in the vector as shown, was not present in the vectors of the expression study.

Our experiments in murine hematopoietic cells showing high-level lentiviral vector transgene expression from the EF-1α promoter were confirmed in human hematopoietic cells (198, 211). In addition to the other promoters tested, the murine stem cell virus LTR promoter (MSCV U3), and the gibbon ape leukemia promoter (GALV U3) were tested, but shown not to express at as high a level as the EF-1α promoter. Although these transgene expression studies were performed on human $CD34^+$ cells *in vitro* and showed expression for up to five weeks in differentiated cells, they did not address whether expression was maintained after hematopoietic stem cell differentiation to mature progeny. Therefore, we initiated an *in vivo* transgene expression study whereby the lentiviral vector transgene expression was measured in the in the progeny of NOD/SCID repopulating cells.

As performed in mouse cells in Paper II, vectors driving expression from the muPGK and CMV internal promoters were compared with vectors driving expression from the EF-1α promoter. In addition, two vectors driving expression from the MESV LTR promoter placed either internally of the lentiviral vector or substituting the U3 portion of the HIV LTR in the vector as well as vectors containing the *woodchuck posttranscriptional regulatory element* (WPRE) (288) and the self-inactivating (SIN)

deletion (289) were compared (vector design shown in Figure 5). As in the mouse and human *in vitro* studies, the EF-1α promoter yielded significantly higher levels of transgene expression *in vivo* than the vectors driving expression from any of the other promoters tested. Surprisingly, the WPRE, which served to increase the level of expression when placed in the context of the lower expressing vectors *in vitro*, did not increase the expression level from the EF-1α vector *in vivo*. However, the SIN deletion more than doubled the expression level in compared to the non-SIN vectors for both the EF-1α vector and the CMV vector in the human cells. The MESV LTR promoter replacing the HIV LTR promoter also yielded high-level expression, however, with great variability among the cells. The EF-1α SIN vector expressed as our best vector in the progeny of human hematopoietic stem cells, at levels 8-fold higher levels compared to the standard muPGK vector used in Papers I, II, and III. The lineage analysis of expression from EF-1α SIN vector in the NOD/SCID mice revealed that both the myeloid and lymphoid cells expressed similar high-levels. An *in vivo* expression study recently performed by Sirven *et al*. demonstrated expression from an EF-1α promoter having had the intron removed. However, expression from that vector was not substantially higher than that of the control CMV vector (226). Recently, an alternative promoter derived from the spleen focus forming virus has shown high-level expression in the progeny of NOD/SCID mice. However, direct comparisons with EF-1α vector have not been performed (59, 274).

The finding that the self-inactivating EF-1α and CMV vectors resulted in higher transgene expression than their non-SIN variants is important as it demonstrates the ability for high-level transgene expressing lentiviral vectors to be developed possessing safety features possibly acceptable for use in the gene therapy clinic. One possible explanation for the increased transgene expression found using the SIN vectors may be that the intron containing EF-1α promoter/enhancer and the full length U3-LTR from the vector both contain multiple SP1 binding sites, and hence may compete with each other for the SP1 transcription factor (102, 259). The SIN vector, in which the LTR promoter and a total of six SP1 sites are removed, may free up the SP1 transcription factor in the vicinity of the EF-1α promoter allowing the EF-1α promoter, which possesses five SP1 binding sites, to express more optimally (249). Just recently, the CMV promoter including its enhancer has also been shown to harbor SP1 binding sites (149, 157).

Interestingly, in cases where transgene expression is low, such as the expression from the muPGK, CMV, or CAG promoters, the WPRE increases the expression in human cells (211). There is new evidence that suggests that the influence on expression by the WPRE is also context dependent; i.e. dependent on the particular transgene being expressed (213). Therefore, lentiviral vector expression of a therapeutic gene regulated by the EF-1α promoter, may in fact be further increased using the WPRE, although the WPRE yields no increase in GFP expression here. The precise mechanisms that determine the expression in the context of WPRE remain to be elucidated.

The position of the promoter within the vector also played a role in the expression potential in the progeny of hematopoietic stem cells differentiated *in vivo*. The MESV LTR promoter when replacing the U3 portion of the HIV LTR expressed significantly higher levels than the same promoter placed internally. This study comparing expression of multiple vectors *in vivo* demonstrates for the first time a lentiviral vector generating high-level transgene expression in hematopoietic cells capable of being generated by a stable packaging cell line. The generation of the stable packaging system for the vector enables high titers to be generated with a reduced likelihood of generating replication competent viral particles.

Interestingly, all the vectors tested lead to similar proportions of GFP positive cells within the lymphoid and myeloid progeny of the transduced repopulating cells. This suggests that on average, the transduced SRC have an equal lymphoid and myeloid repopulating ability and that both promoters continue to express throughout differentiation into both lineages with similar efficiency. The results of the study in paper IV demonstrate that high-level, multilineage transgene expression from lentiviral vectors is achievable in the progeny of NOD/SCID repopulating cells.

Tissue-Specific and Regulated Transgene Expression

The correction of β-thalassemia in a murine model of human disease has been demonstrated following transplantation of lentiviral vector transduced hematopoietic stem cells (154). The vector contained the corrective *β-globin* gene under the regulation of the erythroid specific locus control region modified from the endogenous *β-globin* gene locus control region which expands 40 kb. The functionally critical elements of the locus control region have been identified and sequences between them removed to enable placement within insert size constraints of the vector. However, the splicing signals had to be maintained to preserve the high-level erythroid specific expression, and therefore the expression cassette was placed within the lentiviral vector in the reverse orientation. Using the same locus control region Pawliuk *et al.* were able to demonstrate the correction of two sickle cell anemia mouse models (185). Interestingly, previous attempts at expressing *β-globin* from within oncoretroviral vectors resulted in low-level expression that lasted only short-term *in vivo* (206). The successful results of the lentiviral vector expression suggest an intrinsic advantage to using lentiviral vectors as expression at high-levels can be maintained long-term after transplantation.

Other studies have been performed where tissue-specific promoters have resulted in specific expression in the target cell. For example, a T-lymphocyte-specific proximal *lck* promoter has succeeded in driving expressing only in the thymus, similarly the *myogenin* promoter resulted in expression exclusively in muscle cells (145). Transgene expression specific to antigen presenting cells has also been demonstrated in *in vivo*

experiments in the progeny of SRC were lentiviral vector transduced repopulating cells were into NOD/SCID mice (49).

In addition to tissue-specific expression, regulated transgene expression has also been proposed as a requirement for some disease therapies, whereby patient having undergone gene therapy can be administered a drug and regulate the expression of the transgene in a controllable manner. For example, the a tetracycline (Tet)-regulated promoter within a lentiviral vector has been demonstrated to be able to regulate GFP expression *in vivo* in rat brains by feeding rats with tetracycline in their water (90, 122).

CONCLUSIONS

Paper I

Lentivirus Vector Gene Expression during ES Cell-Derived Hematopoietic Development *In Vitro*

The results generated in this study demonstrate for the first time the ability for lentiviral vectors to efficiently transduced ES cells and to maintain their capability for transgene expression throughout differentiation to hematopoietic cell. Although both the lentiviral vector and oncoretroviral vectors tested underwent some degree of transcriptional inactivation after differentiation, transgene expression was significantly better maintained in the lentiviral vector transduced cells. These results also demonstrate that using a high MOI resulted in high vector copy number in ES cell clones. The efficiency of the ES cell transduction and expression allows for the easy assessment of efficiency of novel expression cassettes throughout differentiation. In addition, it opens possibilities for the creation of animal models of disease or the creation of gain of function animal models as ES cells could be used to generate viable organisms. It also opens new possibilities for the gene therapy as, theoretically, any desired tissue could be derived from the primitive cells and that tissue could contain the relevant gene for the correction of a disease.

Paper II

Lentivirus Gene Transfer in Murine Hematopoietic Progenitor Cells is Compromised by a Delay in Proviral Integration and Results in Transduction Mosaicism and Heterogeneous Gene Expression in Progeny Cells

The results from this study reveal that lentiviral vectors can efficiently transduce primitive murine hematopoietic progenitors (Lin- c-kit+ Sca-1+) under a range of stimulatory conditions. It was also demonstrated that there was delay in the lentiviral vector integration in these cells that can be overcome by the addition of dNTP or increased cytokine stimulation during, but not after, the transduction. In addition, it was found that the transduction efficiency was dependant on the ability of the virus to integrate prior to cell-division, and therefore strategies of minimal stimulation immediately following transduction may prove to be the most efficient conditions for maximal transduction. From these data, the it may be possible to define the conditions for the efficient transduction murine hematopoietic stem cells to test gene therapy as modality for the treatment of disease in murine models of human disease.

Paper III

Efficient Lentiviral Vector Transduction of Human Hematopoietic Candidate Stem Cells

These results demonstrate that lentiviral vectors efficiently transduce NOD/SCID repopulating cells capable of reconstituting both the lymphoid and myeloid compartments in primary and secondary transplant recipient mice. These efficient transduction results could be achieved under minimal stimulatory conditions without the loss of repopulating potential. The ability of the lentiviral vector to transduce human repopulating cells with both lymphomyeloid repopulating ability was demonstrated by limiting cell dose analysis, which was then confirmed molecularly using LAM-PCR as described in Paper IV. These results suggest a positive role for lentiviral vectors in the transduction of human hematopoietic stem cells in clinical protocols.

Paper IV

High-Level Lentiviral Vector Transgene Expression Requires Optimal Vector Design and Multiple Vector Copy Integration into Repopulating Cells: Risk for Insertional Mutagenesis

The results of this study demonstrate that ubiquitous high-level transgene expression can be achieved using lentiviral vectors driving expression from the EF-1 promoter within the context of the theoretically safer SIN vector. However, the finding that lentiviral vectors regularly transduce multiple vector copies into primitive human repopulating cells increases the risk of insertional mutagenesis. This was demonstrated by the integration of a vector integrant into the known tumor suppressor gene BRCA1. The high MOI used during the transductions is a possible cause for the high copy numbers achieved. The development of transduction conditions that result in fewer copies per transduced cell is warranted, however, a loss in transgene expression level is predicted.

GENE THERAPY SUCCESSES

Recently, there have been two successful gene therapy clinical trials where patients have undergone correction of deficiencies of the hematopoietic system. These clinical trials have been carried out on patients with either the X-linked or adenosine deaminase (ADA) form of severe combined immune deficiency (SCID), and now after autologous transplantation of oncoretroviral vector modified stem cells, all treated patients have shown near complete normalization of immune function (1, 37). X-linked SCID results in immune deficiency due to a mutation in the gamma chain gene required for the interleukin (IL) receptors -2, -4, -7, -15, and –21, for immune cell proliferation, differentiation, and response. Because the IL receptors are required for differentiation and activation of early and late lymphoid cells, when the transduced □-chain gene is expressed modified lymphoid cells proliferate and differentiate, while the unmodified cells are left unable to do so. This was demonstrated by the fact that in the patients, while virtually all of the lymphoid cells contained the corrected gene, only a small fraction of the myeloid cells contained the gene. Therefore, there was selective growth advantage for modified cells of the lymphoid lineage, which resulted in the ability for correcting the disease phenotypes despite the low-level transduction of stem cells (as determined by the low frequency of modified myeloid cells).

ADA SCID is a metabolic disorder, which results in the accumulation of toxic metabolites that primarily effect lymphoid cell differentiation and function, however also results in non-immunological abnormalities. Hematopoietic stem cell correction has been demonstrated to successfully reverse the disease phenotype with full restoration of the immunological function. The selective advantage for the transduced cells is based on the ability to prevent the accumulation of toxic substances that cause the death of unmodified cells. The selective advantage of the corrected cells is thought to be weaker in ADA SCID compared to X-linked SCID, which was the reason for the weak non-myeloablative treatment of the patients with low-dose busulfan prior to stem cell transplantation; to give a further advantage to the transplanted cells. The stem cell ablation was not required in the treatment of X-linked SCID to achieve the therapeutic benefit. As in X-linked SCID, there appeared to be a selective advantage for ADA corrected cells of the lymphoid lineage. However, the analysis of the blood levels of ADA SCID have shown increased levels of non-transduced myeloid cells likely as a result of the systemic detoxification by the corrected cells. Moreover, the normalization of erythrocytic and other non-lymphoid lineages were achieved due to the correction of the multipotent hematopoietic stem cell. Previous gene therapy trials for ADA SCID may have failed due to the simultaneous use of the drug PEG-ADA, which corrects the deficiency in patients and thereby removes the selective advantage of the corrected cells. The patients mentioned in the above studies have been treated successfully and lead relatively normal lives free from immune isolation. This is as a testament to the potential of hematopoietic stem cell mediated gene therapy for hematological disorders.

FUTURE STUDIES

Optimization of Transduction Conditions to Reduce Multiple Vector Copies per Cell

The results described in Paper IV demonstrate that a potential risk for insertional mutagenesis exists using lentiviral vector mediated genetic correction in hematopoietic stem cells. Based on these findings further optimization of transduction conditions to reduce the vector copy number integrated per cell is warranted. It may be possible to reduce the lentiviral vector copy number without compromising the percentage of vector containing cells simply by lowering the MOI. In the recent study by Lois *et al.* the number of vector copies integrated into single cell embryos could be reduced by the lowering of virus titer used during transduction (145). Furthermore, recently studies in NOD/SCID using low MOI of 5-10 TU/cell could efficiently transduce SRC (25-40%) (82, 214). These results suggest that the transduction efficiency may not be severely hampered by the reduced MOI. However, we expect that the transgene expression level will be diminished as the copy number is decreased. In comparison, thorough analysis of retroviral vector integration has shown usually only one vector integration event per cell. However, these analyses should now be repeated using more sensitive integration site determination techniques based on LAM-PCR.

Identification of Candidate Diseases for Gene Therapy

Identification of suitable target disorders for hematopoietic stem cell gene therapy is critical to the success of gene therapy. Life threatening diseases, that would potentially be cured by the insertion of a single or very few corrective genes, and where no other medical treatments exist, would be ideal candidates. Moreover, diseases where there is a near complete molecular understanding of the mechanisms of pathogenicity should be considered. The genetic mutations causing the disease cannot possess dominant negative effects that would compete out the newly inserted correcting gene, since the original mutant gene would remain. Furthermore, as there is a limitation in the insert size in the vectors the transcription regulatory elements and the transgene would need to be less than 7 kb long. Ideal candidate disease would also result in the selective survival advantage of the corrected cells, with particular emphasis on the survival of the stem cell. This is necessary to overcome the low efficiency of transduction currently seen in human stem cells. The candidate diseases proposed to be clinically applicable for gene therapy trials thus far are relatively rare in the population, e.g. SCID, lysozomal storage disorders, Diamond-Blackfan anemia, Fanconi anemia, Fabry disease to name a few (68) (197) (205) (98) (2), however, continued research is making new disease candidates available.

Generation of Animal Models of Disease

The development of animal models of human disease will play a critical role to determine the feasibility of the gene therapy strategies for correction of hematological disorders. In generated models of disease, issues regarding the levels and specificity of the therapeutic gene expression within a particular hematopoietic lineage can be assessed. In addition, the levels of engrafting cells, and the efficiency of transduction of engrafting cells required to generate the therapeutic response in a patient can be estimated from the models. One excellent example of the use of an animal model for this purpose is by Pawliuk *et al.* where they use two sickle cell anemia mouse models, BERK (183), and SAD (246) to test the feasibility to cure the disease using lentiviral vectors encoding a modified human *β-globin* gene (185). Both the Berkley and SAD models showed reductions in the numbers of sickle celled erythrocytes after over expression of a *β-globin* mutant gene whose product is more likely to be incorporated in to the hemoglobin structure than the sickling form. The reduction in sickle cell counts in the peripheral blood is the direct result of the high expression tissue-specific *β-globin* gene expression from within the lentiviral vector. The expression resulted in that the symptoms of the disease in those mice could be virtually eliminated, demonstrating the high likelihood of curing sickle cell anemia in patients, provided that sufficient engraftment of transduced stem cells could be achieved.

Vector Improvements

While the generation of lentiviral vector with tissue-specific promoters is critical to the successful treatment of many diseases, so too is the ability to express multiple transgenes within the vector. Many diseases may prove to require multiple transgenes to be expressed in order to elicit the desired therapeutic effect. However, very few of such lentiviral vectors have been generated. Attempts at using the internal ribosomal entry site (IRES) sequences for the generation of bicistronic lentiviral vectors, as commonly used in oncoretroviral vectors, have yielded very low expression levels of the downstream gene, which may be problematic for treatment of many candidate diseases. Therefore, novel multiple gene expression systems will need to be devised and tested within the context of the target cell, and may contain such features as dual promoters, E2A protease, and novel splicing features(122, 130, 146, 200, 287). Furthermore, dual gene vectors will be required for therapies in which an induced selective advantage for the corrected cells is desirable, such that a gene inferring a selectable advantage as well as the therapeutic gene are expressed, as described in Figure 6.

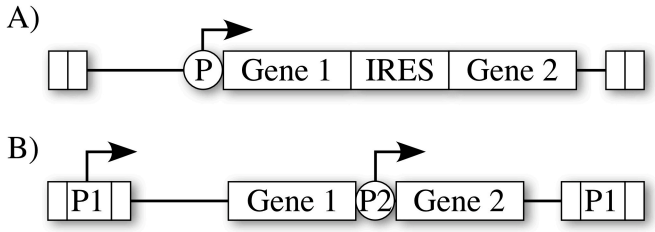

Figure 6. Examples of vectors (shown as integrated proviruses) expressing two genes, for example a therapeutic gene and a selectable marker gene. A) A self-inactivating (SIN) vector where most of the U3 region of the LTR has been deleted. An internal promoter (P) drives the expression of two genes, 1 and 2. The advantage with this design is that the SIN design offers increased safety, but the disadvantage is that the expression of gene 2 is going to be reduced since it is placed downstream of an internal ribosomal entry site (IRES) which allows the translation of the second gene from the bicistronic mRNA. The SIN vector cannot be produced in the permanent packaging cell line shown in Figure 3. B). This vector contains two promoters, one within the LTR (not the HIV-1 promoter for safety reasons) and another promoter (P2) internally. The advantage with this design is that the expression level will be as high as expected from the two individual promoters and here the vector can be produced in the permanent packaging cell line shown in Figure 3. Since the LTR contains an active promoter, there is increased risk from insertional mutagenesis compared with the construct in A).

In vivo Selection of Modified cells

As was demonstrated in the study by Cavazzana-Calvo *et al*. in the successful curing of patients with X-linked SCID (37), although the transduction efficiency into hematopoietic stem cells was very low, the there was the possibility for the success of the treatment. This was based on the selective advantage of the genetically corrected cells compared to non-transduced cells. Therefore, there have been attempts made to artificially induce a survival or proliferative advantage to the transduced cells. One study has attempted to provide an inducible proliferative advantage to the transduced cells by coupling the intracytoplasmic domain of the c-mpl receptor to a mutated FKBP12 protein capable of dimerization and c-mpl signal transduction upon induction with an inert chemical. The results of their numerous studies showed a predominant myeloid cell expansion in human cells and no detectable expansion of stem cells *in vivo* (118, 119, 144, 169, 203). However, other signaling molecules that require dimerization for activation could be used within this system, and may prove more successful at expanding stem cells.

Another strategy for the selective expansion of transduced hematopoietic stem cells could be to overexpress the *HOXB4* transcription factor. As previously described oncoretroviral vector driven *HOXB4* overexpression in hematopoietic stem cells is able to expand the hematopoietic stem cell compartment *in vitro* and *in vivo* without

apparent deleterious effects on their subsequent differentiation or regulation of mature progeny output (10, 33, 243). However, high-level *HOXB4* expression has been demonstrated to induce myeloid cell differentiation *in vitro* and have negative effects on lymphoid reconstitution *in vivo* (28, 137, 204), and therefore it may be desirable to design therapeutic vectors with regulated *HOXB4* expression, using for example the tetracycline regulated expression system from within the lentiviral vector (90, 122).

Other strategies of providing selective advantage to the transduced cell population are based on providing transduced cells with a gene for resistance to a cytotoxic drug. For example vectors containing dihydrofolate reductase (6, 48), p-glycoprotein pumps (such as multidrug resistance 1 (MDR1)) (192, 215, 221), or MGMT (5, 155), among others have been tested. However, none of these systems has provided consistent evidence for stem cell survival advantage.

Merging Gene Therapies and Cell therapies

A new avenue and as yet still theoretical means of treatment of disease is derived from the ability to differentiate genetically corrected embryonic stem (ES) cells into cells of the desired lineage and transfer these into the patient. Since ES cells can differentiate into all tissues of the organism, virtually any tissue would be theoretically possible to generate *in vitro* and be able to be applied to the a patient. This includes the generation of hematopoietic stem cells as recently described by Kyba *et al.* (137) by over expression of the *HOXB4* transcription factor during ES cell differentiation *in vitro*. If the technology of nuclear transfer (268) from a patient cell into an ES cell demonstrates to be a viable means of generating new tissue from the patient itself, then new tissue will be immunologically recognized as self and not undergo rejection by the patient. While the proof of principle has been demonstrated in the genetic correction of immune compromised Rag-2 deficient mice (204), obstacles must first be surpassed. For example, the low level of MHC class 1 found on hematopoietic cells derived from the ES cells is low and causes an NK cell mediated immune response against the corrected cells. While this, the ultimate form of Gene Therapy just described, could result in the ability to correct defective genes in virtually any tissue and, therefore, result in potential cures for many diseases in patients, there is still much work to be done. The infancy of this field also suggests that for the near future, successes in gene therapy will be the result of gene transfer into adult hematopoietic stem cells.

CONCLUDING REMARKS

Although the high transduction efficiencies regularly achieved using the lentiviral vectors on primitive repopulating cells of NOD/SCID mice are occasionally reported using oncoretroviral vectors, the ability to efficiently transduce SRC using lentiviral vectors under conditions that are significantly shorter than those required for oncoretroviral vector transduction suggest that the repopulating potential of these cells will be better maintained. In addition, integrated lentiviral vectors are relatively insensitivity to transcriptional inactivation in comparison to oncoretroviral based vectors, and hence have the propensity for long-term transgene expression in the progeny of the transduced cells. However, the true efficacy of the vectors to transduced true human stem cells capable of long-term reconstitution in a patient can only be determined in the clinical setting. Unfortunately, the issue raised in the Paper IV with regards to the multiple copy integration suggests that for the moment lentiviral vectors should not advance to the clinic. However, we (unpublished data) and others have demonstrated that high efficiency transduction of hematopoietic repopulating cells can be achieved using significantly lower MOI, suggesting that the problem of multiple copy integration can be overcome. This combined with advances in transduction protocols, tissue-specific and regulated expression, selective expansion of transduced cells, the molecular understandings of disease, all suggest the success of gene therapy is of the hematopoietic system is imminent. Furthermore the new advances in ES cell differentiation and modification will add a new and exciting component to treating disease, with more diseases potentially curable than ever before.

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Isao Hamaguchi, **Niels-Bjarne Woods,** Ioannis Panagopoulos, Elisabet Andersson, Cecilia Fahlman, Hanna Mikkola, Romain Zufferey, Didier Trono, Stefan Karlsson: Lentiviral Vector Gene Expression During ES Cell Derived Hematopoietic Development *In Vitro*. **J of Virol, Nov. 2000; Vol. 74(22) p. 10778-10784.**

Hanna Mikkola, **Niels-Bjarne Woods**, Marketa Sjögren, Hildur Helgadottir, Isao Hamaguchi, Sten-Eirik Jacobsen, Didier Trono, Stefan Karlsson: Lentiviral Gene Transfer in Murine Hematopoietic Stem Cells is Compromised by a Delay in Proviral Integration and Results in Transduction Mosaicism and Heterogeneous Gene Expression in Progeny Cells. **J of Virol, Dec. 2000; Vol. 74(24) p.11911.**

Niels-Bjarne Woods, Cecilia Fahlman, Hanna Mikkola, Isao Hamaguchi, Karin Olsson, Romain Zufferey, Didier Trono, Stefan Karlsson: Lentiviral Gene Transfer into Primary and Secondary NOD/SCID Repopulating Cells. **Blood, Dec. 1 2000; Vol. 93(12) p.3725**.

Niels-Bjarne Woods, Eva Nilsson, Karin Olsson, Stefan Karlsson: Lentiviral-Mediated Gene Transfer into Hematopoietic Cells. **J Int Med, 2001; 249:1-5.**

Hanna Mikkola, Hildur Helgadottir, **Niels-Bjarne Woods**, Patrick Salmon, Didier Trono, Stefan Karlsson: Efficient Lentiviral Gene Transfer into Murine Hematopoietic Stem Cells and Stable Expression of GFP in Myeloid and Lymphoid Cells in Serially Transplanted Recipients. **(Submitted, 2002).**

Niels-Bjarne Woods, Andreas Ooka, Stefan Karlsson: Development of Gene Therapy for Hematopoietic Stem Cells Using Lentiviral Vectors. **Leukemia, 2002 Apr; 16(4):563-9. Review.**

Stefan Karlsson, Andreas Ooka, **Niels-Bjarne Woods**: Development of Gene Therapy for Blood Disorders by Gene Transfer into Hematopoietic Stem Cells. **Haemophilia, 2002 May; 8(3):255-60. Review.**

Niels-Bjarne Woods, Arne Muessig, Manfred Schmidt, Johan Flygare, Karin Olsson, Patrick Salmon, Didier Trono, Christof von Kalle, Stefan Karlsson: Lentiviral Vector Transduction of NOD/SCID Repopulating Cells Results in Multiple Vector Integrations per Transduced Cell: Risk of Insertional Mutagenesis**. (Blood in press, 2002).**

Niels-Bjarne Woods, Johan Flygare, Hanna Mikkola, Eva Nilsson, Karin Olsson, Thomas Relander, Johan Richter, Patrick Salmon, Didier Trono, Stefan Karlsson. Optimal Lentiviral Vector Design for High-Level Transgene Expression in the Progeny of NOD/SCID repopulating cells. (manuscript, 2002).

REFERENCES

1. Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A, Morecki S, Andolfi G, Tabucchi A, Carlucci F, Marinello E, Cattaneo F, Vai S, Servida P, Miniero R, Roncarolo MG, Bordignon C. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Science 296 (5577): 2410-3. 2002.
2. Aiuti A, Vai S, Mortellaro A, Casorati G, Ficara F, Andolfi G, Ferrari G, Tabucchi A, Carlucci F, Ochs HD, Notarangelo LD, Roncarolo MG, Bordignon C. Immune reconstitution in ADA-SCID after PBL gene therapy and discontinuation of enzyme replacement. Nat Med 8 (5): 423-5. 2002.
3. Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med 185 (1): 111-20. 1997.
4. Akkina RK, Walton RM, Chen ML, Li QX, Planelles V, Chen IS. High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. J Virol 70 (4): 2581-5. 1996.
5. Allay JA, Davis BM, Gerson SL. Human alkyltransferase-transduced murine myeloid progenitors are enriched in vivo by BCNU treatment of transplanted mice. Exp Hematol 25 (10): 1069-76. 1997.
6. Allay JA, Persons DA, Galipeau J, Riberdy JM, Ashmun RA, Blakley RL, Sorrentino BP. In vivo selection of retrovirally transduced hematopoietic stem cells. Nat Med 4 (10): 1136-43. 1998.
7. An DS, Wersto RP, Agricola BA, Metzger ME, Lu S, Amado RG, Chen IS, Donahue RE. Marking and gene expression by a lentivirus vector in transplanted human and nonhuman primate CD34(+) cells. J Virol 74 (3): 1286-95. 2000.
8. Andreadis ST, Brott D, Fuller AO, Palsson BO. Moloney murine leukemia virus-derived retroviral vectors decay intracellularly with a half-life in the range of 5.5 to 7.5 hours. J Virol 71 (10): 7541-8. 1997.
9. Antonchuk J, Sauvageau G, Humphries RK. HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation. Exp Hematol 29 (9): 1125-34. 2001.
10. Antonchuk J, Sauvageau G, Humphries RK. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell 109 (1): 39-45. 2002.
11. Ash RC, Detrick DA, Zanjani ED. Studies of human pluripotential hemopoietic stem cells (CFU-GEMM) in vitro. Blood 58 (2): 309-16. 1981.
12. Barrette S, Douglas JL, Seidel NE, Bodine DM. Lentivirus-based vectors transduce mouse hematopoietic stem cells with similar efficiency to moloney murine leukemia virus-based vectors. Blood 96 (10): 3385-91. 2000.
13. Becker PS, Nilsson SK, Li Z, Berrios VM, Dooner MS, Cooper CL, Hsieh CC, Quesenberry PJ. Adhesion receptor expression by hematopoietic cell lines and murine progenitors: modulation by cytokines and cell cycle status. Exp Hematol 27 (3): 533-41. 1999.
14. Bensinger WI, Clift R, Martin P, Appelbaum FR, Demirer T, Gooley T, Lilleby K, Rowley S, Sanders J, Storb R, Buckner CD. Allogeneic peripheral blood stem cell transplantation in patients with advanced hematologic malignancies: a retrospective comparison with marrow transplantation. Blood 88 (7): 2794-800. 1996.
15. Berardi AC, Meffre E, Pflumio F, Katz A, Vainchenker W, Schiff C, Coulombel L. Individual CD34+CD38lowCD19-CD10- progenitor cells from human cord blood generate B lymphocytes and granulocytes. Blood 89 (10): 3554-64. 1997.

16. Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD, Bernstein ID. Antigen CD34+ marrow cells engraft lethally irradiated baboons. J Clin Invest 81 (3): 951-5. 1988.
17. Berkowitz R, Ilves H, Lin WY, Eckert K, Coward A, Tamaki S, Veres G, Plavec I. Construction and molecular analysis of gene transfer systems derived from bovine immunodeficiency virus. J Virol 75 (7): 3371-82. 2001.
18. Berrios VM, Dooner GJ, Nowakowski G, Frimberger A, Valinski H, Quesenberry PJ, Becker PS. The molecular basis for the cytokine-induced defect in homing and engraftment of hematopoietic stem cells. Exp Hematol 29 (11): 1326-35. 2001.
19. Bhardwaj G, Murdoch B, Wu D, Baker DP, Williams KP, Chadwick K, Ling LE, Karanu FN, Bhatia M. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. Nat Immunol 2 (2): 172-80. 2001.
20. Bhatia M, Bonnet D, Kapp U, Wang JC, Murdoch B, Dick JE. Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. J Exp Med 186 (4): 619-24. 1997.
21. Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. A newly discovered class of human hematopoietic cells with SCID- repopulating activity. Nat Med 4 (9): 1038-45. 1998.
22. Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci U S A 94 (10): 5320-5. 1997.
23. Björnsson JM, Brun A, Larsson N, Repetowska E, Karlsson S. HoxB4 Deficiency Causes Reduced Cellularity in Hematopoietic Organs and Diminished Proliferative Response of Primitive Hematopoietic Progenitors [abstract]. Blood 98(11) Suppl. 1: Abstract # 281. 2001.
24. Boheler KR, Czyz J, Tweedie D, Yang HT, Anisimov SV, Wobus AM. Differentiation of pluripotent embryonic stem cells into cardiomyocytes. Circ Res 91 (3): 189-201. 2002.
25. Bonini C, Ferrari G, Verzeletti S, Servida P, Zappone E, Ruggieri L, Ponzoni M, Rossini S, Mavilio F, Traversari C, Bordignon C. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. Science 276 (5319): 1719-24. 1997.
26. Borge OJ, Ramsfjell V, Cui L, Jacobsen SE. Ability of early acting cytokines to directly promote survival and suppress apoptosis of human primitive CD34+CD38- bone marrow cells with multilineage potential at the single-cell level: key role of thrombopoietin. Blood 90 (6): 2282-92. 1997.
27. Brugarolas J, Jacks T. Double indemnity: p53, BRCA and cancer. p53 mutation partially rescues developmental arrest in Brca1 and Brca2 null mice, suggesting a role for familial breast cancer genes in DNA damage repair. Nat Med 3 (7): 721-2. 1997.
28. Brun A, Fan X, Humphries RK, Karlsson S. The Effects of HoxB4 in Human Primitive Hematopoietic Progenitors are Concentration Dependent: High Levels of HoxB4 Lead to Increased Myeloid Differentiation and a Reduction in Proliferative Capacity [abstract]. Blood 98(11): Suppl. 1a: Abstract # 267. 2001.
29. Brustle O, Jones KN, Learish RD, Karram K, Choudhary K, Wiestler OD, Duncan ID, McKay RD. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science 285 (5428): 754-6. 1999.
30. Bukrinsky MI, Haggerty S, Dempsey MP, Sharova N, Adzhubel A, Spitz L, Lewis P, Goldfarb D, Emerman M, Stevenson M. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature 365 (6447): 666-9. 1993.

31. Bukrinsky MI, Sharova N, Dempsey MP, Stanwick TL, Bukrinskaya AG, Haggerty S, Stevenson M. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc Natl Acad Sci U S A 89 (14): 6580-4. 1992.
32. Burns J, Friedmann T, Driever W, Burrascano M, Yee J. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: Concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc Natl Acad Sci USA 90 (17): 8033-8037. 1993.
33. Buske C, Feuring-Buske M, Abramovich C, Spiekermann K, Eaves CJ, Coulombel L, Sauvageau G, Hogge DE, Humphries RK. Deregulated expression of HOXB4 enhances the primitive growth activity of human hematopoietic cells. Blood 100 (3): 862-8. 2002.
34. Case SS, Price MA, Jordan CT, Yu XJ, Wang L, Bauer G, Haas DL, Xu D, Stripecke R, Naldini L, Kohn DB, Crooks GM. Stable transduction of quiescent CD34(+)CD38(-) human hematopoietic cells by HIV-1-based lentiviral vectors. Proc Natl Acad Sci U S A 96 (6): 2988-93. 1999.
35. Cashman JD, Lapidot T, Wang JC, Doedens M, Shultz LD, Lansdorp P, Dick JE, Eaves CJ. Kinetic evidence of the regeneration of multilineage hematopoiesis from primitive cells in normal human bone marrow transplanted into immunodeficient mice. Blood 89 (12): 4307-16. 1997.
36. Cassel A, Cottler-Fox M, Doren S, Dunbar C. Retroviral-mediated gene transfer into CD34-enriched human peripheral blood stem cells. Exp Hematol 21 (4): 585-591. 1993.
37. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 288 (5466): 669-72. 2000.
38. Cepko CL, Roberts BE, Mulligan RC. Construction and applications of a highly transmissible murine retrovirus shuttle vector. Cell 37 (3): 1053-62. 1984.
39. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science 263 (5148): 802-5. 1994.
40. Challita PM, Kohn DB. Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. Proc Natl Acad Sci U S A 91 (7): 2567-71. 1994.
41. Chen W, Wu X, Levasseur DN, Liu H, Lai L, Kappes JC, Townes TM. Lentiviral vector transduction of hematopoietic stem cells that mediate long-term reconstitution of lethally irradiated mice. Stem Cells 18 (5): 352-9. 2000.
42. Cheng L, Du C, Murray D, Tong X, Zhang YA, Chen BP, Hawley RG. A GFP reporter system to assess gene transfer and expression in human hematopoietic progenitor cells. Gene Ther 4 (10): 1013-22. 1997.
43. Cheng L, Fu J, Tsukamoto A, Hawley RG. Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells. Nat Biotechnol 14 (5): 606-9. 1996.
44. Cherry SR, Biniszkiewicz D, van Parijs L, Baltimore D, Jaenisch R. Retroviral expression in embryonic stem cells and hematopoietic stem cells. Mol Cell Biol 20 (20): 7419-26. 2000.
45. Civin CI, Almeida-Porada G, Lee MJ, Olweus J, Terstappen LW, Zanjani ED. Sustained, retransplantable, multilineage engraftment of highly purified adult human bone marrow stem cells in vivo. Blood 88 (11): 4102-9. 1996.
46. Clapp DW, Freie B, Lee WH, Zhang YY. Molecular evidence that in situ-transduced fetal liver hematopoietic stem/progenitor cells give rise to medullary hematopoiesis in adult rats. Blood 86 (6): 2113-22. 1995.
47. Conneally E, Cashman J, Petzer A, Eaves C. Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay

of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. Proc Natl Acad Sci U S A 94 (18): 9836-41. 1997.
48. Corey CA, DeSilva AD, Holland CA, Williams DA. Serial transplantation of methotrexate-resistant bone marrow: protection of murine recipients from drug toxicity by progeny of transduced stem cells. Blood 75 (2): 337-43. 1990.
49. Cui Y, Golob J, Kelleher E, Ye Z, Pardoll D, Cheng L. Targeting transgene expression to antigen-presenting cells derived from lentivirus-transduced engrafting human hematopoietic stem/progenitor cells. Blood 99 (2): 399-408. 2002.
50. Danos O, Mulligan RC. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc Natl Acad Sci U S A 85 (17): 6460-4. 1988.
51. Dao MA, Hannum CH, Kohn DB, Nolta JA. FLT3 ligand preserves the ability of human CD34+ progenitors to sustain long-term hematopoiesis in immune-deficient mice after ex vivo retroviral-mediated transduction. Blood 89 (2): 446-56. 1997.
52. Dao MA, Hashino K, Kato I, Nolta JA. Adhesion to fibronectin maintains regenerative capacity during ex vivo culture and transduction of human hematopoietic stem and progenitor cells. Blood 92 (12): 4612-21. 1998.
53. Dao MA, Nolta JA. CD34: to select or not to select? That is the question. Leukemia 14 (5): 773-6. 2000.
54. Dao MA, Nolta JA. Immunodeficient mice as models of human hematopoietic stem cell engraftment. Curr Opin Immunol 11 (5): 532-7. 1999.
55. Dao MA, Shah AJ, Crooks GM, Nolta JA. Engraftment and retroviral marking of CD34+ and CD34+CD38- human hematopoietic progenitors assessed in immune-deficient mice. Blood 91 (4): 1243-55. 1998.
56. Dao MA, Yu XJ, Nolta JA. Clonal diversity of primitive human hematopoietic progenitors following retroviral marking and long-term engraftment in immune-deficient mice. Exp Hematol 25 (13): 1357-66. 1997.
57. Darlix JL, Lapadat-Tapolsky M, de Rocquigny H, Roques BP. First glimpses at structure-function relationships of the nucleocapsid protein of retroviruses. J Mol Biol 254 (4): 523-37. 1995.
58. de Wynter EA, Heyworth CM, Mukaida N, Matsushima K, Testa NG. NOD/SCID repopulating cells but not LTC-IC are enriched in human CD34+ cells expressing the CCR1 chemokine receptor. Leukemia 15 (7): 1092-101. 2001.
59. Demaison C, Parsley K, Brouns G, Scherr M, Battmer K, Kinnon C, Grez M, Thrasher AJ. High-level transduction and gene expression in hematopoietic repopulating cells using a human imunodeficiency virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. Hum Gene Ther 13 (7): 803-13. 2002.
60. Denning-Kendall PA, Evely R, Singha S, Chapman M, Bradley BA, Hows JM. In vitro expansion of cord blood does not prevent engraftment of severe combined immunodeficient repopulating cells. Br J Haematol 116 (1): 218-28. 2002.
61. Dick JE, Bhatia M, Gan O, Kapp U, Wang JC. Assay of human stem cells by repopulation of NOD/SCID mice. Stem Cells 15 (Suppl 1): 199-203; discussion 204-7. 1997.
62. Dick JE, Kamel-Reid S, Murdoch B, Doedens M. Gene transfer into normal human hematopoietic cells using in vitro and in vivo assays. Blood 78 (3): 624-34. 1991.
63. Donahue RE, Sorrentino BP, Hawley RG, An DS, Chen IS, Wersto RP. Fibronectin fragment CH-296 inhibits apoptosis and enhances ex vivo gene transfer by murine retrovirus and human lentivirus vectors independent of viral tropism in nonhuman primate CD34+ cells. Mol Ther 3 (3): 359-67. 2001.

64. Donovan PJ, Gearhart J. The end of the beginning for pluripotent stem cells. Nature 414 (6859): 92-7. 2001.
65. Dorie MJ, Maloney MA, Patt HM. Turnover of circulating hematopoietic stem cells. Exp Hematol 7 (9): 483-9. 1979.
66. Douglas J, Kelly P, Evans JT, Garcia JV. Efficient transduction of human lymphocytes and CD34+ cells via human immunodeficiency virus-based gene transfer vectors. Hum Gene Ther 10 (6): 935-45. 1999.
67. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. A third-generation lentivirus vector with a conditional packaging system. J Virol 72 (11): 8463-71. 1998.
68. Dunbar CE, Kohn DB, Schiffmann R, Barton NW, Nolta JA, Esplin JA, Pensiero M, Long Z, Lockey C, Emmons RV, Csik S, Leitman S, Krebs CB, Carter C, Brady RO, Karlsson S. Retroviral transfer of the glucocerebrosidase gene into CD34+ cells from patients with Gaucher disease: in vivo detection of transduced cells without myeloablation. Hum Gene Ther 9 (17): 2629-40. 1998.
69. Dunbar CE, Takatoku M, Donahue RE. The impact of ex vivo cytokine stimulation on engraftment of primitive hematopoietic cells in a non-human primate model. Ann N Y Acad Sci 938: 236-44; discussion 244-5. 2001.
70. Eisenman RN, Vogt VM. The biosynthesis of oncovirus proteins. Biochim Biophys Acta 473 (3-4): 187-239. 1978.
71. Ekblad CM, Wilkinson HR, Schymkowitz JW, Rousseau F, Freund SM, Itzhaki LS. Characterisation of the BRCT domains of the breast cancer susceptibility gene product BRCA1. J Mol Biol 320 (3): 431-42. 2002.
72. Emerman M, Malim MH. HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. Science 280 (5371): 1880-4. 1998.
73. Evans JT, Kelly PF, O'Neill E, Garcia JV. Human cord blood CD34+CD38- cell transduction via lentivirus-based gene transfer vectors. Hum Gene Ther 10 (9): 1479-89. 1999.
74. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 292 (5819): 154-6. 1981.
75. Farrington SM, Belaoussoff M, Baron MH. Winged-helix, Hedgehog and Bmp genes are differentially expressed in distinct cell layers of the murine yolk sac. Mech Dev 62 (2): 197-211. 1997.
76. Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. Nat Genet 25 (2): 217-22. 2000.
77. Franz T, Hilberg F, Seliger B, Stocking C, Ostertag W. Retroviral mutants efficiently expressed in embryonal carcinoma cells. Proc Natl Acad Sci U S A 83 (10): 3292-6. 1986.
78. Gallay P, Hope T, Chin D, Trono D. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc Natl Acad Sci U S A 94 (18): 9825-30. 1997.
79. Gallay P, Stitt V, Mundy C, Oettinger M, Trono D. Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import. J Virol 70 (2): 1027-32. 1996.
80. Gan OI, Murdoch B, Larochelle A, Dick JE. Differential maintenance of primitive human SCID-repopulating cells, clonogenic progenitors, and long-term culture-initiating cells after incubation on human bone marrow stromal cells. Blood 90 (2): 641-50. 1997.
81. Gao Z, Golob J, Tanavde VM, Civin CI, Hawley RG, Cheng L. High levels of transgene expression following transduction of long-term NOD/SCID-repopulating human cells with a modified lentiviral vector. Stem Cells 19 (3): 247-59 2001.

82. Gatlin J, Douglas J, Evans JT, Collins RH, Wendel GD, Garcia JV. In vitro selection of lentivirus vector-transduced human CD34+ cells. Hum Gene Ther 11 (13): 1949-57. 2000.
83. Giannoukakis N, Mi Z, Gambotto A, Eramo A, Ricordi C, Trucco M, Robbins P. Infection of intact human islets by a lentiviral vector. Gene Ther 6 (9): 1545-51. 1999.
84. Glimm H, Eaves CJ. Direct evidence for multiple self-renewal divisions of human in vivo repopulating hematopoietic cells in short-term culture. Blood 94 (7): 2161-8. 1999.
85. Glimm H, Oh IH, Eaves CJ. Human hematopoietic stem cells stimulated to proliferate in vitro lose engraftment potential during their S/G(2)/M transit and do not reenter G(0). Blood 96 (13): 4185-93. 2000.
86. Glimm H, Tang P, Clark-Lewis I, von Kalle C, Eaves C. Ex vivo treatment of proliferating human cord blood stem cells with stroma-derived factor-1 enhances their ability to engraft NOD/SCID mice. Blood 99 (9): 3454-7. 2002.
87. Godin I, Cumano A. The hare and the tortoise: an embryonic haematopoietic race. Nat Rev Immunol 2 (8): 593-604. 2002.
88. Goerner M, Horn PA, Peterson L, Kurre P, Storb R, Rasko JE, Kiem HP. Sustained multilineage gene persistence and expression in dogs transplanted with CD34(+) marrow cells transduced by RD114-pseudotype oncoretrovirus vectors. Blood 98 (7): 2065-70. 2001.
89. Goff SP, Lobel LI. Mutants of murine leukemia viruses and retroviral replication. Biochim Biophys Acta 907 (2): 93-123. 1987.
90. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline- responsive promoters. Proc Natl Acad Sci U S A 89 (12): 5547-51. 1992.
91. Gossler A, Doetschman T, Korn R, Serfling E, Kemler R. Transgenesis by means of blastocyst-derived embryonic stem cell lines. Proc Natl Acad Sci U S A 83 (23): 9065-9. 1986.
92. Gothot A, van der Loo JC, Clapp DW, Srour EF. Cell cycle-related changes in repopulating capacity of human mobilized peripheral blood CD34(+) cells in non-obese diabetic/severe combined immune-deficient mice. Blood 92 (8): 2641-9. 1998.
93. Grez M, Akgun E, Hilberg F, Ostertag W. Embryonic stem cell virus, a recombinant murine retrovirus with expression in embryonic stem cells. Proc Natl Acad Sci U S A 87 (23): 9202-6. 1990.
94. Guenechea G, Gan OI, Dorrell C, Dick JE. Distinct classes of human stem cells that differ in proliferative and self-renewal potential. Nat Immunol 2 (1): 75-82. 2001.
95. Guenechea G, Gan OI, Inamitsu T, Dorrell C, Pereira DS, Kelly M, Naldini L, Dick JE. Transduction of human CD34+ CD38- bone marrow and cord blood-derived SCID-repopulating cells with third-generation lentiviral vectors. Mol Ther 1 (6): 566-73. 2000.
96. Haar JL, Ackerman GA. A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse. Anat Rec 170 (2): 199-223. 1971.
97. Haas DL, Case SS, Crooks GM, Kohn DB. Critical factors influencing stable transduction of human CD34(+) cells with HIV-1-derived lentiviral vectors. Mol Ther 2 (1): 71-80. 2000.
98. Hacein-Bey-Abina S, Le Deist F, Carlier F, Bouneaud C, Hue C, De Villartay JP, Thrasher AJ, Wulffraat N, Sorensen R, Dupuis-Girod S, Fischer A, Davies EG, Kuis W, Leiva L, Cavazzana-Calvo M. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. N Engl J Med 346 (16): 1185-93. 2002.

99. Han JJ, Mhatre AN, Wareing M, Pettis R, Gao WQ, Zufferey RN, Trono D, Lalwani AK. Transgene expression in the guinea pig cochlea mediated by a lentivirus- derived gene transfer vector. Hum Gene Ther 10 (11): 1867-73. 1999.
100. Hanenberg H, Xiao XL, Dilloo D, Hashino K, Kato I, Williams DA. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. Nat Med 2 (8): 876-82. 1996.
101. Hao QL, Smogorzewska EM, Barsky LW, Crooks GM. In vitro identification of single CD34+CD38- cells with both lymphoid and myeloid potential. Blood 91 (11): 4145-51. 1998.
102. Harrich D, Garcia J, Wu F, Mitsuyasu R, Gonazalez J, Gaynor R. Role of SP1-binding domains in in vivo transcriptional regulation of the human immunodeficiency virus type 1 long terminal repeat. J Virol 63 (6): 2585-91. 1989.
103. Hawley RG, Fong AZ, Burns BF, Hawley TS. Transplantable myeloproliferative disease induced in mice by an interleukin 6 retrovirus. J Exp Med 176 (4): 1149-63. 1992.
104. Heinzinger NK, Bukinsky MI, Haggerty SA, Ragland AM, Kewalramani V, Lee MA, Gendelman HE, Ratner L, Stevenson M, Emerman M. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc Natl Acad Sci U S A 91 (15): 7311-5. 1994.
105. Hennemann B, Oh IH, Chuo JY, Kalberer CP, Schley PD, Rose-John S, Humphries RK, Eaves CJ. Efficient retrovirus-mediated gene transfer to transplantable human bone marrow cells in the absence of fibronectin. Blood 96 (7): 2432-9. 2000.
106. Hock RA, Miller AD. Retrovirus-mediated transfer and expression of drug resistance genes in human haematopoietic progenitor cells. Nature 320 (6059): 275-7. 1986.
107. Holmes-Son ML, Appa RS, Chow SA. Molecular genetics and target site specificity of retroviral integration. Adv Genet 43: 33-69 2001.
108. Horwitz ME, Malech HL, Anderson SM, Girard LJ, Bodine DM, Orlic D. Granulocyte colony-stimulating factor mobilized peripheral blood stem cells enter into G1 of the cell cycle and express higher levels of amphotropic retrovirus receptor mRNA. Exp Hematol 27 (7): 1160-7. 1999.
109. Illmensee K, Hoppe PC. Nuclear transplantation in Mus musculus: developmental potential of nuclei from preimplantation embryos. Cell 23 (1): 9-18. 1981.
110. Iwakuma T, Cui Y, Chang LJ. Self-inactivating lentiviral vectors with U3 and U5 modifications. Virology 261 (1): 120-32. 1999.
111. Jacobsen SE, Borge OJ, Ramsfjell V, Cui L, Cardier JE, Veiby OP, Murphy MJ, Jr., Lok S. Thrombopoietin, a direct stimulator of viability and multilineage growth of primitive bone marrow progenitor cells. Stem Cells 14 (Suppl 1): 173-80. 1996.
112. Jahner D, Stuhlmann H, Stewart CL, Harbers K, Lohler J, Simon I, Jaenisch R. De novo methylation and expression of retroviral genomes during mouse embryogenesis. Nature 298 (5875): 623-8. 1982.
113. Jang SK, Pestova TV, Hellen CU, Witherell GW, Wimmer E. Cap-independent translation of picornavirus RNAs: structure and function of the internal ribosomal entry site. Enzyme 44 (1-4): 292-309. 1990.
114. Jang SK, Wimmer E. Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein. Genes Dev 4 (9): 1560-72. 1990.
115. Jetmore A, Plett PA, Tong X, Wolber FM, Breese R, Abonour R, Orschell-Traycoff CM, Srour EF. Homing efficiency, cell cycle kinetics, and survival of quiescent and cycling human CD34(+) cells transplanted into conditioned NOD/SCID recipients. Blood 99 (5): 1585-93. 2002.

116. Jetzt AE, Yu H, Klarmann GJ, Ron Y, Preston BD, Dougherty JP. High rate of recombination throughout the human immunodeficiency virus type 1 genome. J Virol 74 (3): 1234-40. 2000.
117. Jiang Y, Prosper F, Verfaillie CM. Opposing effects of engagement of integrins and stimulation of cytokine receptors on cell cycle progression of normal human hematopoietic progenitors. Blood 95 (3): 846-54. 2000.
118. Jin L, Siritanaratkul N, Emery DW, Richard RE, Kaushansky K, Papayannopoulou T, Blau CA. Targeted expansion of genetically modified bone marrow cells. Proc Natl Acad Sci U S A 95 (14): 8093-7. 1998.
119. Jin L, Zeng H, Chien S, Otto KG, Richard RE, Emery DW, Blau CA. In vivo selection using a cell-growth switch. Nat Genet 26 (1): 64-6. 2000.
120. Jordan CT, Yamasaki G, Minamoto D. High-resolution cell cycle analysis of defined phenotypic subsets within primitive human hematopoietic cell populations. Exp Hematol 24 (11): 1347-55. 1996.
121. Kafri T, Blomer U, Peterson DA, Gage FH, Verma IM. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat Genet 17 (3): 314-7. 1997.
122. Kafri T, van Praag H, Gage FH, Verma IM. Lentiviral vectors: regulated gene expression. Mol Ther 1 (6): 516-21. 2000.
123. Kansas GS. Selectins and their ligands: current concepts and controversies. Blood 88 (9): 3259-87. 1996.
124. Kavanaugh MP, Miller DG, Zhang W, Law W, Kozak SL, Kabat D, Miller AD. Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent phosphate symporters. Proc Natl Acad Sci U S A 91 (15): 7071-5. 1994.
125. Kelly PF, Vandergriff J, Nathwani A, Nienhuis AW, Vanin EF. Highly efficient gene transfer into cord blood nonobese diabetic/severe combined immunodeficiency repopulating cells by oncoretroviral vector particles pseudotyped with the feline endogenous retrovirus (RD114) envelope protein. Blood 96 (4): 1206-14. 2000.
126. Kim DW, Uetsuki T, Kaziro Y, Yamaguchi N, Sugano S. Use of the human elongation factor 1 alpha promoter as a versatile and efficient expression system. Gene 91 (2): 217-23. 1990.
127. Klages N, Zufferey R, Trono D. A stable system for the high-titer production of multiply attenuated lentiviral vectors. Mol Ther 2 (2): 170-6. 2000.
128. Klug CA, Cheshier S, Weissman IL. Inactivation of a GFP retrovirus occurs at multiple levels in long-term repopulating stem cells and their differentiated progeny. Blood 96 (3): 894-901. 2000.
129. Kluge KA, Bonifacino AC, Sellers S, Agricola BA, Donahue RE, Dunbar CE. Retroviral transduction and engraftment ability of primate hematopoietic progenitor and stem cells transduced under serum-free versus serum-containing conditions. Mol Ther 5 (3): 316-22. 2002.
130. Klump H, Schiedlmeier B, Vogt B, Ryan M, Ostertag W, Baum C. Retroviral vector-mediated expression of HoxB4 in hematopoietic cells using a novel coexpression strategy. Gene Ther 8 (10): 811-7. 2001.
131. Kobari L, Pflumio F, Giarratana M, Li X, Titeux M, Izac B, Leteurtre F, Coulombel L, Douay L. In vitro and in vivo evidence for the long-term multilineage (myeloid, B, NK, and T) reconstitution capacity of ex vivo expanded human CD34(+) cord blood cells. Exp Hematol 28 (12): 1470-80. 2000.
132. Kohn DB. Gene therapy for haematopoietic and lymphoid disorders. Clin Exp Immunol 107 Suppl 1: 54-7. 1997.
133. Korin YD, Zack JA. Nonproductive human immunodeficiency virus type 1 infection in nucleoside-treated G0 lymphocytes. J Virol 73 (8): 6526-32. 1999.

134. Korin YD, Zack JA. Progression to the G1b phase of the cell cycle is required for completion of human immunodeficiency virus type 1 reverse transcription in T cells. J Virol 72 (4): 3161-8. 1998.
135. Kronenwett R, Martin S, Haas R. The role of cytokines and adhesion molecules for mobilization of peripheral blood stem cells. Stem Cells 18 (5): 320-30. 2000.
136. Kurre P, Morris J, Horn PA, Harkey MA, Andrews RG, Kiem HP. Gene transfer into baboon repopulating cells: A comparison of Flt-3 Ligand and megakaryocyte growth and development factor versus IL-3 during ex vivo transduction. Mol Ther 3 (6): 920-7. 2001.
137. Kyba M, Perlingeiro RC, Daley GQ. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. Cell 109 (1): 29-37. 2002.
138. Laker C, Meyer J, Schopen A, Friel J, Heberlein C, Ostertag W, Stocking C. Host cis-mediated extinction of a retrovirus permissive for expression in embryonal stem cells during differentiation. J Virol 72 (1): 339-48. 1998.
139. Lapidot T, Pflumio F, Doedens M, Murdoch B, Williams DE, Dick JE. Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. Science 255 (5048): 1137-41. 1992.
140. Larochelle A, Vormoor J, Hanenberg H, Wang J, Bathia M, Lapidot T, Moritz T, Murdoch B, Xiao X, Kato I, Williams D, Dick J. Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: Implications for gene therapy. Nature Med 2 (12): 1329-1337. 1996.
141. Lewis ID, Almeida-Porada G, Du J, Lemischka IR, Moore KA, Zanjani ED, Verfaillie CM. Umbilical cord blood cells capable of engrafting in primary, secondary, and tertiary xenogeneic hosts are preserved after ex vivo culture in a noncontact system. Blood 97 (11): 3441-9. 2001.
142. Lewis P, Hensel M, Emerman M. Human immunodeficiency virus infection of cells arrested in the cell cycle. Embo J 11 (8): 3053-8. 1992.
143. Li Z, Dullmann J, Schiedlmeier B, Schmidt M, von Kalle C, Meyer J, Forster M, Stocking C, Wahlers A, Frank O, Ostertag W, Kuhlcke K, Eckert HG, Fehse B, Baum C. Murine leukemia induced by retroviral gene marking. Science 296 (5567): 497. 2002.
144. Li ZY, Otto K, Richard RE, Ni S, Kirillova I, Fausto N, Blau CA, Lieber A. Dimerizer-induced proliferation of genetically modified hepatocytes. Mol Ther 5 (4): 420-6. 2002.
145. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science 295 (5556): 868-72. 2002.
146. Lotti F, Menguzzato E, Rossi C, Naldini L, Ailles L, Mavilio F, Ferrari G. Transcriptional targeting of lentiviral vectors by long terminal repeat enhancer replacement. J Virol 76 (8): 3996-4007. 2002.
147. Luens KM, Travis MA, Chen BP, Hill BL, Scollay R, Murray LJ. Thrombopoietin, kit ligand, and flk2/flt3 ligand together induce increased numbers of primitive hematopoietic progenitors from human CD34+Thy-1+Lin- cells with preserved ability to engraft SCID-hu bone. Blood 91 (4): 1206-15. 1998.
148. Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 292 (5520): 1389-94. 2001.
149. Luu P, Flores O. Binding of SP1 to the immediate-early protein-responsive element of the human cytomegalovirus DNA polymerase promoter. J Virol 71 (9): 6683-91. 1997.

150. Magnuson T, Epstein CJ, Silver LM, Martin GR. Pluripotent embryonic stem cell lines can be derived from tw5/tw5 blastocysts. Nature 298 (5876): 750-3. 1982.
151. Markowitz D, Goff S, Bank A. A safe packaging line for gene transfer: separating viral genes on two different plasmids. J Virol 62 (4): 1120-4. 1988.
152. Marsh M, Helenius A. Virus entry into animal cells. Adv Virus Res 36: 107-51. 1989.
153. Matsuoka S, Tsuji K, Hisakawa H, Xu M, Ebihara Y, Ishii T, Sugiyama D, Manabe A, Tanaka R, Ikeda Y, Asano S, Nakahata T. Generation of definitive hematopoietic stem cells from murine early yolk sac and paraaortic splanchnopleures by aorta-gonad-mesonephros region-derived stromal cells. Blood 98 (1): 6-12. 2001.
154. May C, Rivella S, Callegari J, Heller G, Gaensler KM, Luzzatto L, Sadelain M. Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. Nature 406 (6791): 82-6. 2000.
155. Maze R, Kapur R, Kelley MR, Hansen WK, Oh SY, Williams DA. Reversal of 1,3-bis(2-chloroethyl)-1-nitrosourea-induced severe immunodeficiency by transduction of murine long-lived hemopoietic progenitor cells using O6-methylguanine DNA methyltransferase complementary DNA. J Immunol 158 (2): 1006-13. 1997.
156. Medvinsky AL, Samoylina NL, Muller AM, Dzierzak EA. An early pre-liver intraembryonic source of CFU-S in the developing mouse. Nature 364 (6432): 64-7. 1993.
157. Meier JL, Keller MJ, McCoy JJ. Requirement of multiple cis-acting elements in the human cytomegalovirus major immediate-early distal enhancer for viral gene expression and replication. J Virol 76 (1): 313-26. 2002.
158. Meslin FX, Stohr K, Heymann D. Public health implications of emerging zoonoses. Rev Sci Tech 19 (1): 310-7. 2000.
159. Meyerhans A, Vartanian JP, Hultgren C, Plikat U, Karlsson A, Wang L, Eriksson S, Wain-Hobson S. Restriction and enhancement of human immunodeficiency virus type 1 replication by modulation of intracellular deoxynucleoside triphosphate pools. J Virol 68 (1): 535-40. 1994.
160. Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol 10 (8): 4239-42. 1990.
161. Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. J Virol 72 (10): 8150-7. 1998.
162. Miyoshi H, Smith KA, Mosier DE, Verma IM, Torbett BE. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. Science 283 (5402): 682-6. 1999.
163. Miyoshi H, Takahashi M, Gage F, Verma I. Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. Proc Natl Acad Sci USA 94: 10319-10323. 1997.
164. Moritz T, Dutt P, Xiao X, Carstanjen D, Vik T, Hanenberg H, Williams DA. Fibronectin improves transduction of reconstituting hematopoietic stem cells by retroviral vectors: evidence of direct viral binding to chymotryptic carboxy-terminal fragments. Blood 88 (3): 855-62. 1996.
165. Moritz T, Williams DA. Gene transfer into the hematopoietic system. Curr Opin Hematol 1 (6): 423-8. 1994.
166. Moynahan ME, Chiu JW, Koller BH, Jasin M. Brca1 controls homology-directed DNA repair. Mol Cell 4 (4): 511-8. 1999.
167. Muller AM, Dzierzak EA. ES cells have only a limited lymphopoietic potential after adoptive transfer into mouse recipients. Development 118 (4): 1343-51. 1993.
168. Murray LJ, Young JC, Osborne LJ, Luens KM, Scollay R, Hill BL. Thrombopoietin, flt3, and kit ligands together suppress apoptosis of human

mobilized CD34+ cells and recruit primitive CD34+ Thy-1+ cells into rapid division. Exp Hematol 27 (6): 1019-28. 1999.
169. Neff T, Horn PA, Valli VE, Gown AM, Wardwell S, Wood BL, Von Kalle C, Schmidt M, Peterson LJ, Morris JC, Richard RE, Clackson T, Kiem HP, Blau CA. Pharmacologically regulated in vivo selection in a large animal. Blood 100 (6): 2026-2031. 2002.
170. Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith A. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95 (3): 379-91. 1998.
171. Nie Z, Bergeron D, Subbramanian RA, Yao XJ, Checroune F, Rougeau N, Cohen EA. The putative alpha helix 2 of human immunodeficiency virus type 1 Vpr contains a determinant which is responsible for the nuclear translocation of proviral DNA in growth-arrested cells. J Virol 72 (5): 4104-15. 1998.
172. Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev 12 (13): 2048-60. 1998.
173. Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 24 (4): 372-6. 2000.
174. Nolta JA, Hanley MB, Kohn DB. Sustained human hematopoiesis in immunodeficient mice by cotransplantation of marrow stroma expressing human interleukin-3: analysis of gene transduction of long-lived progenitors. Blood 83 (10): 3041-51. 1994.
175. Novelli EM, Cheng L, Yang Y, Leung W, Ramirez M, Tanavde V, Enger C, Civin CI. Ex vivo culture of cord blood CD34+ cells expands progenitor cell numbers, preserves engraftment capacity in nonobese diabetic/severe combined immunodeficient mice, and enhances retroviral transduction efficiency. Hum Gene Ther 10 (18): 2927-40. 1999.
176. O'Shea KS. Embryonic stem cell models of development. Anat Rec 257 (1): 32-41. 1999.
177. Orkin SH. Diversification of haematopoietic stem cells to specific lineages. Nat Rev Genet 1 (1): 57-64. 2000.
178. Ory D, Neugeboren B, Muligan R. A stable cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc Natl Acad Sci USA 93 (21): 11400-11406. 1996.
179. Osawa M, Hanada K-i, Hamada H, Nakauchi H. Long-Term Lymphohematopoietic Reconstitution by a Single CD34-Low/Negative Hematopoietic Stem Cell. <EM>Science</EM> 273 (5272): 242-245. 1996.
180. Pannell D, Osborne CS, Yao S, Sukonnik T, Pasceri P, Karaiskakis A, Okano M, Li E, Lipshitz HD, Ellis J. Retrovirus vector silencing is de novo methylase independent and marked by a repressive histone code. Embo J 19 (21): 5884-94. 2000.
181. Papayannopoulou T, Priestley GV, Nakamoto B. Anti-VLA4/VCAM-1-induced mobilization requires cooperative signaling through the kit/mkit ligand pathway. Blood 91 (7): 2231-9. 1998.
182. Papayannopoulou T, Priestley GV, Nakamoto B, Zafiropoulos V, Scott LM, Harlan JM. Synergistic mobilization of hemopoietic progenitor cells using concurrent beta1 and beta2 integrin blockade or beta2-deficient mice. Blood 97 (5): 1282-8. 2001.
183. Paszty C, Brion CM, Manci E, Witkowska HE, Stevens ME, Mohandas N, Rubin EM. Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell disease. Science 278 (5339): 876-8. 1997.
184. Pawliuk R, Eaves C, Humphries RK. Evidence of both ontogeny and transplant dose-regulated expansion of hematopoietic stem cells in vivo. Blood 88 (8): 2852-8. 1996.

185. Pawliuk R, Westerman KA, Fabry ME, Payen E, Tighe R, Bouhassira EE, Acharya SA, Ellis J, London IM, Eaves CJ, Humphries RK, Beuzard Y, Nagel RL, Leboulch P. Correction of sickle cell disease in transgenic mouse models by gene therapy. Science 294 (5550): 2368-71. 2001.
186. Pedersen L, van Zeijl M, Johann SV, O'Hara B. Fungal phosphate transporter serves as a receptor backbone for gibbon ape leukemia virus. J Virol 71 (10): 7619-22. 1997.
187. Peled A, Petit I, Kollet O, Magid M, Ponomaryov T, Byk T, Nagler A, Ben-Hur H, Many A, Shultz L, Lider O, Alon R, Zipori D, Lapidot T. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 283 (5403): 845-8. 1999.
188. Persons DA, Allay JA, Allay ER, Smeyne RJ, Ashmun RA, Sorrentino BP, Nienhuis AW. Retroviral-mediated transfer of the green fluorescent protein gene into murine hematopoietic cells facilitates scoring and selection of transduced progenitors in vitro and identification of genetically modified cells in vivo. Blood 90 (5): 1777-86. 1997.
189. Pfeifer A, Ikawa M, Dayn Y, Verma IM. Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc Natl Acad Sci U S A 99 (4): 2140-5. 2002.
190. Piacibello W, Sanavio F, Severino A, Dane A, Gammaitoni L, Fagioli F, Perissinotto E, Cavalloni G, Kollet O, Lapidot T, Aglietta M. Engraftment in nonobese diabetic severe combined immunodeficient mice of human CD34(+) cord blood cells after ex vivo expansion: evidence for the amplification and self-renewal of repopulating stem cells. Blood 93 (11): 3736-49. 1999.
191. Ploemacher RE, van der Sluijs JP, Voerman JS, Brons NH. An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. Blood 74 (8): 2755-63. 1989.
192. Podda S, Ward M, Himelstein A, Richardson C, de la Flor-Weiss E, Smith L, Gottesman M, Pastan I, Bank A. Transfer and expression of the human multiple drug resistance gene into live mice. Proc Natl Acad Sci U S A 89 (20): 9676-80. 1992.
193. Poeschla EM, Wong-Staal F, Looney DJ. Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. Nat Med 4 (3): 354-7. 1998.
194. Ponder KP. An Introduction to Molecular Medicine and Gene Therapy. Wiley-Liss : 77-91. 2001.
195. Prosper F, Verfaillie CM. Regulation of hematopoiesis through adhesion receptors. J Leukoc Biol 69 (3): 307-16. 2001.
196. Punzel M, Wissink SD, Miller JS, Moore KA, Lemischka IR, Verfaillie CM. The myeloid-lymphoid initiating cell (ML-IC) assay assesses the fate of multipotent human progenitors in vitro. Blood 93 (11): 3750-6. 1999.
197. Qin G, Takenaka T, Telsch K, Kelley L, Howard T, Levade T, Deans R, Howard BH, Malech HL, Brady RO, Medin JA. Preselective gene therapy for Fabry disease. Proc Natl Acad Sci U S A 98 (6): 3428-33. 2001.
198. Ramezani A, Hawley TS, Hawley RG. Lentiviral vectors for enhanced gene expression in human hematopoietic cells. Mol Ther 2 (5): 458-69. 2000.
199. Reiser J, Harmison G, Kluepfel-Stahl S, Brady R, Karlsson S, Schubert M. Transduction of nondividing cells using pseudotyped defecctive high-titer HIV type 1 particles. Proc Natl Acad Sci USA 93 (26): 15266-15271. 1996.
200. Reiser J, Lai Z, Zhang XY, Brady RO. Development of multigene and regulated lentivirus vectors. J Virol 74 (22): 10589-99. 2000.
201. Relander T, Karlsson S, Richter J. Oncoretroviral gene transfer to NOD/SCID repopulating cells using three different viral envelopes. J Gene Med 4 (2): 122-32. 2002.

202. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18 (4): 399-404. 2000.
203. Richard RE, Wood B, Zeng H, Jin L, Papayannopoulou T, Blau CA. Expansion of genetically modified primary human hemopoietic cells using chemical inducers of dimerization. Blood 95 (2): 430-6. 2000.
204. Rideout WM, 3rd, Hochedlinger K, Kyba M, Daley GQ, Jaenisch R. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell 109 (1): 17-27. 2002.
205. Rio P, Segovia JC, Hanenberg H, Casado JA, Martinez J, Gottsche K, Cheng NC, Van De Vrugt HJ, Arwert F, Joenje H, Bueren JA. In vitro phenotypic correction of hematopoietic progenitors from Fanconi anemia group A knockout mice. Blood 100 (6): 2032-2039. 2002.
206. Rivella S, Sadelain M. Genetic treatment of severe hemoglobinopathies: the combat against transgene variegation and transgene silencing. Semin Hematol 35 (2): 112-25. 1998.
207. Roberts PC, Kipperman T, Compans RW. Vesicular stomatitis virus G protein acquires pH-independent fusion activity during transport in a polarized endometrial cell line. J Virol 73 (12): 10447-57. 1999.
208. Roe T, Reynolds TC, Yu G, Brown PO. Integration of murine leukemia virus DNA depends on mitosis. Embo J 12 (5): 2099-108. 1993.
209. Rohwedel J, Maltsev V, Bober E, Arnold HH, Hescheler J, Wobus AM. Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. Dev Biol 164 (1): 87-101. 1994.
210. Rosu-Myles M, Gallacher L, Murdoch B, Hess DA, Keeney M, Kelvin D, Dale L, Ferguson SS, Wu D, Fellows F, Bhatia M. The human hematopoietic stem cell compartment is heterogeneous for CXCR4 expression. Proc Natl Acad Sci U S A 97 (26): 14626-31. 2000.
211. Salmon P, Kindler V, Ducrey O, Chapuis B, Zubler RH, Trono D. High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors. Blood 96 (10): 3392-8. 2000.
212. Sauvageau G, Thorsteinsdottir U, Eaves CJ, Lawrence HJ, Largman C, Lansdorp PM, Humphries RK. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. Genes Dev 9 (14): 1753-65. 1995.
213. Schambach A, Wodrich H, Hildinger M, Bohne J, Krausslich HG, Baum C. Context dependence of different modules for posttranscriptional enhancement of gene expression from retroviral vectors. Mol Ther 2 (5): 435-45. 2000.
214. Scherr M, Battmer K, Blomer U, Schiedlmeier B, Ganser A, Grez M, Eder M. Lentiviral gene transfer into peripheral blood-derived CD34+ NOD/SCID-repopulating cells. Blood 99 (2): 709-12. 2002.
215. Schilz AJ, Schiedlmeier B, Kuhlcke K, Fruehauf S, Lindemann C, Zeller WJ, Grez M, Fauser AA, Baum C, Eckert HG. MDR1 gene expression in NOD/SCID repopulating cells after retroviral gene transfer under clinically relevant conditions. Mol Ther 2 (6): 609-18. 2000.
216. Schmidt M, Hoffmann G, Wissler M, Lemke N, Mussig A, Glimm H, Williams DA, Ragg S, Hesemann CU, von Kalle C. Detection and direct genomic sequencing of multiple rare unknown flanking DNA in highly complex samples. Hum Gene Ther 12 (7): 743-9. 2001.

217. Schmidt M, Zickler P, Hoffmann G, Haas S, Wissler M, Mussig A, Tisdale JF, Andrews RG, Wu T, Kiem H-P, Dunbar CE, von Kalle C. Polyclonal long-term repopulating stem cell clones in a primate model. Blood (in press) 2002.
218. Schnell T, Foley P, Wirth M, Munch J, Uberla K. Development of a self-inactivating, minimal lentivirus vector based on simian immunodeficiency virus. Hum Gene Ther 11 (3): 439-47. 2000.
219. Schroder A, Shinn P, Chen H, Berry C, Ecker J, Bushman F. HIV-1 Integration in the Human Genome Favors Active Genes and Local Hotspots. Cell 110 (4): 521. 2002.
220. Schuler W, Weiler IJ, Schuler A, Phillips RA, Rosenberg N, Mak TW, Kearney JF, Perry RP, Bosma MJ. Rearrangement of antigen receptor genes is defective in mice with severe combined immune deficiency. Cell 46 (7): 963-72. 1986.
221. Sellers SE, Tisdale JF, Agricola BA, Metzger ME, Donahue RE, Dunbar CE, Sorrentino BP. The effect of multidrug-resistance 1 gene versus neo transduction on ex vivo and in vivo expansion of rhesus macaque hematopoietic repopulating cells. Blood 97 (6): 1888-91. 2001.
222. Shimizu Y, Ogawa M, Kobayashi M, Almeida-Porada G, Zanjani ED. Engraftment of cultured human hematopoietic cells in sheep. Blood 91 (10): 3688-92. 1998.
223. Shinkai Y, Rathbun G, Lam KP, Oltz EM, Stewart V, Mendelsohn M, Charron J, Datta M, Young F, Stall AM, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell 68 (5): 855-67. 1992.
224. Shultz LD, Schweitzer PA, Christianson SW, Gott B, Schweitzer IB, Tennent B, McKenna S, Mobraaten L, Rajan TV, Greiner DL, et al. Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. J Immunol 154 (1): 180-91. 1995.
225. Sirven A, Pflumio F, Zennou V, Titeux M, Vainchenker W, Coulombel L, Dubart-Kupperschmitt A, Charneau P. The human immunodeficiency virus type-1 central DNA flap is a crucial determinant for lentiviral vector nuclear import and gene transduction of human hematopoietic stem cells. Blood 96 (13): 4103-10. 2000.
226. Sirven A, Ravet E, Charneau P, Zennou V, Coulombel L, Guetard D, Pflumio F, Dubart-Kupperschmitt A. Enhanced transgene expression in cord blood CD34(+)-derived hematopoietic cells, including developing T cells and NOD/SCID mouse repopulating cells, following transduction with modified trip lentiviral vectors. Mol Ther 3 (4): 438-48. 2001.
227. Speck NA, Baltimore D. Six distinct nuclear factors interact with the 75-base-pair repeat of the Moloney murine leukemia virus enhancer. Mol Cell Biol 7 (3): 1101-10. 1987.
228. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76 (2): 301-14. 1994.
229. Srour EF, Jetmore A, Wolber FM, Plett PA, Abonour R, Yoder MC, Orschell-Traycoff CM. Homing, cell cycle kinetics and fate of transplanted hematopoietic stem cells. Leukemia 15 (11): 1681-4. 2001.
230. Steffen D. Proviruses are adjacent to c-myc in some murine leukemia virus-induced lymphomas. Proc Natl Acad Sci U S A 81 (7): 2097-101. 1984.
231. Stein BS, Gowda SD, Lifson JD, Penhallow RC, Bensch KG, Engleman EG. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell 49 (5): 659-68. 1987.
232. Stevenson M, Stanwick TL, Dempsey MP, Lamonica CA. HIV-1 replication is controlled at the level of T cell activation and proviral integration. Embo J 9 (5): 1551-60. 1990.

233. Stewart CL, Stuhlmann H, Jahner D, Jaenisch R. De novo methylation, expression, and infectivity of retroviral genomes introduced into embryonal carcinoma cells. Proc Natl Acad Sci U S A 79 (13): 4098-102. 1982.
234. Stocking C, Bergholz U, Friel J, Klingler K, Wagener T, Starke C, Kitamura T, Miyajima A, Ostertag W. Distinct classes of factor-independent mutants can be isolated after retroviral mutagenesis of a human myeloid stem cell line. Growth Factors 8 (3): 197-209. 1993.
235. Suemori H, Tada T, Torii R, Hosoi Y, Kobayashi K, Imahie H, Kondo Y, Iritani A, Nakatsuji N. Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. Dev Dyn 222 (2): 273-9. 2001.
236. Sutherland HJ, Lansdorp PM, Henkelman DH, Eaves AC, Eaves CJ. Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. Proc Natl Acad Sci U S A 87 (9): 3584-8. 1990.
237. Sutton RE, Reitsma MJ, Uchida N, Brown PO. Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. J Virol 73 (5): 3649-60. 1999.
238. Szilvassy SJ, Humphries RK, Lansdorp PM, Eaves AC, Eaves CJ. Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. Proc Natl Acad Sci U S A 87 (22): 8736-40. 1990.
239. Tahara-Hanaoka S, Sudo K, Ema H, Miyoshi H, Nakauchi H. Lentiviral vector-mediated transduction of murine CD34(-) hematopoietic stem cells. Exp Hematol 30 (1): 11-7. 2002.
240. Tajima F, Deguchi T, Laver JH, Zeng H, Ogawa M. Reciprocal expression of CD38 and CD34 by adult murine hematopoietic stem cells. Blood 97 (9): 2618-24. 2001.
241. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science 282 (5391): 1145-7. 1998.
242. Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, Hearn JP. Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A 92 (17): 7844-8. 1995.
243. Thorsteinsdottir U, Sauvageau G, Humphries RK. Enhanced in vivo regenerative potential of HOXB4-transduced hematopoietic stem cells with regulation of their pool size. Blood 94 (8): 2605-12. 1999.
244. Trono D. HIV accessory proteins: leading roles for the supporting cast. Cell 82 (2): 189-92. 1995.
245. Trono D. Lentiviral vectors: turning a deadly foe into a therapeutic agent. Gene Ther 7 (1): 20-3. 2000.
246. Trudel M, Saadane N, Garel MC, Bardakdjian-Michau J, Blouquit Y, Guerquin-Kern JL, Rouyer-Fessard P, Vidaud D, Pachnis A, Romeo PH, et al. Towards a transgenic mouse model of sickle cell disease: hemoglobin SAD. Embo J 10 (11): 3157-65. 1991.
247. Uchida N, He D, Friera AM, Reitsma M, Sasaki D, Chen B, Tsukamoto A. The unexpected G0/G1 cell cycle status of mobilized hematopoietic stem cells from peripheral blood. Blood 89 (2): 465-72. 1997.
248. Uchida N, Sutton RE, Friera AM, He D, Reitsma MJ, Chang WC, Veres G, Scollay R, Weissman IL. HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. Proc Natl Acad Sci U S A 95 (20): 11939-44. 1998.
249. Uetsuki T, Naito A, Nagata S, Kaziro Y. Isolation and characterization of the human chromosomal gene for polypeptide chain elongation factor-1 alpha. J Biol Chem 264 (10): 5791-8. 1989.

250. Vallon-Christersson J, Cayanan C, Haraldsson K, Loman N, Bergthorsson JT, Brondum-Nielsen K, Gerdes AM, Moller P, Kristoffersson U, Olsson H, Borg A, Monteiro AN. Functional analysis of BRCA1 C-terminal missense mutations identified in breast and ovarian cancer families. Hum Mol Genet 10 (4): 353-60. 2001.
251. van Hennik PB, Verstegen MM, Bierhuizen MF, Limon A, Wognum AW, Cancelas JA, Barquinero J, Ploemacher RE, Wagemaker G. Highly efficient transduction of the green fluorescent protein gene in human umbilical cord blood stem cells capable of cobblestone formation in long-term cultures and multilineage engraftment of immunodeficient mice. Blood 92 (11): 4013-22. 1998.
252. VandenDriessche T, Thorrez L, Naldini L, Follenzi A, Moons L, Berneman Z, Collen D, Chuah MK. Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells in vivo. Blood 100 (3): 813-22. 2002.
253. Varnum-Finney B, Xu L, Brashem-Stein C, Nourigat C, Flowers D, Bakkour S, Pear WS, Bernstein ID. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat Med 6 (11): 1278-81. 2000.
254. Vijaya S, Steffen DL, Kozak C, Robinson HL. Dsi-1, a region with frequent proviral insertions in Moloney murine leukemia virus-induced rat thymomas. J Virol 61 (4): 1164-70. 1987.
255. Vijaya S, Steffen DL, Robinson HL. Acceptor sites for retroviral integrations map near DNase I- hypersensitive sites in chromatin. J Virol 60 (2): 683-92. 1986.
256. von Kalle C, Kiem HP, Goehle S, Darovsky B, Heimfeld S, Torok-Storb B, Storb R, Schuening FG. Increased gene transfer into human hematopoietic progenitor cells by extended in vitro exposure to a pseudotyped retroviral vector. Blood 84 (9): 2890-7. 1994.
257. von Schwedler U, Kornbluth RS, Trono D. The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. Proc Natl Acad Sci U S A 91 (15): 6992-6. 1994.
258. Wagemaker G, Vriesendorp HM, van Bekkum DW. Successful bone marrow transplantation across major histocompatibility barriers in rhesus monkeys. Transplant Proc 13 (1 Pt 2): 875-80. 1981.
259. Wakabayashi-Ito N, Nagata S. Characterization of the regulatory elements in the promoter of the human elongation factor-1 alpha gene. J Biol Chem 269 (47): 29831-7. 1994.
260. Walsh CE, Mann MM, Emmons RV, Wang S, Liu JM. Transduction of CD34-enriched human peripheral and umbilical cord blood progenitors using a retroviral vector with the Fanconi anemia group C gene. J Investig Med 43 (4): 379-85. 1995.
261. Wang J, Doedens M, Dick J. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood 89 (11): 3919-3924. 1997.
262. Wang X, Appukuttan B, Ott S, Patel R, Irvine J, Song J, Park JH, Smith R, Stout JT. Efficient and sustained transgene expression in human corneal cells mediated by a lentiviral vector. Gene Ther 7 (3): 196-200. 2000.
263. Weissman IL, Anderson DJ, Gage F. Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. Annu Rev Cell Dev Biol 17: 387-403. 2001.
264. Wichterle H, Lieberam I, Porter J, Jessell T. Directed differentiation of embryonic stem cells into motor neurons. Cell 110 (3): 385. 2002.
265. Wiles MV. Embryonic stem cell differentiation in vitro. Methods Enzymol 225: 900-918. 1995.

266. Wiles MV, Keller G. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. Development 111 (2): 259-67. 1991.
267. Williams DA. Ex vivo expansion of hematopoietic stem and progenitor cells--robbing Peter to pay Paul? Blood 81 (12): 3169-72. 1993.
268. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature 385 (6619): 810-3. 1997.
269. Wilpshaar J, Bhatia M, Kanhai HH, Breese R, Heilman DK, Johnson CS, Falkenburg JH, Srour EF. Engraftment potential of human fetal hematopoietic cells in NOD/SCID mice is not restricted to mitotically quiescent cells. Blood 100 (1): 120-7. 2002.
270. Wilpshaar J, Falkenburg JH, Tong X, Noort WA, Breese R, Heilman D, Kanhai H, Orschell-Traycoff CM, Srour EF. Similar repopulating capacity of mitotically active and resting umbilical cord blood CD34(+) cells in NOD/SCID mice. Blood 96 (6): 2100-7. 2000.
271. Wong PM, Chung SW, Chui DH, Eaves CJ. Properties of the earliest clonogenic hemopoietic precursors to appear in the developing murine yolk sac. Proc Natl Acad Sci U S A 83 (11): 3851-4. 1986.
272. Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL. Physiological migration of hematopoietic stem and progenitor cells. Science 294 (5548): 1933-6. 2001.
273. Xu K, Ma H, McCown TJ, Verma IM, Kafri T. Generation of a Stable Cell Line Producing High-Titer Self-Inactivating Lentiviral Vectors. Mol Ther 3 (1): 97-104. 2001.
274. Yam PY, Li S, Wu J, Hu J, Zaia JA, Yee JK. Design of HIV Vectors for Efficient Gene Delivery into Human Hematopoietic Cells. Mol Ther 5 (4): 479-84. 2002.
275. Yamada K, Olsen JC, Patel M, Rao KW, Walsh CE. Functional correction of fanconi anemia group C hematopoietic cells by the use of a novel lentiviral vector. Mol Ther 3 (4): 485-90. 2001.
276. Yamaguchi M, Ikebuchi K, Hirayama F, Sato N, Mogi Y, Ohkawara J, Yoshikawa Y, Sawada K, Koike T, Sekiguchi S. Different adhesive characteristics and VLA-4 expression of CD34(+) progenitors in G0/G1 versus S+G2/M phases of the cell cycle. Blood 92 (3): 842-8. 1998.
277. Yamashita T, Nakahata T. Current knowledge on the pathophysiology of Fanconi anemia: from genes to phenotypes. Int J Hematol 74 (1): 33-41. 2001.
278. Ye ZQ, Qiu P, Burkholder JK, Turner J, Culp J, Roberts T, Shahidi NT, Yang NS. Cytokine transgene expression and promoter usage in primary CD34+ cells using particle-mediated gene delivery. Hum Gene Ther 9 (15): 2197-205. 1998.
279. Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13 (8): 335-40. 1997.
280. Yoder MC. Introduction: spatial origin of murine hematopoietic stem cells. Blood 98 (1): 3-5. 2001.
281. Yonemura Y, Ku H, Hirayama F, Souza LM, Ogawa M. Interleukin 3 or interleukin 1 abrogates the reconstituting ability of hematopoietic stem cells. Proc Natl Acad Sci U S A 93 (9): 4040-4. 1996.
282. Yoshida K, Chambers I, Nichols J, Smith A, Saito M, Yasukawa K, Shoyab M, Taga T, Kishimoto T. Maintenance of the pluripotential phenotype of embryonic stem cells through direct activation of gp130 signalling pathways. Mech Dev 45 (2): 163-71. 1994.
283. Young JC, Lin K, Travis M, Hansteen G, Abitorabi A, Sirenko O, Murray L, Hill B. Investigation into an engraftment defect induced by culturing primitive hematopoietic cells with cytokines. Cytotherapy 3 (4): 307-20. 2001.
284. Zanjani ED, Flake AW, Almeida-Porada G, Tran N, Papayannopoulou T. Homing of human cells in the fetal sheep model: modulation by antibodies activating

or inhibiting very late activation antigen-4- dependent function. Blood 94 (7): 2515-22. 1999.
285. Zanjani ED, Pallavicini MG, Harrison MR, Tavassoli M. Successful stable xenograft of human fetal hemopoietic cells in preimmune fetal sheep. Trans Assoc Am Physicians 104: 181-6. 1991.
286. Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L, Charneau P. HIV-1 genome nuclear import is mediated by a central DNA flap. Cell 101 (2): 173-85. 2000.
287. Zhu Y, Feuer G, Day SL, Wrzesinski S, Planelles V. Multigene lentiviral vectors based on differential splicing and translational control. Mol Ther 4 (4): 375-82. 2001.
288. Zufferey R, Donello JE, Trono D, Hope TJ. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J Virol 73 (4): 2886-92. 1999.
289. Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol 72 (12): 9873-9880. 1998.
290. Zufferey R, Nagy D, Mandel R, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol 15 (9): 871-875. 1997.