Retroviral gene transfer to repopulating hematopoietic cells

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Less is more…

Contents

Abbreviations

293GPG	Packaging cell line producing VSV-G pseudotyped vectors
3T3	Mouse fibroblast cell line
5-FU	5-fluorouracil (cytostatic drug)
AAV	Adeno-associated virus
ADA-SCID	Severe combined immune deficiency due to lack of adenosine deaminase
BM	Bone marrow
BMP	Bone morphogenetic protein
BMT	Bone marrow transplantation
CB	Human umbilical cord blood
CD	Cluster of differentiation; system defining hematopoietic cells based on their function and expression of surface markers
CFU	Colony forming unit
CFU-GM	CFU-granulocyte/macrophage
CGD	Chronic granulomatous disease
c-kit	The receptor for kit ligand (SCF)
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CXCR4	Receptor for the chemokine SDF-1
DNA	Deoxyribonucleic acid
EGFP	Enhanced Green fluorescent protein
ELTC-IC	Extended long-term culture-initiating cell
Env	Retroviral envelope protein
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf (bovine) serum
FeLV	Feline leukemia virus
FL	Fetal liver tyrosine kinase 3 ligand (cytokine); Flt-3 ligand
FV	Foamy virus
G418	Aminoglycoside antibiotic, toxic to cells not expressing neo gene
GALV	Gibbon ape leukemia virus
G-CSF	Granulocyte colony stimulating factor (cytokine)
GFP	Green fluorescent protein
GLVR1	GALV receptor
GP+envAM12	Packaging cell line producing vectors with amphotropic envelope
HFV	Human foamy virus
HIV-1	Human immunodeficiency virus 1
HOXB4	Homeobox B4 gene. Transcription factor involved in primitive hematpoiesis
HSC	Hematopoietic stem cell
HT1080	Human fibrosarcoma cell line
IL	Interleukin (3, 6…)
IRES	Internal ribosomal entry site
K562	Hematopoietic cell line (chronic myelogenous leukemia)
Lin	Hematopoietic lineage marker
LMO2	Master gene involved in primitive hematopoiesis and playing a role in T cell leukemia
LTBMC	Long-term bone marrow culture
LTC-IC	Long-term culture-initiating cell
LTR	Long terminal repeat. Promoter sequences in retroviruses
MDR	Multiple drug resistance
MGDF	Megakaryocyte growth and development factor, equivalent to TPO
MLVAR	Murine leukemia virus amphotropic receptor (=Pit-2)
MOI	Multiplicity of infection; the ratio between the number of vector particles and the number of target cells at transduction
MPB	Mobilized peripheral blood
MSCV	Murine stem cell virus

Neo	Neomycin phosphotransferase; neomycin resistance gene
NK cell	Natural killer cell
NOD/SCID	Non-obese diabetic/severe combined immune deficiency (mouse strain)
PCR	Polymerase chain reactin
PG13	Packaging cell line producing GALV pseudotyped vectors
Pit-1	GALV receptor (=GLVR1)
Pit-2	Amphotropic receptor; MLVAR
PKC	Protein kinase C
PL	Preload (of vector onto the surface of a cell culture well)
PMA	Phorbol 12-myristate 13-acetate (phorbol ester)
Q-RT-PCR	Quantitative reverse transcriptase PCR (real time PCR)
RCR	Replication competent retrovirus/recombinants
RD114	Feline endogenous virus envelope
RDR	RD114 receptor. Receptor for the feline endogenous virus
RNA	Ribonucleic acid
Sca-1	Stem cell antigen
SCF	Stem cell factor (cytokine). Same as Kit ligand
SDF-1	Stromal derived factor 1; chemokine
SIN	Self-inactivating vector. LTR driven expression is blocked.
SP	Side population
SRC	NOD/SCID repopulating (human) cell
TGF-β	Transforming growth factor β
TPO	Thrombopoietin, bioequivalent to MGDF
VCM	Vector containing medium
VEGFR2	Vascular endothelial growth factor receptor 2
VSV-G	Vesicular stomatitis virus G-protein
X-SCID	X-chromosome linked severe combined immune deficiency lacking γc-chain component of multiple cytokine receptors
YFP	Yellow fluorescent protein

List of articles

The present thesis is based on the following articles, which will be referred to in the text by their Roman numerals:

I. *Relander T., Fahlman C., Karlsson S. and Richter J*: Low level of gene transfer to and engraftment of murine bone marrow cells from long-term bone marrow cultures. **Experimental Hematology** 28; 373-381, 2000.

II. *Relander T, Brun A, Hawley RG, Karlsson S and Richter J*: Retroviral transduction of human $CD34^+$ cells on fibronectin fragment CH-296 is inhibited by high concentrations of vector containing medium. **The Journal of Gene Medicine**, 3: 207-218, 2001.

III. *Relander T, Brun ACM, Olsson K, Pedersen L and Richter J*: Overexpression of Gibbon Ape Leukemia virus (GALV) receptor (GLVR1) on human $CD34^+$ cells increases gene transfer mediated by GALV pseudotyped vectors. **Molecular Therapy**, 6:3; 400-406, 2002.

IV. *Relander T, Karlsson S and Richter J*: Oncoretroviral gene transfer to NOD/SCID repopulating cells using three different viral envelopes. **The Journal of Gene Medicine**, 4: 1-11, 2002.

Introduction

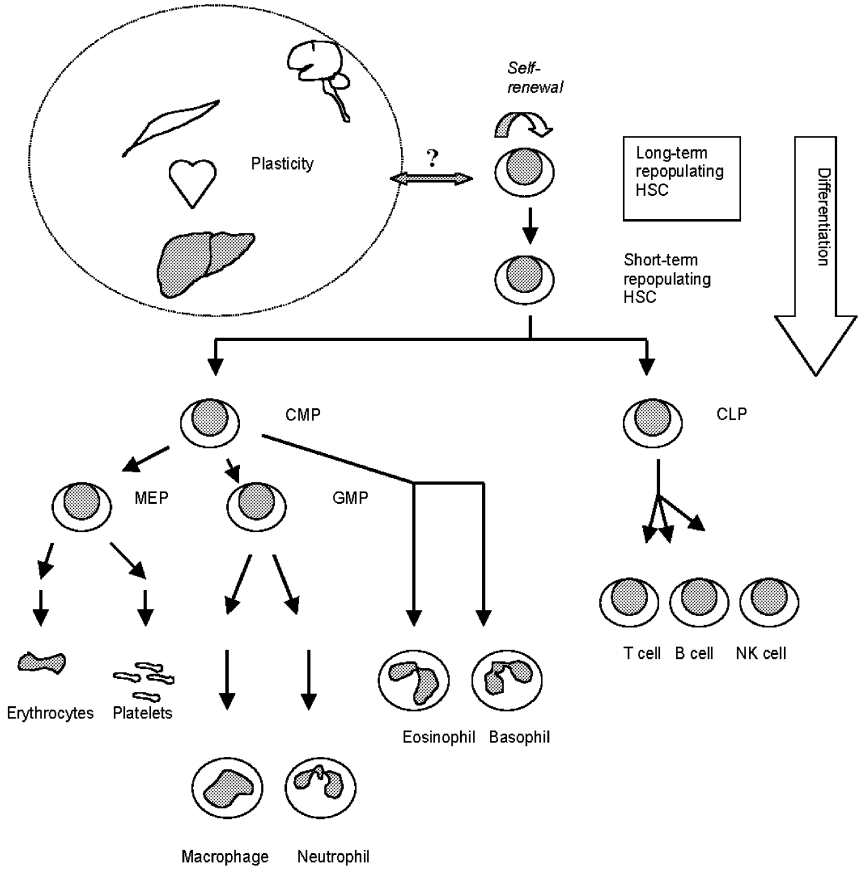
Permanent gene transfer to hematopoietic stem cells (HSCs) has the potential to provide cure for a number of monogenic diseases affecting the hematopoietic system (Karlsson, 1991). Hematopoietic stem cells have the capacity to differentiate into all types of mature blood cells and sustain hematopoiesis throughout life. Murine oncoretroviral based vectors can be used to transfer genes into hematopoietic cells from patients, and after manipulations *in vitro* these cells can be reinfused into the host. In mice, it has been possible to reproducibly transfer genes to repopulating cells of the hematopoietic system since the late 1980´s (Szilvassy, et al., 1989), but translation of these methods for use in humans and large animals has been far less successful (Halene and Kohn, 2000, Richter and Karlsson, 2001) with a few notable exceptions: the clinical trials involving children with severe combined immune deficiencies (SCID) (Aiuti, et al., 2002a, Cavazzana-Calvo, et al., 2000). Therefore, efforts are still needed to improve methods to efficiently transfer genes into repopulating human hematopoietic cells. This forms the background of the work presented in this thesis.

Hematopoiesis

Hematopoiesis is the continuously ongoing process of blood formation. Mature blood cells have a number of functions essential for life: the red blood cells serve as transporters of oxygen to the tissues, the various white blood cells (granulocytes, monocytes, T and B lymphocytes) form the defense against infections, and platelets are involved in the early formation of a blood clot to prevent bleeding. The mature blood cells have a limited life span ranging from hours (granulocytes) to months or years (lymphocytes). Every day, approximately 10^{12} new cells are formed under steady-state conditions (Ogawa, 1993). All these cells originate from a pool of hematopoietic stem cells (HSC), which are located in the bone marrow in adult humans.

The process of hematopoiesis is usually described in a hierarchical fashion with long-term HSCs, which give rise to multipotent primitive progenitors, at the top (***Figure 1***). In mice, these cells can maturate into well-defined lineage restricted progenitors lacking self-renewal capacity, the common lymphoid progenitor (CLP) (Kondo, et al., 1997) and the common myeloid progenitor (CMP) (Akashi, et al., 2000), and then further differentiate to more committed progenitors and finally to mature blood cells (Weissman, 2000). To produce

Figure 1. Hematopoietic stem cells and the hematopoietic hierarchy.



Stem cell self renewal or differentiation to various hematopoietic lineages is shown, as well as the more controversial issues about transdifferentiation into neuronal, liver, myocardial or muscle cells. CMP=common myeloid progentior; CLP=common lymphoid progenitor; MEP=megakaryocyte-erythrocyte progenitor; GMP=granulocyte-macrophage progenitor.

sufficient numbers of mature hematopoietic cells, but not more than needed, hematopoiesis has to be strictly regulated. This includes a balance between lineage commitment and self-renewal of stem cells as well as between proliferation and maturation of lineage restricted progenitors and apoptosis of mature cells.

Extrinsic regulators of hematopoiesis include hematopoietic growth factors such as cytokines and cell-cell and cell-stroma interactions. It is not entirely clear how lineage commitment in primitive hematopoietic cells is regulated (for recent review, see (Zhu and Emerson, 2002)). The exact role of cytokines in the process of lineage commitment has been under some

debate. According to the "stochastic" hypothesis, lineage commitment is a random event followed by differentiation and proliferation supported by cytokines. Signaling through cytokines and their receptors would thus be important for sustaining cell viability and proliferation, but to have a permissive rather than instructive function for differentiation (Socolovsky, et al., 1998, Stoffel, et al., 1999). Others support the hypothesis that cytokines play direct instructive roles in cell fate decisions (Metcalf, 1991), or a combination of both hypotheses. Moreover, intrinsic regulators of hematopoiesis are important in decisions regarding cell fate. During hematopoiesis, nuclear transcription factors play important roles in controlling gene expression (for review, see (Orkin, 2000)). Typically, these factors act in combinations, with expression patterns specific for different lineages, and single factors are not sufficient for determining cell fate. In addition to promoting a certain lineage, these factors also act to suppress other lineages simultaneously. Many transcription factors have been discovered as genes involved in chromosomal translocations in leukemia.

Stem cells

Stem cells are defined as rare clonogenic cells with a capability of self-renewal and differentiation into mature cells of various types, a feature termed pluripotentiality (Weissman, et al., 2001). The mechanisms controlling stem cell fate decisions such as self-renewal, differentiation/expansion or apoptosis are largely unknown. Embryonic stem cells (ES cells) are truly pluripotential and have the capacity to differentiate to any type of tissue in the body, but ES cells are not discussed further here. Most knowledge about adult stem cells is retrieved from studies of the hematopoietic system, where the existence of hematopoietic stem cells relies on firm experimental and clinical evidence from transplantation studies. Individual HSCs can differentiate and give rise to all cells of the blood system (***Figure 1***), i.e. they are pluripotent and, furthermore, have an enormous proliferative potential. Upon transplantation, single murine HSCs have the capacity to repopulate the entire blood system (provide multilineage reconstitution) of lethally irradiated recipient mice (Osawa, et al., 1996, Smith, et al., 1991). HSCs have the ability to self-renew, which means that they following cell division give rise to at least one cell with the same properties and phenotype as the original cell (Domen and Weissman, 1999, Spangrude, et al., 1991). In the adult human, hematopoietic stem cells reside mainly in the bone marrow and are very rare with a frequency of 1 in 10^4-10^5 bone marrow mononuclear cells (Bonnet, 2002).

HSCs for clinical transplantation can be harvested from the bone marrow or mobilized to the peripheral blood using cytokines alone (Duhrsen, et al., 1988), or cytokines in combination with cytotoxic chemotherapy. Because of the faster hematological recovery following autotransplantation with MPB compared with BM cells, MPB has become the most commonly used source for autologous and allogeneic transplantation (To, et al., 1992). Alternatively, HSCs from human umbilical cord blood (CB) can be used (Gluckman, et al., 1989), although low cell numbers in the grafts is a limitation, especially for transplantation to adult patients (for recent review, see (Sanz and Sanz, 2002)).

More recently, the stem cell field has come to general attention and debate following exciting reports on stem cell plasticity. In contrast to what has been believed impossible, reports have claimed transdifferentiation of adult stem cells from one germ layer to another, for example mesodermally derived HSC to endodermally derived mature liver cells (Lagasse, et al., 2000). Other examples of such developmental plasticity include reports according to which genetically labeled neural progenitors could generate hematopoiesis (Bjornson, et al., 1999), and conversely, it has been claimed that hematopoietic stem cells can give rise to neuronal cells (Mezey, et al., 2000). Moreover, bone marrow cells injected into the site of myocardial damage were described to reconstitute the damaged area and acquire muscle cell markers (Orlic, et al., 2001a). Mobilization of hematopoietic progenitors from the bone marrow of mice subjected to myocardial infarction has been associated with improved tissue regeneration and cardiac function (Orlic, et al., 2001b). Results like these have created great enthusiasm over the potentials of stem cell based regenerative medicine for common disorders in the future, possibly also involving genetic modification of stem cells. However, most of the reports claiming transdifferentiation have been questioned in some ways and explanations other than stem cell plasticity may exist (Orkin and Zon, 2002). For example, heterogeneity/impurity of the stem cell sources with stem cells committed to differentiate to a specific tissue mixed with true HSC could account for regeneration of a specific tissue, such as muscle, without involving transdifferentiation. Given the enormous proliferative capacity of true hematopoietic stem cells, a very limited reservoir of HSCs contaminating a population of tissue specific stem cells could clearly give rise to hematopoiesis in a recipient and might incorrectly be interpreted as transdifferentiation. Two important criteria for transdifferentiation experiments have been suggested: the input cells should be purified prospectively and marked to allow clonal analysis, and the function of the terminal cells

should be shown, i.e., not only presence of surface markers characteristic of a specific tissue (Orkin and Zon, 2002). In a recent report from Weissman's group, single GFP^+ murine HSC were transplanted to irradiated recipients. In animals with complete hematopoietic donor cell reconstitution, no appreciable donor cell contribution to non-hematopoietic tissues was seen, not even following radiation induced tissue damage to the bowel (Wagers, et al., 2002). This suggests that stem cell transdifferentiation, if it exists, is an unusual event under normal circumstances.

The phenotype of hematopoietic stem cells

In contrast to the situation in humans, attempts to phenotypically define HSCs in mice have been successful. The long-term repopulating cells in the bone marrow of mice have been shown to have a phenotype negative for mature lineage markers (lin^-) and positive for Sca-1 and Thy-1 (Spangrude, et al., 1988, Uchida and Weissman, 1992). The fraction of this population with expression of c-kit is further enriched for stem cell activity (Ikuta and Weissman, 1992) and transplantation of single $Lin^-c\text{-}kit^+Sca\text{-}1^+CD34^-$ cells can reconstitute hematopoiesis in irradiated mice (Osawa, et al., 1996). Despite considerable efforts to characterize the phenotype of human HSC by analyzing cell surface markers using monoclonal antibodies, it has not yet been possible to determine specific surface markers enabling investigators to prospectively isolate individual human HSCs. Therefore, characterization and enumeration of different HSC fractions relies on their activity in clonogenic or stromal-based long-term culture assays, on xenotransplant experiments or, ultimately, on human clinical trials. However, subpopulations of hematopoietic cells, which are enriched for stem cell activity, have been identified. The sialomucin CD34, which is expressed on 1-5 % of the mononuclear cells in the bone marrow (Andrews, et al., 1986, Civin, et al., 1984), has been widely used as a marker for human hematopoietic progenitors. CD34 is expressed on primitive myeloid cells including almost all human bone marrow cells capable of colony formation in vitro, but also on immature B and T lymphocytes, and is down-regulated as the cells differentiate (for review, see (Krause, et al., 1996)). Among non-hematopoietic cells, endothelial cells and embryonic fibroblasts express CD34 on their surface. The function of the CD34 antigen is not completely known. It is thought to be involved in adhesion molecule interactions and may play a role in cell homing to the bone marrow or to sites of inflammation. Selection of $CD34^+$ cells from bone marrow mononuclear cells has been utilized as a means to enrich for primitive cells for transplantation and ex vivo manipulation including gene transfer approaches. Upon transplantation of autologous $CD34^+$

bone marrow cells to lethally irradiated baboons, cells selected for CD34 expression could engraft and hematologically rescue all animals, whereas animals transplanted with $CD34^-$ cells died from marrow aplasia (Berenson, et al., 1988). Numerous clinical transplantations using selected $CD34^+$ cells have been carried out since more than a decade both in the autologous (Berenson, et al., 1991, Shpall, et al., 1994) and in the allogeneic settings (Bensinger, et al., 1995, Schmitz, et al., 1995). In autologous transplantation following high dose chemotherapy for cancer, the purification procedure has an advantage as the transplant is theoretically purged from contaminating cancer cells by the selection process because most solid tumor or myeloma cells do not express CD34. Although it has been proven that tumor cell contamination of the graft can contribute to relapse of the disease (Rill, et al., 1994), the true clinical relevance of various purging procedures have not been fully established.

Besides CD34 being a marker for primitive hematopoietic cells, the vast majority of the cells expressing the CD34 antigen are, however, progenitors and not true stem cells. Therefore, investigators have attempted to further purify $CD34^+$ cells for stem cell activity. CD38 is an early marker for lineage differentiation and can be used to subdivide $CD34^+$ cells, as $CD34^+CD38^+$ cells (99 % of the $CD34^+$cells) are committed and have reduced ability to form primitive colonies (Terstappen, et al., 1991). $CD34^+CD38^-$ cells on the contrary, can give rise to hematopoietic colonies following long-term culture on stroma (long-term culture-initiating cells, LTC-IC), are mostly not in active cell cycle and are less responsive to cytokine stimulation (Reems and Torok-Storb, 1995). Furthermore, only the $CD34^+CD38^-$ cells are able to give rise to colonies following extended long-term culture (>60 days) on stromal cells (Hao, et al., 1995). Supporting these *in vitro* data, it has been shown that the human cells capable of repopulating the bone marrow of fetal sheep (Civin, et al., 1996) and immunodeficient mice (Bhatia, et al., 1997b, Larochelle, et al., 1996) reside entirely in the very small fraction of $CD34^+$ cells with a low expression of CD38. In vitro, it has been possible to generate both myeloid and lymphoid differentiation from $CD34^+CD38^-$ cells also at the single cell level, proving pluripotentiality within this cell fraction (Hao, et al., 1998). Another marker associated with stem cell phenotype is c-kit, the receptor for stem cell factor (SCF). The stem cell activity in $CD34^+$ cells from human BM resides in the fraction of these cells with low expression of c-kit, as shown in the fetal sheep xenograft model (Kawashima, et al., 1996). Finally, differential expression of HLA-DR can also be used for purification of human HSCs($CD34^+HLA\text{-}DR^-$) (Lu, et al., 1987). However, despite extensive preclinical and

clinical experience from using $CD34^+$ as a surrogate marker for human stem cells in vitro and in vivo, concern has been raised whether selection for $CD34^+$ cells would deplete the transplant from important stem cells lacking CD34 expression. In mice, unlike the situation in humans, Osawa and colleagues found long-term hematopoietic reconstitution in irradiated mice following transplantation of single cells with the Lin^- c-Kit^+ Sca-1^+ $CD34^-$ phenotype only, which occurred in 20 % of the mice, whereas $CD34^+$ cells could only give rise to short-term hematopoiesis (Osawa, et al., 1996). In a very clear-cut and convincing paper from Ogawa´s group, it was shown that the majority of the stem cells in steady state mouse BM were $CD34^-$, but acquired CD34 expression upon activation and could, following transplantation, revert to the $CD34^-$ phenotype at steady state (Sato, et al., 1999). However, others have found a superior or similar long-term repopulating ability among the $CD34^+$ compared to the $CD34^-$ cells (Donnelly, et al., 1999, Morel, et al., 1998). Also in humans, there is considerable evidence for hematopoietic activity also in the $CD34^-$ fraction of hematopoietic cells. Bhatia and co-workers were the first to show engraftment of human CB $CD34^-CD38^-Lin^-$ cells in the bone marrow of NOD/SCID mice (Bhatia, et al., 1998). Furthermore, they demonstrated that the $CD34^-$ cells differed from their $CD34^+$ counterparts in their very limited potential to form colonies and their low frequency of long term culture-initiating cells (LTC-IC), despite possessing NOD/SCID repopulating capacity. They could also show induction of $CD34^+$ cells from $CD34^-$ cells *in vitro* and *in vivo* suggesting that the $CD34^-$ cells were developmentally at an earlier stage in the hematopoietic hierarchy. This hypothesis was further supported by Nakamura *et al.*, who demonstrated acquisition of CD34 expression in $CD34^-$ cells during ex vivo culture, which was paralleled by a gain of colony forming and NOD/SCID repopulating capability (Nakamura, et al., 1999). Finally, Zanjani and colleagues showed that human BM $CD34^-Lin^-$ cells (as well as $CD34^+$ cells) could engraft the bone marrow of preimmune fetal sheep and give rise to all lympho-hematopoietic lineages and also to $CD34^+$ cells in the bone marrow of recipients of primary and secondary transplants (Zanjani, et al., 1998). In their report, the frequency of repopulating stem cells was at least 2-3 fold lower in the $CD34^-$ compartment compared to their $CD34^+$ counterparts, as judged by transplantation experiments at limiting dilution. Experiments in the NOD/SCID xenotransplant model support the rare existence of $CD34^-$ SRC, but these were shown to have a 100-fold lower engrafting capacity than $CD34^+$ cells (Gao, et al., 2001). Similar to the report by Sato in the murine system, Dao and colleagues recently demonstrated reversible expression of CD34 in human $CD34^+CD38^-$ BM cells transplanted to *bnx* mice (Dao, et al.,

2002). $CD34^-$ cells recovered from these mice retained the capability of secondary reconstitution and could give rise to $CD34^+$ progeny. The issue of CD34 expression on human hematopoietic stem cells have been reviewed lately (Dao and Nolta, 2000, Engelhardt, et al., 2002, Goodell, 1999).

An alternative way to enrich HSCs is based on the ability of primitive cells to exclude the fluorescent DNA binding dye Hoechst 33342. This property relies on the presence of an MDR-like pump mechanism present in candidate stem cells (Zhou, et al., 2001). Using dual-wavelength FACS, Goodell *et al.* isolated a population of cells from mouse bone marrow having the capacity to exclude Hoechst 33342. They called this population "side population" (SP) cells and showed that they possessed long-term multi-lineage engraftment capability (Goodell, et al., 1996). In a subsequent report from the same group, cells with the SP phenotype were identified among cells from human and rhesus monkey bone marrow as well and were shown to be $CD34^-$ and Lin^- just as their murine counterparts (Goodell, et al., 1997). Only 0.05 % of human bone marrow cells exhibited the SP phenotype. Primarily these SP cells were unable to form hematopoietic colonies, but after long-term culture they gained this function as well as CD34 expression. The frequency of LTC-ICs was higher among SP cells than among $CD34^+CD38^-$ cells and altogether the authors concluded that SP cells might represent primitive precursors to $CD34^+$ cells.

CD133 (AC133) is an alternative, more recently described marker for human primitive hematopoietic cells. It is a transmembrane polypeptide expressed on 20-60 % of human $CD34^+$ cells and antibodies to CD133 stain committed as well as non-committed progenitors. $CD133^+$ cells were shown to be capable to engraft the bone marrow in the human/fetal sheep xenograft model (Yin, et al., 1997). This marker may be useful when aiming at obtaining higher purity of progenitor cells compared to selection based on CD34 expression only. However, considerably higher purification of HSCs is thought to be the result if the selection is based on KDR receptor expression (Ziegler, et al., 1999). This receptor, which is also called vascular endothelial growth factor receptor 2 (VEGFR 2), is expressed on less than 1 % of the $CD34^+$ hematopoietic progenitor cells and on endothelial cells as well. However, to date, other groups have not reproduced the results reported by Ziegler *et al.*

Cell cycle status, expansion and engraftment of HSCs

The issues of cell cycle status and engraftment capability are of great importance for the field of stem cell based gene therapy. Successful gene therapy targeting HSCs with retroviral vectors requires that the target cells pass through mitosis (Miller, et al., 1990). As primitive hematopoietic cells are largely non-cycling, cytokine stimulation using cytokines is needed to push the cells into cell cycle. This carries the risk that cells differentiate, thereby losing their stem cell properties, and may also negatively affect their ability to home to and engraft into the bone marrow.

Given the enormous proliferative potential of individual HSCs, it is somewhat paradoxical that these cells are mainly quiescent under normal conditions. However, it is generally believed that steady-state hematopoiesis is maintained by a limited number of active clones at any given time-point. Over time, different clones are successively recruited into cell cycle allowing the majority of the stem cells to remain dormant (clonal succession model). As retroviral vectors integrate semi-randomly into their target cell genome, insertion site analysis can be used to quantify the numbers of clones involved in active hematopoiesis at a given time-point. In non-human primate models of hematopoiesis, long-term tracking of individual retrovirally marked clones has shown that hematopoiesis in large animals is polyclonal, with different clones contributing to hematopoiesis over time (Kim, et al., 2000, Schmidt, et al., 2002)(for review, see (Shi, et al., 2002)). The quiescent nature of primitive hematopoietic progenitors has allowed for enrichment for murine stem cells by pretreating animals with cell cycle specific cytotoxic drugs such as 5-fluorouracil (5-FU), which selectively kills actively cycling cells (Hodgson and Bradley, 1979, Lerner and Harrison, 1990). A similar approach to enrich for human HSCs utilizes stimulation of bone marrow cells with SCF and IL-3 concurrently with long-term 5-FU incubation. This treatment spared approximately 1 in 10^5 cells, which were quiescent and exhibited a phenotype associated with stem cells (Berardi, et al., 1995).

There is a close relationship between the cell cycle status of the HSCs and their ability to engraft into the bone marrow (Habibian, et al., 1998). Treatment with G-CSF results in mobilization of hematopoietic progenitors from the bone marrow to the circulation, where they can be harvested for transplantation or *in vitro* manipulation purposes. In mice, G-CSF stimulation leads to increased cycling of progenitor cells in the bone marrow, but, somewhat surprisingly, a very low proportion of the hematopoietic progenitors released to the

circulation are actively cycling (Roberts and Metcalf, 1995). In humans, hematopoietic progenitors differ considerably in their cell cycle characteristics depending on which source they originate from. Thus, almost all (> 99 %) mobilized PB $CD34^+$ cells, 97 % of CB $CD34^+$ cells and approximately 85-95 % of BM $CD34^+$ cells reside in the G_0/G_1 phase of the cell cycle (Gothot, et al., 1997, Traycoff, et al., 1994, Uchida, et al., 1997). It has been suggested that the most primitive hematopoietic cells, which are slow responders to cytokine stimulation, reside in G_0 and early G_1, whereas more committed progenitors preferentially are in the G_1 phase of the cell cycle. When further subdividing the quiescent cells based on RNA content, Gothot *et al.* (Gothot, et al., 1998) could demonstrate a severalfold superior repopulating capability of human $CD34^+$ BM or MPB cells in G_0 compared to G_1 in the NOD/SCID model. Interestingly, they also found a nearly complete loss of repopulating capacity in cells moving from G_0 to G_1 following cytokine stimulation, whereas those remaining in G_0 retained their repopulating ability. However, the mechanisms underlying these findings were not explained. Did the cells passing from G_0 to G_1 lose their stem cell properties, or could the engraftment defect be explained by impaired homing to the bone marrow as a result of changes in expression of adhesion molecules on the cell surface? When comparing the cell cycle status of $CD34^+CD38^-$ cells from BM and MPB, a significantly higher proportion of MPB cells were in G_1 than were BM cells, showing a higher degree of activation in MPB cells despite a similar proportion of cells residing outside of $S/G_2/M$ (Horwitz, et al., 1999). In contrast to the results obtained with BM or MPB cells, activation of hematopoietic cells from CB or fetal liver into the G_1 phase of cell cycle did not change the ability of these cells to repopulate the bone marrow of NOD/SCID mice significantly (Wilpshaar, et al., 2002, Wilpshaar, et al., 2000). In accordance with these latter findings, Glimm and Eaves could show, that following short-term liquid culture with multiple cytokine stimulation, the majority of engrafting $CD34^+$ cells from fetal liver and CB in had passed through multiple self-renewal divisions with retained, albeit quantitatively reduced, engraftment capability (Glimm and Eaves, 1999).

Homing and engraftment

For HSCs to engraft in the bone marrow following transplantation, they must first home to the BM and thereafter proliferate and differentiate within the BM. Both processes comprise interactions between adhesion molecules in the BM microenvironment and receptors on the surface of the HSCs. The process of homing involves adhesion of transplanted cells to the surface of blood vessels in the bone marrow, followed by extravasation to the hematopoietic

compartment, where interaction with stromal elements takes place. The chemokine stromal derived factor-1 (SDF-1) and its receptor CXCR4 are known to be involved in homing and engraftment of human cells in the BM of NOD/SCID mice, and up-regulation of CXCR4 by incubation of cells with IL-6 and SCF has been suggested as a way to improve engraftment (Peled, et al., 1999)(reviewed in (Lapidot and Kollet, 2002)). SDF-1 is produced in a variety of tissues and by BM stromal cells and most likely plays a role in maintaining a quiescent state of the stem cells (Cashman, et al., 2002) and in retaining them within the BM. Interestingly, exposure of cultured CB or MPB $CD34^+$ cells to SDF-1 has been shown to increase their ability to engraft in the BM of NOD/SCID mice (Glimm, et al., 2002, Plett, et al., 2002) despite down-regulation of CXCR4. Although the mechanisms involved are unknown, this approach has the potential to be utilized to improve engraftment of gene modified cells in human gene transfer protocols. In addition to SDF-1/CXCR4, a number of adhesion molecules, such as integrins and selectins, are involved in interactions between hematopoietic cells and BM stromal cells and play a role in engraftment and maintenance of HSCs in the bone marrow.

Models for assaying hematopoietic stem cell function *in vitro* and *in vivo*

In vitro assays

Various biological assays are needed for enumeration and characterization of human HSCs, as their exact phenotype at the single cell level has not yet been defined. Furthermore, it is also important to study the functional characteristics of candidate stem cells applying various *in vitro* and *in vivo* methods following cell manipulations, such as ex vivo expansion and gene transfer experiments. No single optimal stem cell assay exists, as the available methods measure different primitive populations as a surrogate for stem cells. Till and McCulloch (Till and McCulloch, 1961) pioneered this field describing the first "stem cell" assay, CFU-S (colony forming unit in the spleen), which later has been shown to reflect the contents of primitive progenitors rather than true stem cells. They injected hematopoietic cells intravenously into lethally irradiated mice and could quantitate the progenitor content by counting the nodules, which developed in the spleens of the recipient animals. A very common *in vitro* method to quantitate clonogenic hematopoietic progenitors is to culture them in methylcellulose with cytokines and study the development of different hematopoietic colonies (CFU-C). These assays, however, also reflect short-term progenitors rather than stem cells. By culturing hemopoietic cells in liquid culture on a layer of stromal cells for 5-8

weeks before plating them into methylcellulose, a more primitive cell population can be detected, namely the long-term culture-initiating cell (LTC-IC), which is 30-fold less frequent than clonogenic cells in human BM (Sutherland, et al., 1989, Sutherland, et al., 1990). Transduction of CFU and LTC-IC using retroviral vectors was fairly efficient already early in the development of gene therapy, indicating that these cells could easily be recruited into cell cycle with cytokine stimulation (Moore, et al., 1992). A related in vitro assay for stem cells, also involving long term culture, is the cobblestone area forming cell assay (CAFC), where the read-out is production of clones on a stromal layer, instead of CFU as in the LTC-IC assays (Ploemacher, et al., 1989). By prolonging the culture period to 60-100 days in the extended LTC-IC assay (ELTC-IC), an even more primitive cell population, believed to be closely related to the long-term repopulating stem cells, can be detected (Hao, et al., 1995, Hao, et al., 1996). Retroviral transduction of ELTC-IC was shown to be inefficient as was the situation for long-term repopulating human cells, probably reflecting the higher degree of quiescence in this primitive cell population. More recently, efficient retroviral transduction of human BM and CB ELTC-IC was shown by Björgvinsdóttir *et al.* (Bjorgvinsdottir, et al., 2002). Interestingly, gene marking of ELTC-IC was generally higher than that of SRC, suggesting that the cells represent different stages in the hematopoietic hierarchy.

In vivo models

Although very useful, *in vitro* methods cannot fully replace *in vivo* assays of stem cell function. In mouse to mouse transplantation models using congenic strains, it is possible to determine donor cell contribution to hematopoiesis in a reliable way, thus allowing for studies of the repopulating ability of various cell populations and the effects of cell manipulations including gene transfer. In order to further sharpen the analysis of stem cell self-renewal, secondary transplants can be made (Woods, et al., 2000).

Xenograft models

To assay human hematopoiesis *in vivo*, a number of human-xenograft transplantation models can be used. Various immunodeficient mouse strains capable of supporting human hematopoiesis are available, such as beige-nude-xid (*bnx*) (Nolta, et al., 1994), SCID mice and the non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Prochazka, et al., 1992, Shultz, et al., 1995). The advantages of the *bnx* mice are, among

other things, that they support human T cell development and that transplanted mice can be followed for longer periods of time (almost a year) compared to NOD/SCID mice. However, upon intravenous injection of human hematopoietic cells to sublethally irradiated mice, the NOD/SCID mice yield the highest levels of engraftment of human cells in the bone marrow, which has made it the most commonly used xenograft model for assaying human candidate HSCs. The NOD/SCID mice lack functional lymphoid cells and have very limited natural killer (NK) cell activity (Shultz, et al., 1995), which makes them unable to reject human cell transplants. The NOD/SCID mouse model has proven to be of great importance as a model for human in vivo hematopoiesis for the fields of transplantation biology and gene therapy ever since the first publication reporting on engraftment of primitive human hematopoietic cells in the bone marrow of NOD/SCID mice (Larochelle, et al., 1996). The human cells capable of repopulating NOD/SCID mice, termed SCID-repopulating cells (SRC), are considered to be more primitive in the hematopoietic hierarchy than most LTC-ICs and CFUs, and give rise to both myeloid and B lymphoid cells, but not T cells. However, by combining stromal co-culture and fetal thymic organ culture it is possible to generate T, B and NK cells as well as granulocytes in vitro from human CB cells engrafted in the bone marrow of NOD/SCID mice (Robin, et al., 1999). Larochelle *et al.* showed that the SRC activity was found exclusively in the $CD34^{+}CD38^{-}$ cell fraction and reported very inefficient transduction of SRCs, reminding of the situation for ELTC-ICs to that date. However, more recently, efficient retroviral transduction of SRCs has been demonstrated (see ***Table 2***). There are considerable differences in the frequency of SRCs among human hematopoietic cells depending on the cell source, as calculated from experiments with transplantation of human cells at limiting numbers. Wang *et al.* reported the SRC frequency in CB cells to be 3-fold higher than in BM and 6-fold higher than in MPB mononuclear cells, that is 1 in $9.3x\ 10^{5}$ CB cells, 1 in $3x10^{6}$ BM and 1 in $6x10^{6}$ MPB cells (Wang, et al., 1997). In contrast, van der Loo *et al.* found a much lower SRC frequency, namely 1 in $1.7x10^{6}$ MPB $CD34^{+}$ cells corresponding to 1 in $2.2x10^{8}$ mononuclear cells (van der Loo, et al., 1998). The differing results may be explained by the more sensitive method, Southern blot, used by Wang *et al.* to detect human cell engraftment, compared to flow cytometry used by van der Loo *et al.* Also contributing to a higher engraftment of human cells in the work by Wang may be the administration of human cytokines to the mice post transplantation. As hematopoietic progenitors from CB readily engraft in the bone marrow of NOD/SCID mice following culture, expansion and gene transfer, most information on human hematopoiesis in the

NOD/SCID mouse model has been gained from investigations of CB cells, although the most relevant source of hematopoietic progenitors for clinical use would be the MPB or BM. Human cell engraftment in the bone marrow of NOD/SCID mice, as well as lineage differentiation and proliferation, can be detected by flow cytometry, typically at 6-8 weeks or later after transplantation, and human CFU-Cs can be cultured from chimeric bone marrow using human cell specific cytokines. In order to increase the engraftment of human cells in NOD/SCID mouse bone marrow, some investigators treat the mice with growth factors, co-transplant cell lines expressing human cytokines or administer anti-NK cell antibodies, but transplants can be performed without administration of exogenous cytokines and with CD34 purified cells from CB (Hogan, et al., 1997b) or MPB (Hogan, et al., 1997a).

Although shown to be a very useful and feasible model for studying human hematopoiesis *in vivo*, the NOD/SCID mouse model has some limitations. First, the model is artificial in a number of ways and it is not clear whether mechanisms for homing, engraftment and support of human hematopoiesis from the mouse bone marrow microenvironment are fully relevant for the situation in humans. Second, there is a high degree of variability in human cell engraftment levels among animals injected with the same cells, making it very difficult to draw firm conclusions regarding homing and engraftment without including a large number of mice in the experiments (Ballen, et al., 2001, Hogan, et al., 1997a). Third, the short life span of the mice as they develop thymic lymphomas makes long-term studies of hematopoiesis impossible (Prochazka, et al., 1992). However, although not reflecting steady-state hematopoiesis, secondary transplantation experiments may be valuable to improve evaluation of the self-renewal capacity among human candidate stem cells. Fourth, the NOD/SCID model does not support terminal differentiation of the human erythroid lineage, not even following EPO administration to the mice, despite presence of erythroid progenitors in their bone marrow (Cashman, et al., 1997). Furthermore, some very important concerns have been raised regarding the representativity of the SRCs as surrogates for human stem cells. Does the SRC represent a true human HSC, or rather, a less primitive progenitor cell, which may also be more susceptible to retroviral transduction than the HSCs? Contrary to previous reports, engraftment of less primitive $CD34^{+}CD38^{+}$ cells in NOD/SCID bone marrow for up to 12 weeks has been reported, and may thus be detected as SRCs when assayed under standard conditions 6-8 weeks post transplantation (Hogan, et al., 2002). These cells were not able to engraft in secondary recipients confirming their less primitive position in the hematopoietic hierarchy. In an interesting abstract from Kiem´s group in Seattle,

retroviral transduction of CD34-enriched baboon bone marrow cells was performed before autotransplantation to irradiated recipients concomitant to transplantation to NOD/SCID mice. Gene transfer to 50-60 % of baboon SRCs could be accomplished, compared to a 4-7% transduction efficiency of cells that engrafted in baboon bone marrow, suggesting that SRCs might represent a different, maybe less primitive and more easily transducible subset of hematopoietic progenitors than true large animal HSCs (Horn, et al., 2001). It has not been proved if the conditions when transplanting baboon cells to NOD/SCID mice also reflect the relationship between human cells and NOD/SCID mice, but this seems likely. No direct comparison between gene transfer to repopulating human hematopoietic cells in the autotransplantation setting and gene transfer to SRCs has been published yet.

The human/fetal sheep xenograft model is a highly useful experimental system allowing studies of human hematopoiesis in vivo for long periods of time (for review, see (Zanjani, et al., 1996)). This model takes advantage of the immaturity of the immune system in fetal sheep, allowing for engraftment of human cells without bone marrow conditioning, if transplantation takes place in utero when fetuses are 50-60 days of age. The ovine bone marrow microenvironment can support maintenance and differentiation of human hematopoietic progenitors (albeit at low levels of chimerism) from various sources, and also allows secondary transplantations. A similar human/fetal dog xenotransplant model has been reported by Dubé's group in Toronto, which was utilized to assay human hematopoietic progenitors subjected to LTBMC transduction (Omori, et al., 1999).

Large animal models of hematopoiesis

The disappointing results from early gene marking studies in humans, despite reproducible and efficient gene transfer in mouse models, has highlighted the need for more representative in vivo models, which could better reflect human hematopoiesis. Large animal models, using dogs or, particularly, non-human primates, have proven to be extremely valuable with gene marking results highly predictive of the situations in human trials (for recent review, see (Donahue and Dunbar, 2001)). Non-human primates (rhesus macaques and baboons) are genetically much more closely related to humans than are mice and are also more similar in their cellular turnover in the hematopoietic system and in retroviral receptor expression levels on HSCs. Furthermore, when it comes to other large animal models, including the human/fetal sheep xenograft model, these species allow for hematological sampling during

years. Finally, these animals are not inbred, and the results obtained are more likely to be generally applicable than those involving only one mouse strain. Recent progress in gene marking of HSCs in the rhesus model has resulted in gene marking levels of 15-20 % of primitive progenitors, enabling analysis of vector insertion sites using various PCR based techniques (Shi, et al., 2002). This gene marking level is also encouraging for future clinical gene therapy in humans. Obvious drawbacks of the non-human primate models are extremely high costs associated with keeping the animals and caring for them through ablative marrow conditioning with all the supportive care which is required. Therefore, only a few institutions in the world are doing gene therapy experiments involving non-human primates. As another consequence, the number of animals involved in each experiment has to be limited, and the results must rely on internal controls such as competitive repopulating experiments instead of standard statistical methods.

Gene transfer to HSC

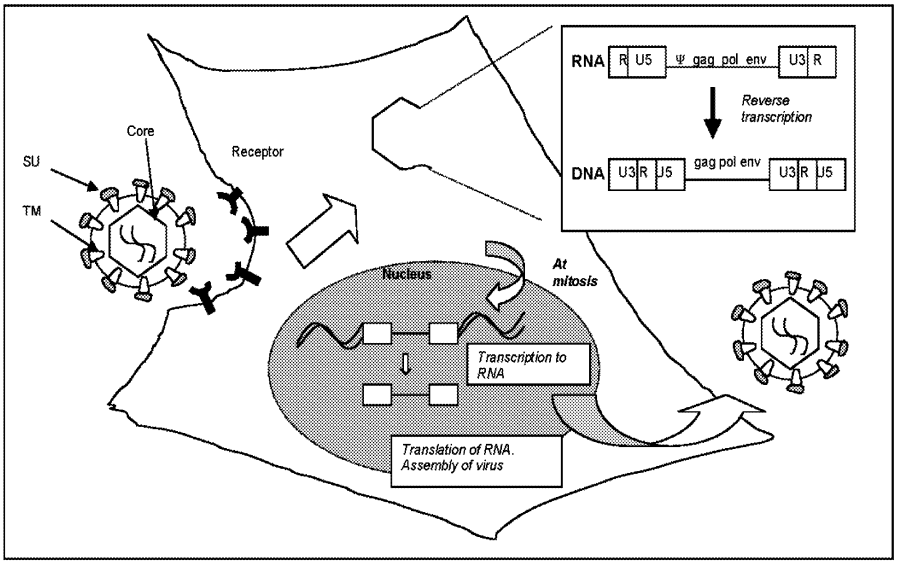
Permanent introduction of new genes into HSCs provides a potential for treating a number of monogenic diseases affecting the hematopoietic system, as the transgene ideally will be expressed in all the progeny of the HSC for a lifetime. To reach this goal, transduction of HSC needs to be efficient as well as engraftment of gene modified cells and expression of the transgene has to be sustained long-term. Most attempts to perform permanent gene transfer to HSC are based on viral vectors. In humans, the goal described remains elusive with few exceptions (Aiuti, et al., 2002a, Cavazzana-Calvo, et al., 2000).

The life cycle of wild-type oncoretroviruses

Wild-type retroviruses comprise a large family of RNA-viruses, including simple retroviruses such as Moloney Murine Leukemia virus, which belongs to the group of oncoretroviruses (Coffin, et al., 1997). Complex retroviruses, such as lentiviruses, include Human immunodeficiency virus-1 (HIV-1). Oncoretroviruses can cause a number of malignant diseases in mammals with early or late onset. The oncoretroviral particles are approximately 70-100 nm in diameter and consist of a viral glycoprotein envelope, structural proteins, enzymes necessary for virus replication and a genome comprising two copies of single stranded RNA of 7-10 kb. The envelope protein (coded by the *env* gene) specifies the host

range of the virus, as it specifically interacts with its receptor on the target cell surface. Following binding to the receptor, fusion of the viral envelope with the target cell membrane

Figure 2. The life cycle of a wild-type oncoretrovirus



The surface component of the retroviral envelope binds specifically to its receptor on the cell surface. The viral particle is taken up into the cell cytoplasm, where the envelope is removed. The viral genome (RNA) is reverse transcribed to DNA whereby two identical sequences, the so called LTRs are formed at each end. Following mitosis, the viral complex relocates to the nucleus and, as an effect of *integrase* enzyme, the virus is integrated into the host cell genome. From its position in the genome, the virus is transcribed as any regular gene to RNA. Following translation of RNA to viral proteins, finally new viral particles are formed. The newly formed virions acquire envelope structures from the host cell membrane. SU=surface component of envelope; TM=transmembrane component; Ψ=packaging signal. Modified from Günzburg and Salmons, 1996.

occurs, whereafter the viral particle is taken up by the cell and the envelope is removed (***Figure 2***). Viral RNA is then transcribed by reverse trancriptase in the cytoplasm to form double-stranded DNA. Breakdown of the nuclear membrane is needed for the provirus to be able to enter the nucleus; therefore, oncoretroviruses can only infect cells which pass mitosis in close timely association with viral infection (Hajihosseini, et al., 1994, Miller, et al., 1990). During reverse transcription, the sequences of the virus located at the 5' and 3' ends are duplicated to form identical sequences termed long terminal repeats (LTR). As the viral double stranded DNA gets in contact with the target cell chromosomes, integration into the host cell genome occurs as a result from integrase action. Integration of the viral genome has been believed to occur mostly at random, but recent data show that retroviral vector

integration in human repopulating hematopoietic cells takes place at preferred integration sites (Laufs, et al., 2003). In case of infection with a wild-type retrovirus, the integrated DNA-form of the virus, termed provirus, is transcribed from the host cell genome to form RNA coding for the viral proteins and viral RNA. Following assembly of viral proteins and genome (RNA), new viral particles are generated and bud off from the cell surface to spread of the viral infection. If, instead, a replication incompetent retroviral vector is integrated, the proviral transgene is expressed from its position in the host cell DNA, driven by LTR or internal promoters. No viral particles can be formed as this provirus lacks the genes for *gag*, *pol* and *env*.

Generation of oncoretroviral vectors

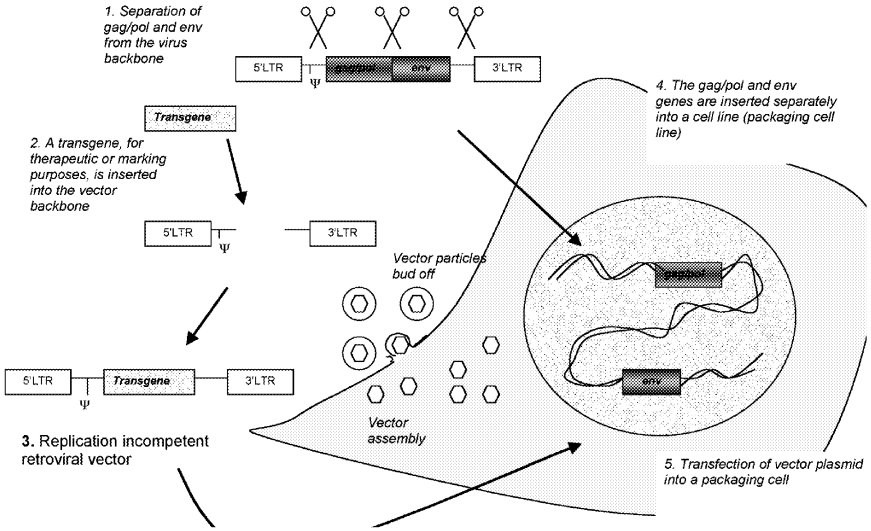
Retroviruses can be engineered to become gene transfer vehicles for permanent gene transfer as they integrate into the target cell genome (***Figure 3***) (for review, see (Günzburg and Salmons, 1996, Kay, et al., 2001)). The oncoretrovirus genome consists of the *gag*, *pol* and *env* genes, which encode for structural proteins, viral enzymes including integrase and reverse transcriptase and envelope proteins, respectively. These genes are flanked by the long terminal repeat sequences (LTR). To generate a vector, the coding sequences and cis-acting sequences are removed and separated into different plasmids in order to avoid recombination, which could lead to production of replication competent virus. A foreign gene of interest, such as a marker gene or a therapeutic gene, can thus replace the gag, pol and env sequences. The viral genes are instead expressed in a cell line, termed packaging cell line. A retroviral vector carrying a transgene can be introduced into the genome of the packaging cell, which will then produce infection-competent, but replication-defective vector particles for gene transfer purposes. The abortive infection of a target cell by a retroviral vector is termed transduction.

Oncoretroviral vectors

Most oncoretroviral vectors are based on the Moloney Murine Leukemia Virus (Cepko, et al., 1984). These vectors can carry transgene inserts of a size up to 8 kb. Transcription of the provirus is driven from the 5' LTR, but internal promoters for gene expression may also be used. Sustained high levels of retroviral gene expression are crucial for successful HSC based gene therapy strategies. Limitations of standard retroviral vectors include lack of expression in embryonic stem cells, believed to be caused by methylation of the provirus (Jahner and

Jaenisch, 1985). This phenomenon leads to silencing of gene expression despite the presence of the provirus, and is important also for retroviral gene expression in adult murine

Figure 3. Generation of a retroviral vector and a packaging cell line



The DNA form of a retrovirus is shown schematically. LTR=long terminal repeat; Ψ=packaging signal; *gag/pol* and *env*=retroviral genes

hematopoietic cells (Challita and Kohn, 1994), but the significance of silencing in the human setting is not completely established. To ensure sufficient retroviral vector expression, improvements in vector design have been made (for review, see (Hawley, 2001). The murine embryonic stem cell virus (MESV), created by Ostertag's group in Hamburg, is capable of expressing in ES cells due to mutations affecting LTR and enhancer regions (Grez, et al., 1990). Hawley and colleagues developed the Murine stem cell virus (MSCV) vector, partly based on the MESV vector (Hawley, et al., 1992). The MSCV vector has been widely used and has been shown to express in murine and human hematopoietic cells. In order to optimize vector driven gene expression in hematopoietic stem cells and primitive myeloid cells, Baum *et al.* developed hybrid vectors with LTR sequences (U3) containing enhancer/promoter regions from the murine spleen focus-forming virus combined with MESV derived primer binding site sequences (Baum, et al., 1995). Efficient gene marking of human CB derived SRCs has been shown using these vectors (van Hennik, et al., 1998). Kohn's group in Los Angeles has also developed improved retroviral vectors with a number of changes to the

original Moloney murine leukemia virus, including modifications in the LTR and primer binding sites to achieve improved gene expression (Robbins, et al., 1998). These vectors mediated a more consistent gene expression in murine hematopoietic cells in vivo, with less risk of silencing following serial bone marrow transplantation. Significantly higher GFP expression levels were obtained with this modified vector (MND) compared to standard Moloney murine leukemia vector in a mouse bone marrow transplant model (Halene, et al., 1999).

Retroviruses are thus susceptible to transcriptional silencing and position effects, the latter meaning that expression of the provirus may be decreased due to influences from the surrounding chromatin, and thus specific to the integration site of the provirus. Inserting DNA sequences with insulator properties flanking the retroviral genome can reduce positional effects, improve expression and decrease methylation of the 5' LTR (Emery, et al., 2000, Rivella, et al., 2000). This may also be beneficial from a safety point of view, as insultors reduce the risk of interactions between vector LTR and neighboring genes (Baum, et al., 2003).

Viral envelopes and receptors

The viral envelope proteins are encoded by the *env* gene and comprise one transmembrane (TM) and one surface (SU) component, which together form a knob on the surface of the viral particle. Viral tropism is determined by specific interactions between the SU component and its receptor. Retroviruses can be grouped into different interference groups based on the receptors they use for cell entry (Miller, 1996). Retroviral vectors can be generated with envelope proteins from different viruses, termed pseudotyping, by exchanging the *env* gene in a packaging cell line. A summary of commonly used viral envelopes in retroviral vectors, their receptor and examples of packaging cell lines are shown in ***Table 1***. For transduction of human cells, vectors with the wild-type amphotropic envelope have been most widely used, also in clinical studies. Vectors with an envelope derived from the Gibbon Ape Leukemia Virus (GALV) may have some advantages in transduction of human primitive hematopoietic cells (see below).

Vectors pseudotyped with VSV-G envelope interact with membrane phospholipids to gain entry into the target cells (Burns, et al., 1993, Emi, et al., 1991). These are abundantly present and thus lack of receptor expression is not considered a problem, and the host range of VSV-

G vectors is wide. Moreover, these vector particles are stable, allowing concentration by ultracentrifugation, which may be an advantage as transduction using VSV-G vectors is dose-dependent, in contrast to the situation with amphotropic particles (Arai, et al., 1999).

Vectors pseudotyped with the feline endogenous virus envelope RD114 can be used as alternatives for transduction of human cells (Cosset, et al., 1995). The receptor for RD114 retrovirus (RDR) is a neutral amino acid transporter and was cloned in 1999 (Rasko, et al., 1999). RDR expression has been reported to be high in primitive human hematopoietic progenitors, and transduction of human hematopoietic cells capable of engrafting in the BM of fetal sheep has been demonstrated (Lee Lucas, et al., 2002). Concentrated (Gatlin, et al., 2001) as well as non-concentrated (Kelly, et al., 2000) RD114 pseudotyped vectors can efficiently transduce SRC from human CB.

Table 1.

Viral envelope	Receptor	Receptor function	Tropism		Packaging cell lines, examples
			Human cells	Murine cells	
Ecotropic	Slc7a1 (Rec-1; mCAT)	Amino acid transporter	-	+	GP+E86
Amphotropic	MLVAR (Pit-2)	Phosphate transporter	+	+	GP+envAM12; PA317
GALV	GLVR1 (Pit-1)	Phosphate transporter	+	-	PG13
VSV-G	Membrane phospholipids	Cell membrane component	+	+	293GPG
RD114	RDR	Amino acid tranporter	+	-	FLYRD18

Transduction of human HSCs – the role of retroviral receptor expression

As a result of the poor gene transfer levels obtained in early clinical trials using amphotropic vectors to transduce HSCs, much interest has focused on the expression levels of the cognate receptor, MLVAR (Pit-2). It was cloned in 1994 (Miller, et al., 1994, van Zeijl, et al., 1994) and is expressed at low levels on primitive human hematopoietic progenitors as analyzed by semiquantitative RT-PCR (Orlic, et al., 1996). The MLVAR and GALV receptors are closely related sodium-dependent phosphate symporters with more than 60 % sequence identity (Kavanaugh, et al., 1994). Although GLVR1 was initially believed to be expressed at higher levels than MLVAR on primitive hematopoietic cells, later reports have shown low expression of both receptors on $CD34^{+}CD38^{-}$ cells (Orlic, et al., 1998). However, culturing

of $CD34^+$ cells with IL-3, IL-6 and SCF may make them more susceptible to retroviral transduction, not only by promoting cell cycling, but also by increasing specific binding of amphotropic particles to hematopoietic cells. This was determined using flow cytometry, but it was not distinguished whether increased receptor expression or higher receptor affinity was underlying the effect (Crooks and Kohn, 1993). Efforts have been made to increase MLVAR expression levels. Orlic and co-workers showed that progenitors isolated from frozen CB samples had 12-fold higher mRNA levels for MLVAR compared to fresh cells and, in addition, showed increase of MLVAR in mouse BM following cytokine treatment (Orlic, et al., 1998). Using G-CSF to mobilize hematopoietic progenitors to peripheral blood, Horwitz *et al.* showed that $CD34^+CD38^-$ cells in MPB had 4-fold higher levels of the amphotropic receptor than had their counterparts in steady-state BM; however, they did not report the receptor levels in cytokine treated BM (Horwitz, et al., 1999). In cell lines, a 3-fold increased expression of GLVR-1 and MLVAR was seen following culture in phosphate-free medium (Kavanaugh, et al., 1994). Phosphate depletion can probably not be utilized for primitive hematopoietic cells (unpublished observations from our group), but works in protocols developed to transduce lymphocytes (Bunnell, et al., 1995).

A correlation has been shown between expression levels of MLVAR or GLVR1 and efficiency of retrovirus transcduction of hematopoietic cells (Orlic, et al., 1996) or cell lines (Sabatino, et al., 1997). However, others found no correlation between expression levels of GLVR1 or MLVAR and the transduction efficiency obtained with GALV or amphotropic vectors in a number of human cell lines (Uckert, et al., 1998). Interestingly, they also studied vectors with the 10A1 envelope, which are capable of entering cells by GLVR1 or MLVAR receptors (Miller and Chen, 1996) and thus could be expected to have an advantage. However, the 10A1 vectors were not to superior to amphotropic or GALV vectors individually.

Forced expression of MLVAR has been shown to increase amphotropic retroviral vector mediated transduction of cell lines (Kurre, et al., 1999, Macdonald, et al., 2000). Adenoviral vector mediated overexpression of MLVAR in cell lines naturally resistant to amphotropic transduction has been shown to overcome that barrier, but not to improve transduction of cells already permissive at start (Lieber, et al., 1995). The Seattle group used a system with tetracycline regulated expression of GLVR1 in cell lines and found correlation between

receptor expression levels and susceptibility to infection by GALV vectors (Kurre, et al., 2001). In order to analyze GLVR1 expression in cell lines and $CD34^+$ cells they generated a fusion protein consisting of a part of GALV envelope protein linked to a human IgG Fc, thus making FACS analysis of GLVR1 expression possible. They demonstrated that culture of $CD34^+$ BM cells for 72 hours with multiple cytokines lead to increased expression of MLVAR as well as GLVR1.

Thus, the reports regarding retroviral vector mediated transduction of cells in relation to their expression of the retroviral receptor are somewhat contradictory. This may in part be due to methodological difficulties in determining levels of retroviral receptors, which have to rely on measurement of receptor RNA content by RT-PCR, or on flow cytometric measurement of viral binding to its receptor, as antibodies against GLVR1 or MLVAR not are available to date. Kiem *et al.* showed superior transduction of repopulating hematopoietic cells by GALV pseudotyped compared to amphotropic vectors in baboons and found a correlation to higher expression of GLVR1 than MLVAR in baboon bone marrow cells (Kiem, et al., 1997). However, although their model of studying gene transfer to HSCs is highly relevant, only 4 animals were studied, gene transfer rates were highly variable and receptor levels were determined by semiquantitative RT-PCR. Altogether, it seems likely that low expression levels of MLVAR and GLVR1 receptors limit transduction of primitive human hematopoietic cells.

Alternative vector systems

Foamy virus vectors

Human foamy virus (HFV) is a complex retrovirus of the spumavirus family. Foamy viruses have some properties making them attractive as gene transfer vectors: they are not associated with any human disease, they have a large packaging capacity (at least 9.2 kb of foreign sequences) and replication-incompetent HFV vectors can integrate into the genome of non-dividing cells more efficiently than oncoretroviruses (Russell and Miller, 1996). In addition to the retroviral *gag*, *pol* and *env* sequences, foamy virus genome contains an additional gene termed *bel1*, which encodes for the transactivator (Tas) needed for transcription from the viral LTR. The foamy virus genome undergoes reverse transcription already in the virions, and therefore the infectious virus is mostly present in the form of double-stranded DNA. Early HFV vectors could not be produced at a high titer without the *gag* and *pol* genes, which

were unwanted for safety reasons and because they occupied space from inserted transgenes. However, more recently, improved FV vectors have been developed, which can be produced at high titer and allow concentration by ultracentrifugation (Trobridge, et al., 2002). Foamy viruses can infect a number of different cell types from many species. Nevertheless, using a 10 hour transduction protocol, FV vectors have been used to efficiently transduce human hematopoietic progenitors capable of repopulating the bone marrow of NOD/SCID mice with persistent transgene expression (Josephson, et al., 2002). Human SRCs have also been transduced using simian FV vectors (Zucali, et al., 2002). Thus, for the future, FV based vectors hold promise for gene therapy approaches targeting HSCs, but current experiences from use of FV vectors are limited compared to oncoretroviral or lentiviral based vector systems.

Lentiviral vectors

Human immunodeficiency virus-1 (HIV-1) is a complex retrovirus belonging to the lentivirus family. Wild-type HIV causes a slowly developing immunodeficiency, eventually leading to acquired immunodeficiency syndrome (AIDS) in humans. Lentiviral based vectors are promising and powerful tools under preclinical development for human stem cell based gene transfer approaches (for review, see (Trono, 2000, Woods, et al., 2002)). Lentiviral vectors have an important potential advantage over oncoretroviral vectors in their ability to transduce non-dividing cells, such as primitive hematopoietic cells and neural cells (Naldini, et al., 1996). The lentiviral preintegration complex, which consists of the viral genome, structural proteins and the enzymes reverse transcriptase and integrase, is actively transported through nucleopores into the cell nucleus and thus does not require breakdown of the nuclear membrane to enable transduction (Bukrinsky, et al., 1992). The exact mechanisms underlying nuclear import of the preintegration complex are not known, but the presence of a central DNA flap (central polypurine tract, CPPT) is believed to be involved, and lentiviral vectors containing this component were shown to mediate increased gene transfer (Zennou, et al., 2000). Moreover, the lentiviral preintegration complex may persist stably for weeks in the cytoplasm and can transduce cells upon their activation (Stevenson, et al., 1990). In contrast, oncoretroviral vectors have an intracellular half-life of approximately 5-7,5 hours, implicating that infection needs to take place shortly before mitosis for integration to occur (Andreadis, et al., 1997).

The genome of wild-type HIV contains 9 genes, but not all of them are necessary for infection and in the development of third generation lentiviral vectors, 60 % of the HIV genome has been removed. Three out of nine genes are necessary for production of functional vector: *gag*, *pol* and *rev*; the latter coding for a post-transcriptional regulator of *gag* and *pol* function. Deletion of major parts of the HIV genome from the lentiviral vector is an important step forward from a biosafety standpoint. To further increase safety, vectors with a modification in the U3 region of LTR have been made, thereby blocking transcription driven from LTR following reverse transcription in self-inactivating (SIN) vectors.

As wild-type HIV primarily targets CD4 expressing cells, lentiviral vectors have been pseudotyped, mostly with the VSV-G envelope, to be able to target a number of different cell types. Using VSV-G pseudotyped lentiviral vectors, human $CD34^+$ (Akkina, et al., 1996) and $CD34^+CD38^-$ cells (Case, et al., 1999, Uchida, et al., 1998) have been successfully transduced under conditions with minimal stimulation, which do not allow oncoretroviral gene transfer. This may be an advantage, as reduced manipulation of primitive hematopoietic cells is likely to avoid disturbance of stem cell functions. Furthermore, gene transfer to CB derived NOD/SCID repopulating cells has been achieved (Miyoshi, et al., 1999), also to cells capable of secondary reconstitution (Woods, et al., 2000). In rhesus monkeys, persistent lentiviral vector mediated gene marking of MPB $CD34^+$ cells has been shown, whereas expression declined over time in animals transplanted with gene marked autologous BM cells (An, et al., 2001).

In summary, lentiviral vectors are promising tools for stem cell based gene transfer applications. However, biosafety issues are currently limiting their use in a clinical setting, although it has been claimed that third-generation lentiviral vectors are at least as safe as vectors derived from oncoretroviruses regarding the risk for emergence of recombinant replication competent recombinants (RCR). Permanent packaging cell lines producing lentiviral vectors have recently been developed (Klages, et al., 2000). This is important progress, as vector production can be better characterized and monitored compared to production involving transient transfection of plasmids. However, a major biosafety issue, shared with onocoretroviral vectors, regards the risks of insertional mutagenesis, which is particularly relevant when vectors are used at high MOIs, thereby increasing risks of multiple vector integration events per cell (Woods, et al., 2003). To date, no clinical studies using lentiviral vectors have been reported.

Adenoviral vectors

Adenoviruses are DNA viruses causing common upper respiratory tract infections. Recombinant vectors based on adenoviruses have certain attractive properties: they can be generated at high titer mediating high levels of transgene expression, and they can infect non-dividing cells (Kay, et al., 2001). However, as these vectors do not integrate into the host cell genome, expression is lost within 3-4 weeks, making them unsuitable for permanent gene correction, but they may be useful when short-term transgene expression is desired, e.g. in treatment of malignant tumors. Moreover, most people have antibodies against adenoviruses, which may cause immune reactions and can be dangerous and/or lead to loss of gene modified cells.

Adeno-associated virus vectors

Adeno-associated viruses (AAVs) are human parvoviruses containing single-stranded DNA, which normally require a helper virus (adenovirus) to produce infection. AAV is not associated with any human disease, which makes it attractive as a gene transfer vector (for review, see (Russell and Kay, 1999)). Gene transfer mediated by AAV vectors is both episomal and integrating. Most reports show inefficient gene transfer to primitive hematopoietic cells and much interest for use of AAV vectors has instead focused on liver targeted in vivo gene transfer approaches, such as for treatment of hemophilia.

Oncoretroviral gene transfer to hematopoietic cells

In 1983, the first report was published showing retroviral-mediated transfer of a bacterial neomycin resistance gene (*neo*) into murine hematopoietic progenitors using replication competent virus (Joyner, et al., 1983). Later, retroviral gene transfer to mouse CFU-S (Williams, et al., 1984) and long-term repopulating hematopoietic cells was shown (Keller, et al., 1985). Early transduction protocols involved co-culture of the hematopoietic target cells on top of vector producing cells, a procedure deemed inappropriate for clinical use. Cell-free methods were therefore developed (Bordignon, et al., 1989) and transduction of hematopoietic progenitors in the presence of stromal support was found to enhance gene transfer efficiency (Moore, et al., 1992, Wells, et al., 1995, Xu, et al., 1995). Importantly, the group of David Williams showed that a recombinant carboxy-terminal fibronectin fragment CH-296 (Retronectin) could increase gene transfer to murine and human hematopoietic cells using cell-free ampho- or ecotropic vector supernatants, most likely by co-localizing cells and

viral particles, obviating the use of polycations (Hanenberg, et al., 1997, Hanenberg, et al., 1996, Moritz, et al., 1996, Moritz, et al., 1994). In a competitive gene transfer study in non-human primates, gene transfer efficiency was equal in the presence of stroma or CH-296 (Wu, et al., 2000). Furthermore, human hematopoietic progenitors, which were cultured and transduced for 72 hours could engraft in the bone marrow of *bnx* mice if cultured upon fibronectin fragment or stroma, whereas few mice exhibited long-term engraftment of human cells following liquid culture, showing that fibronectin support maintained engraftment capacity of the cells, possibly by affecting adhesion molecules (Dao, et al., 1998). Interestingly, others have shown efficient gene transfer to hematopoietic cells in the absence of fibronectin by preloading vector particles to plain tissue culture dishes, with (Kuhlcke, et al., 2002) or without centrifugation of the vector (Hennemann, et al., 1999, Hennemann, et al., 2000).

Cytokine stimulation of primitive hematopoietic cells

The area of stem cell expansion *ex vivo* is of great interest for the field of gene therapy. As oncoretroviral gene transfer requires division of the target cells and HSCs are largely quiescent, cytokine stimulation is included in most gene transfer experiments with these vectors in order to promote cell division. Early protocols generally utilized the cytokines IL-3, IL-6 and SCF. Flt-3 ligand (FL) has been shown to stimulate the proliferation of primitive hematopoietic cells (Gabbianelli, et al., 1995) and preserve the ability of human cells to engraft in the bone marrow of immune-deficient mice following culture, similar to stromal support, and has therefore been added to transduction protocols (Dao, et al., 1997). Another early acting cytokine, thrombopoietin (TPO), has in combination with SCF and FL been shown to enhance growth of human $CD34^{+}CD38^{-}$ BM cells and to expand ELTC-ICs (Ramsfjell, et al., 1997, Ramsfjell, et al., 1999). The cytokines SCF, FL and TPO form the basis for most current protocols for cell expansion or oncoretroviral gene transfer. However, there is insufficient knowledge regarding regulation of stem cell self-renewal, and despite methods to achieve very considerable expansion of cell numbers, colony forming cells and LTC-ICs (Petzer, et al., 1996), increasing the numbers of long-term repopulating cells has been more problematic. Cytokine stimulation may cause an engraftment defect of the expanded cells and/or drive primitive cells to differentiation as demonstrated in mice (Peters, et al., 1996). It has been shown, that inclusion of IL-3 in the cytokine stimulation may negatively affect long-term repopulating hematopoietic cells (Ueda, et al., 2000, Yonemura,

et al., 1996). On the other hand, IL-3 was recently shown to support long-term repopulating murine hematopoietic cells under serum-free conditions; however, in the presence of serum, loss of long-term reconstitution was seen (Bryder and Jacobsen, 2000). Conneally *et al.* demonstrated a 2-fold expansion of repopulating human cells in the NOD/SCID model following 5-8 days of culture (Conneally, et al., 1997). Similarly, Bhatia *et al.* demonstrated a 2-4-fold increase of CB derived SRC following 4-day serum free culture with multiple cytokines, but complete loss of repopulating activity if culture time was extended to 9 days (Bhatia, et al., 1997a), and similar results have later been reported (Ueda, et al., 2000). Interestingly, Piacibello *et al.* reported 70-fold expansion of CB SRC following up to 10 weeks expansion culture with FL, MGDF(TPO), SCF and IL-6 (Piacibello, et al., 1999) and later the same group showed lentiviral transduction of CB cells capable of expansion and engraftment in primary, secondary and tertiary NOD/SCID mice with preserved multilineage transgene expression (Piacibello, et al., 2002). In contrast, additional expansion culture of rhesus monkey MPB progenitors after a 4-day transduction led to considerable loss of engraftment capacity in vivo (Tisdale, et al., 1998). An alternative approach to improve engraftment, namely by avoiding active cell cycling of cultured cells at time of injection, was presented by Dunbar's group. After a 4-day transduction, cells were maintained for two additional days with minimal cytokine stimulation (SCF only) on fibronectin, yielding consistently higher engraftment levels compared to cells transplanted after a 4 day culture only (Takatoku, et al., 2001).

Long-term bone marrow culture (LTBMC) transduction

The beneficial effects on hematopoietic cell transduction from adding stromal support to the cultured cells probably include cytokine production by stromal cells and direct cell-cell interactions similar to the bone marrow microenvironment *in vivo*. The group of Ian Dubé took this approach further by adopting the LTBMC method described by Dexter *et al.* (Dexter, et al., 1977) for gene transfer purposes. In these cultures, initiated without addition of exogenous cytokines, a layer of adherent cells is gradually formed, giving support to adherent and non-adherent hematopoietic cells. Thus, an in vitro hematopoietic microenvironment is created, which supports the maintenance of CFU-C and CFU-S for weeks, during which proliferation of hematopoietic progenitors takes place. Their proliferation can further be promoted by weekly changes of half of the medium, which was done prior to addition of vector containing medium at repeated occasions in the protocol

developed by Dubé's group. It was believed that this method would provide more physiological recruitment of stem cells into proliferation compared to standard transduction protocols with strong cytokine stimulation, and therefore would better preserve stem cell functions. Promising results from LTBMC transduction of canine hematopoietic cells followed by autotransplantation to myeloablated or non-myeloablated recipients were published in 1992 (Carter, et al., 1992). The same group reported 2 years later very encouraging gene transfer efficiency to repopulating hematopoietic cells using their 21-day three-cycle LTBMC transduction protocol in the same large animal model (Bienzle, et al., 1994). Gene transfer to CFU-GM day 21 reached 44-67 % depending on the method of analysis and maximal gene transfer efficiency to CFU-GM in vivo was 10-30 % during the first 3-6 months with persistence of 5 % gene marked cells at 24 months post transplantation in 2 animals. These results were achieved without any myeloablative treatment of the transplant recipients, which made them even more remarkable, and follow-up was long, up to 2 years. Using human BM cells, gene transfer to 28 % (G418 resistance) or 53 % of CFU-GM (PCR) was achieved (Dube, et al., 1996). These promising results formed the basis for a human clinical phase I gene marking trial recruiting myeloma patients undergoing autologous transplantation in Toronto and was the background also for our interest in exploring the LTBMC transduction method (*Paper I*).

Clinical gene therapy

The clinical experiences from gene transfer trials targeting hematopoietic stem cells can be grouped into gene marking trials, trials with transfer of chemoresistance genes and therapeutic trials in monogenic hereditary diseases (for review, see (Richter and Karlsson, 2001)).

Gene marking trials

Early gene transfer trials involving humans were mostly gene marking studies, with the objectives to test feasibility and safety of oncoretroviral gene transfer to repopulating hematopoietic cells in patients undergoing high dose cancer chemotherapy with autologous stem cell rescue. The *neo* resistance gene, which can be detected by PCR, or by determining the frequency of hematopoietic colonies resistant to the neomycin analog G418, was used as a marker gene in most trials. Gene marking strategies were successfully used to answer the question whether autologous bone marrow cells, harvested from patients in remission from

malignant disease and reinfused as stem cell rescue following high-dose chemotherapy, could contribute to relapse of disease, or whether the relapse originated exclusively from residual malignant cells in the patients. Retroviral gene marking of bone marrow cells harvested from patients with neuroblastoma (Rill, et al., 1994), acute myeloid leukemia (Brenner, et al., 1993b) and chronic myeloid leukemia (Deisseroth, et al., 1994) clearly showed that the harvests were contaminated by malignant cells capable of contributing to a relapse, as a proportion of the recurrent malignant cells carried the *neo* gene. Moreover, gene marking of BM or MPB $CD34^+$ cells from patients with breast cancer or multiple myeloma showed that gene modified normal hematopoietic cells from both sources could contribute to hematopoiesis and could be detected for up to 18 months in patient bone marrow, but the marking levels were low, approximately 1:1000 to 1:10000 bone marrow cells (Dunbar, et al., 1995). The authors concluded that the disappointingly low marking levels, although detectable by molecular methods, were too low to be useful in a potential therapeutic setting. However, marking of up to 15 % of hematopoietic colonies at 18 months post transplant was reported in a pediatric gene marking trial (Brenner, et al., 1993a).

Stem cell protection/selection with chemotherapy resistance genes

Bone marrow toxicity is one of the factors which limit efficient cancer chemotherapy using cytotoxic drugs. The multiple drug resistance gene 1 (MDR1) encodes for the P-glycoprotein, a transmembrane pump with ability to actively transport a variety of cytotoxic drugs such as anthracyclins, vinca alcaloids, taxanes and epipodophyllotoxins out of the cells. P-glycoprotein is expressed in primitive hematopoietic cells and in chemoresistant malignant cells. Retroviral transduction of HSCs has been suggested as a way to protect these cells from cancer chemotherapy and could perhaps be a method to selectively expand transduced cells in vivo. A number of clinical studies have been reported showing that MDR marked cells could contribute to hematopoiesis, but very low levels of gene transfer to repopulating hematopoietic cells have been accomplished. In one study, where only cells subjected to retroviral transduction were infused following high-dose chemotherapy, without the presence of competing unmanipulated cells, marking levels were similarly too low to enable evaluation of a possible myeloprotective effect from the gene transfer procedure (Moscow, et al., 1999). More recently, gene transfer to $CD34^+$ enriched MPB cells from patients undergoing autologous transplantation for germ cell tumors was reported. Transductions were performed in the presence of fibronectin fragment and with SCF/IL-6 or SCF/MGDF/G-CSF. Gene

transfer efficiency to colonies prior to retransplantation was on average 14 %, the transgene could be detected for more than a year, and there were signs of in vivo selection of transduced cells following further chemotherapy. However, MDR-1 expression was inconsistent due to aberrant splicing of the gene product, leading to truncated, non-functional P-glycoprotein. (Abonour, et al., 2000). In mice, development of a myeloproliferative syndrome following transplantation with MDR-1 transduced hematopoietic cells has been described (Bunting, et al., 1998); however, there were no signs of such adverse events in the human study.

Alternative preclinical strategies to expand gene modified cells in vivo

As current methods to transfer genes to human HSCs are fairly inefficient and the engraftment capacity of cultured cells may be compromised, one strategy to overcome this problem is to utilize a system where transduced cells are selected in vivo (for review, see (Persons and Nienhuis, 2002)). Transfer of drug resistance genes such as MDR-1 to HSCs may lead to enrichment of transduced cells following myelotoxic chemotherapy. Variants of dihydrofolate reductase (DHFR) genes, conferring resistance to methotrexate, may offer advantages, especially as the drug is not mutagenic, and considerable enrichment for transduced cells in vivo has been shown in mice (Allay, et al., 1998). The O^6 methylguanin DNA methyltransferase (MGMT) functions in DNA repair, and can be inhibited by O^6 benzylguanin (6-BG). Following transplantation of HSCs transduced to express a 6-BG resistant MGMT mutant to irradiated mice, significant in vivo selection of transduced cells (8-90 % transduced cells in PB) was accomplished by treatment with the alkylating agent BCNU and 6-BG, and persisted after secondary transplantation (Ragg, et al., 2000). Limitations of this approach include the risks of mutagenesis as a result of treatment with alkylating agents. An alternative strategy involves retroviral overexpression of the HOXB4 gene, which is known to be an important regulator of primitive hematopoiesis. Human hematopoietic cells transduced with a HOXB4 coding retroviral vector had a selective growth advantage in vivo when transplanted to NOD/SCID mice; however, the expression levels of HOXB4 may need to be strictly regulated (Buske, et al., 2002, Schiedlmeier, et al., 2002). Blau and colleagues have described other strategies for in vivo selection without cytotoxic chemotherapy using a fusion protein consisting of a growth factor receptor signaling domain, which can be reversibly activated through dimerization by a specific molecule (Jin, et al., 2000).

Treatment of monogenic diseases

Obvious candidate diseases which could benefit from permanent HSC based gene therapy approaches include disorders affecting the hematopoietic system characterized by a defined genetic mutation, and for which allogeneic bone marrow transplantation is a potential treatment. However, bone marrow transplantation is associated with risks, and suitable donors can not be found for all patients; in these situations gene therapy may be a possible treatment option. In contrast to the situation for patients with cancer, full myeloablation is no advantage as such when treating genetic disorders, but adds seriously to the risks of the gene transfer procedure. Consequently, attempts have been made to reduce or avoid myeloablation. Examples of disorders where gene therapy approaches have been tested clinically include Gaucher's disease, ADA-SCID, X-linked SCID, chronic granulomatous disease (CGD) and Fanconi anemia.

Gaucher's disease

Gauchers's disease is an autosomal recessive disorder caused by deficiency of the enzyme glucocerebrosidase, which leads to a lysosomal storage disease. β-glucoside is accumulated in various tissues, most pronouncedly in macrophages. Enzyme replacement therapy and BMT are available treatment options. Following extensive preclinical gene transfer experiments, a human gene therapy trial targeting MPB or BM $CD34^+$ cells was started. Cells were transduced in the presence of stroma, in two patients with addition of IL-3, IL-6 and SCF, for 72 hours and subsequently reinfused to the patients without prior myeloablation. In two out of three patients the transgene could be detected at low frequency for up to three months after transplantation, but there were no signs of clinical benefit or increase of glucocerebrosidase activity (Dunbar, et al., 1998).

ADA-SCID

Severe combined immunodeficiency due to lack of adenosine deaminase (ADA-SCID) is a disorder with B and T cell dysfunction which can be treated with BMT and in which a number of gene therapy trials have been performed. The first trial, instituted at NIH in 1990, involved retroviral gene transfer of the ADA gene to T-lymphocytes. This approach does not lead to permanent cure of the disease, but can give long-lasting correction thanks to the long life-span of lymphocytes (several months, even years), and therefore, repeated infusions of

gene-corrected cells were given to the patients (Blaese, et al., 1995). The number of T cells normalized, immune responses improved and, interestingly, ADA activity in T cells persisted for at least 4 years after the last infusion of T cells. Kohn and co-workers have reported retroviral gene transfer of the ADA gene to autologous CB cells in three newborn children with ADA-SCID resulting in long-term persistence of corrected T cells, despite low level engraftment of gene-modified myeloid cells (Kohn, et al., 1998). Accumulation of toxic metabolites due to the enzyme deficiency in non-corrected cells may offer a selective survival advantage for gene-corrected cells. However, as it was deemed unsafe and unethical to withhold treatment with PEG-ADA from these patients, the effect of gene therapy may have been blunted by the enzyme substitution. In one patient in a previous Italian trial, tapering of PEG-ADA led to increased levels of gene corrected lymphocytes and restoration of immune functions (Aiuti, et al., 2002b). Recently, a new Italian trial enrolling two patients with ADA-SCID was reported, comprising retroviral gene-correction of HSCs followed by autotransfusion after nonmyeloablative conditioning with busulfan (Aiuti, et al., 2002a). In these patients enzyme replacement therapy was not an available option. Both patients underwent the procedure without complications and recovered well as judged clinically and by numerous laboratory investigations. As a sign of selective advantage, the frequency of transgene containing cells was higher among lymphocytes than myeloid cells and increased to 70 % of the B cells in one of the patients. The patients have been followed for a year without signs of silencing of the vector or decreased therapeutic effects.

X-linked SCID

The most common variant of the SCID disorders is the X-linked SCID caused by a mutation affecting the γ-chain, which normally serves as a common subunit of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. This leads to a complete absence of mature T and NK cells, and the affected individuals die from infections in early life unless BMT can be performed. In 2000, Alain Fischer's group in Paris reported the first successful clinical gene therapy trial in two patients (Cavazzana-Calvo, et al., 2000). Autologous BM $CD34^+$ cells were transduced on fibronectin with a retroviral vector coding for the γ-chain in the presence of SCF, MGDF(TPO), FL and IL-3, and were reinfused without marrow conditioning. In both patients, lymphocyte counts and T and B cell functions were normalized, as was the clinical course. As a sign of strong selective advantage for the gene-corrected lymphocytes due to restored cytokine signaling, almost 100 % of the T cells detectable in vivo carried the

transgene despite low level transduction of myeloid cells and B cells. Although the transduction protocol was improved compared to early clinical trials, the strong selection of transduced cells most likely underlies most of the success of gene therapy of this disorder and contrasts to the situation in most other diseases considered for gene therapy. In general, cultured cells, besides representing a small fraction of the total pool of hematopoietic cells, have a disadvantage when competing with unmanipulated cells. Later, the results from the first 5 patients treated with gene therapy were reported with similarly successful results in 4 patients (Hacein-Bey-Abina, et al., 2002). These results, demonstrating for the first time unequivocal proof of principle that integrating stem cell based gene therapy could work in humans and produce substantial clinical improvement of the treated patients, were encouraging for the field of gene therapy. Currently, 11 patients have been enrolled in the Paris trial. However, follow-up recently revealed serious treatment related side effects in two patients, who developed T-cell malignancies (Cavazzana-Calvo, 2002, Check, 2002, Gänsbacher, 2003, Marshall, 2003). In October 2002, one patient (patient 4), who was subjected to gene therapy at the age of one month, was reported to have developed a rapidly progressive lymphoproliferative syndrome 30 months following gene transfer. The initial clinical course of this patient was uncomplicated with polyclonal T-cell proliferation, and the patient did recover from a varicella-zoster infection. When the patient developed splenomegaly and anemia, chemotherapy was instituted. Detailed investigations showed that the malignant clone was of γδ T cell phenotype and expressed the γ-chain. Integration site analysis by Christoph von Kalle, Freiburg, Germany, revealed that vector integration had occurred into the LMO2 gene (LIM domain only 2) on chromosome 11 and could be traced back to 1 year after treatment, with gradual increase between months 17 and 24 after transplantation. The LMO2 gene interacts with Tal-1/SCL and GATA-1 transcription factors, is necessary for normal hematopoiesis and plays a role in T cell acute leukemia (Rabbitts, 2001). There were no signs of replication competent retrovirus. A chromosomal translocation of unknown significance was detected starting month 34. The other patient (patient 5) was diagnosed in December 2002 with splenomegaly, enlarged mediastinum, anemia, thrombocytopenia and high numbers of circulating blast cells of T cell phenotype, all expressing the transgenic protein. In this patient as well, vector integration into LMO2 has been demonstrated (Gänsbacher, 2003).

Thus most likely, retroviral vector insertion into the gene coding for the transcription factor LMO2 at least initiated the T-cell malignancy in the first patient. It has not been established whether secondary events contributed to the malignant course. Additional factors could possibly also include exposure to varicella-zoster and a familial susceptibility to develop malignant tumors. Further analyses of the second patient are pending.

Insertional mutagenesis and gene therapy

The risk of inducing a malignancy as a result of integration of a gene transfer vectors is a common feature of oncoretroviral and lentiviral vectors (for recent review, see (Baum, et al., 2003)). HSCs are long-lived and thus prone to accumulate mutations during life, in addition to the vector integration event, and are at risk for malignant transformation as discussed in the context of cancer stem cells (Reya, et al., 2001). Wild-type murine leukemia viruses transform their host cells by repeated integration as a result of virus replication and reinfection, leading to accumulation of mutations eventually leading to malignant transformation. This may also be a risk associated to gene therapy in case replication competent recombinants develop. Aggressive T-cell lymphoma developed in non-human primates following RCR emergence, but only in animals not capable of forming an antibody response against the virus (Donahue, et al., 1992).

Retroviral vectors (including lentiviral) are believed to integrate semi-randomly into the genome of the target cells, preferentially targeting euchromatin (Rohdewohld, et al., 1987). As demonstrated for HIV and lentiviral vectors, integration occurs preferentially in active genes and identified hot spots, thus increasing the risks of disturbing normal gene function, compared to integration taking place at random (Schroder, et al., 2002). Similarly, oncoretroviral vector integration into human NOD/SCID repopulating cells was shown to favor certain regions (Laufs, et al., 2003). The mechanisms behind preferential integration into certain areas of the genome are largely unknown.

Recent data from our lab give support to the view of preferential vector insertion into genes, as lentiviral vector integration into a tumor suppressor gene (BRCA1) of human SRC was shown (Woods, et al., 2003). In this report, high levels of transgene expression in NOD/SCID repopulating cells was shown to rely on the presence of multiple vector copies per cell. This may be a undesired situation, as the risk of insertional mutagenesis is believed to be

proportional to the number of vector integration events; instead, a vector copy number of 1-2 per cell should be the goal for clinical gene therapy in most situations (Wahlers, et al., 2001).

A number of modifications of retroviral vectors could contribute to a reduced risk of malignant transformation as suggested by Baum *et al.* (Baum, et al., 2003). Using vectors with internal promoter and enhancer sequences instead of LTR driven expression, reduced interaction with neighboring genetic material in the transduced cells could be expected, and maybe further decreased by insulator sequences in the LTRs (Emery, et al., 2000). In addition, if vectors were equipped with a strong RNA termination signal and a strong internal splice acceptor to prevent interaction with neighboring splice acceptors in the target cell genome, at best, the risks of mutagenesis would be restricted to disruption of the gene at which integration occurs.

Despite more than a decade of experience from human clinical gene therapy studies, no cases of insertional malignant transformation in patients have been described until recently. Even more surprisingly, no cases of malignancy in mice or other animals, caused by insertional mutagenesis from integrating gene transfer vectors, were reported until last year (Li, et al., 2002). After long-term follow up and secondary transplantation of BM cells transduced with a vector encoding for the truncated nerve growth factor receptor (dLNGFR), a cohort of mice developed leukemia. Molecular analysis revealed vector insertion into the *Evi-1* gene, which was expressed by the vector LTR in all leukemic cells. In this case of insertional mutagenesis, the transgene product was hypothesized to affect cell growth signaling in cooperation with other receptors.

The lack of mutational events in the patients involved in earlier gene transfer trials may be related to the inefficiency of the gene transfer methods, as well as lack of strong selective expansion of gene modified cells in other situations than X-linked SCID. Also, if a mutation leads to extinction of the gene-modified cell, no harmful effects develop. Moreover, as retroviral insertion is monoallelic, recessive mutations will not penetrate; only more rare dominant effects may be seen. As single mutational events are not known to induce overt malignancy, vector induced mutations, even if affecting genes involved in malignancy, most likely need other pre-existing or accumulating mutations to produce a full malignant phenotype. To draw conclusion on mutational risks from integrating gene therapy therefore requires years of follow-up. Currently, the two tragical cases of treatment induced leukemia

in children participating in an otherwise successful gene therapy trial for X-linked SCID make it necessary to perform detailed studies of these cases of leukemia, as well as of the other patients in the trial before stem cell based clinical trials using integrating vectors for other diagnoses can be considered.

Aims of the present studies

The general aim of the present studies was to develop methods to efficiently transfer genes to hematopoietic stem cells using oncoretroviral vectors. The specific aims were the following:

- To determine whether the long-term bone marrow culture transduction system could be efficiently used for oncoretroviral gene transfer to murine long-term repopulating hematopoietic stem cells, particularly when transplanting gene marked cells to non-myeloablated recipients.

- To optimize conditions for oncoretroviral gene transfer to primitive human hematopoietic cells.

- To reveal mechanisms interfering with efficient gene transfer to primitive human hematopoietic cells.

- To compare oncoretroviral vectors with three different envelopes regarding their ability to transfer genes to primitive human hematopoietic cells assayed as NOD/SCID repopulating cells.

Summary of papers

Long-term bone marrow culture (LTBMC) transduction (Paper I)

The promising results of efficient gene transfer to primitive hematopoietic cells reported by Ian Dubé's group in Toronto using human cells in vitro (Dube, et al., 1996) and, particularly, in their canine autotransplant model (Bienzle, et al., 1994) formed the background for our interest in exploring this transduction method. Moreover, the theoretical advantages of this method (as discussed earlier) seemed attractive in the perspective of the low efficiency of gene transfer to repopulating cells achieved in clinical trials with standard cytokine stimulation using IL-3, IL-6 and SCF (Dunbar, et al., 1995).

We adopted the LTBMC transduction method from Dubé for use in the murine system as described in *Paper I.* Briefly, bone marrow cells from mice were harvested and long-term cultures established. A retroviral vector encoding for EGFP, MGiRL22Y (Persons, et al., 1997), with an ecotropic envelope, was used to transduce the hematopoietic cells. These were cultured for 3 weeks without addition of exogenous cytokines and exposed to vector-containing medium twice, as modified by Dubé and coworkers in their clinical study (Stewart, et al., 1999). However, our results showed disappointingly low gene transfer efficiency in vitro with 22.2 % of CFU-GM expressing EGFP. Furthermore, transgene expression in vivo was seen only up to 4 weeks after transplantation into myeloablated animals, and at very low levels. When large numbers of LTBMC transduced cells (20 or $100x10^6$ input cells) were transplanted to unconditioned recipients, no engraftment of these cells was seen, whereas transplantation of corresponding doses of fresh cells yielded donor cell chimerism up to 20 %. In addition, a few mice died from respiratory distress following intravenous injection of large numbers of LTBMC derived cells. Autopsy revealed abundant thromboembolic material in central blood vessels of the lungs. Competitive repopulation experiments comparing LTBMC cells to fresh cells showed a marked loss of repopulating activity following 3-week culture. When the low recovery of cells from the LTBMCs was taken into account, the long-term repopulating ability of these cells was reduced to 7 % compared to fresh cells. This result was in close concordance with a report from similar experiments with murine bone marrow cells (van der Sluijs, et al., 1993). The low recovery of cells in our experiments (mean 21.6 %) from the LTBMCs was very similar to what was reported from Dubé's group using canine (12 and 15 %) (Bienzle, et al., 1994, Lutzko, et al., 1999) or human cells (28 and 25%) (Dube, et al., 1996, Stewart, et al., 1999). Most

importantly, their clinical gene marking trial ended up with disappointing results showing only low levels of gene marked cells in the bone marrow, too low for therapeutic use in gene correction trials, but detectable by molecular methods (Stewart, et al., 1999). In that trial, proviral DNA could be detected by PCR in 9.8 % (3 months) and 2.3 % (24 months post transplantation) of CFU-GM from bone marrow in a minority of the patients. In the hematopoietic pool at large, the frequency of gene modified cells was between 0.01 % and 1 % as determined by semi-quantitative PCR. In summary, in our experiments, the LTBMC based gene transfer method lead to low level transduction of progenitors, no gene transfer to in vivo repopulating cells and a reduced long-term repopulating ability of transduced cells in both myeloablated and non-ablated recipient animals, respectively. It is not entirely clear why the results were greatly superior in early reports utilizing the canine model (Bienzle, et al., 1994, Carter, et al., 1992). Later experiences in α-L-iduronidase deficient dogs showed low engraftment levels of LTBMC transduced BM cells (Lutzko, et al., 1999). Although the provirus could be detected in up to 6 % of BM derived CFU-GM by PCR, no expression of the therapeutic transgene was seen in peripheral blood or in the bone marrow of transplanted animals. This latter phenomenon was considered to be caused partly by an immune response against the transgene product.

What paper I adds:
In contrast to what was believed when our study started, we could show that the long-term bone marrow culture transduction method led to a low level of gene transfer to hematopoietic progenitors in vitro. Furthermore, transgene expression in vivo was detected only short-term and the procedure dramatically decreased the repopulating ability of cells subjected to LTBMC both in myeloablated and non-myeloablated recipients.

Retroviral transduction of CD34$^+$ cells is inhibited by vector containing medium

*(*Paper II*)*

Our aim was to study oncoretroviral vectors with different envelopes in their ability to transfer genes to primitive human hematopoietic cells. For this purpose, we used the MGIN vector (Cheng, et al., 1997), an MSCV based vector encoding for the gene for EGFP, and made packaging cells producing vector with the amphotropic (GP+envAM12 cells) (Markowitz, et al., 1988), VSV-G (293GPG cells) (Ory, et al., 1996) or the GALV envelope

(PG13 cells) (Miller, et al., 1991). $CD34^+$ cells from umbilical cord blood were cultured with the cytokines FL, SCF and MGDF (TPO) throughout the 96-hour culture, exposed to vector in wells coated with the fibronectin fragment CH-296 (Retronectin) at 48 hrs and analyzed for GFP expression by flow cytometry another 48 hrs later. Surprisingly, if transduction was carried out with the cells suspended in vector containing medium (VCM), optimal transduction was achieved when the vector was diluted approximately 10-fold, yielding a multiplicity of infection (MOI) of 1-3 with a bell-shaped curve shown in Fig. 1 of *Paper II*. This effect was most prominent for the GALV pseudotyped vector. When transduction was carried out in Retronectin-coated wells preloaded with vector (PL), the most efficient transduction was seen if cells were added in medium without vector. The transduction efficiency (TE) was gradually reduced if more vector was added in the medium and reaching the lowest level if cells were added in undiluted VCM. This inhibitory effect from VCM was again most pronounced for GALV vectors (TE 68 vs. 25 % of total cells) but significant also for amphotropic preparations. We could show that the inhibitory effect was envelope-receptor specific; i.e. PL transduction with GALV vector was inhibited only by GALV VCM and not by vectors with amphotropic or VSV-G envelopes. Indeed, using vector with different envelopes simultaneously in one and the same transduction did increase gene transfer, just as could be expected when different receptors were utilized for gaining entry into the cell. There were no signs of non-specific toxic effects from VCM underlying the inhibitory effect on the target cells. Interestingly, supernatants from PG13 packaging cell lines not containing vector, and thus producing only empty viral particles, caused an identical inhibition of gene transfer as did GALV VCM.

What was the mechanism underlying the inhibitory effect from VCM?

There could be a number of explanations to the observed inhibition of transduction. Production of TGF-β by vector producing clones has been shown to inhibit gene transfer to $CD34^+$ cells (Xu, et al., 1994), but we found the same inhibitory effect in multiple different clones making this explanation less likely. Inhibition of gene transfer caused by proteoglycans, as described by Le Doux *et al.* (Le Doux, et al., 1996), was also considered but found unlikely. Instead, the results from our experiments, particularly the cross-inhibition experiments, strongly suggest that the inhibitory phenomenon involved interactions between vector envelopes and their receptor. As amphotropic receptors (Orlic, et al., 1996) and, to some extent, GALV receptors, are expressed at low levels on primitive human hematopoietic

progenitors (Kavanaugh, et al., 1994, Kurre, et al., 2001), competition at the receptor level between functional and non-functional vector particles could most likely contribute to the observed inhibitory effect. Using amphotropic vectors to transduce hematopoietic cells, MacNeill *et al.* reported interference on the receptor level when two different amphotropic vectors were used simultaneously, indicating that the level of MLVAR receptor expression was limiting transduction (MacNeill, et al., 1999). No interference was seen if amphotropic and GALV vectors were used concurrently.

Other groups have shown that non-transducing particles from amphotropic vector producer cells can cause inhibition of gene transfer to cell lines in a way similar to our findings with GALV and amphotropic vectors. Seppen and co-workers found that the amphotropic producer cell PA317 secreted inhibitor(s), which co-purified with vector particles, but did not contain RNA, and interfered with transduction (Seppen, et al., 2000). In a paper by Slingsby *et al.*, amphotropic producers were shown to generate vector preparations with a similar titer but huge differences in their ability to transduce cells at high MOI (Slingsby, et al., 2000). They demonstrated that inhibition of transduction could be explained by high level production of envelope components compared to gag/pol proteins in certain cell lines.

In contrast to the situation for GALV and amphotropic vectors, VSV-G pseudotyped vectors use abundantly present membrane phospholipids for cell entry. As expected, in our experiments, no inhibition of gene transfer was seen from these vectors at high concentrations. In accordance, Arai *et al.* reported dose-dependent gene transfer using VSV-G pseudotyped vectors to transduce cell lines (Arai, et al., 1999). In contrast, our results as well as those reported by others (Arai, et al., 1999, Slingsby, et al., 2000) show an optimal MOI between 1 and 3 when using amphotropic vector particles, whereas at the highest vector concentrations transduction rates decline.

Finally, we could show that the inhibitory effect from high titer GALV VCM was seen not only in the CB $CD34^+$ cells and CFU-GM, but also when transducing more primitive CB $CD34^+CD38^{low}$ cells and $CD34^+$ cells from BM. To study whether the inhibition described was relevant also for primitive human cells with NOD/SCID repopulating ability, we transplanted GALV vector transduced $CD34^+$ CB cells to sublethally irradiated mice. The inhibitory effect from GALV VCM could be demonstrated also at the SRC level, showing that the phenomenon was not restricted to less primitive progenitors in vitro, but relevant at the level of primitive repopulating cells as well. This suggests that the effect may be of

importance for and should be taken into consideration when designing clinical transduction protocols using retroviral vectors.

What paper II adds:

Retroviral vector mediated gene transfer to human hematopoietic cells was inhibited by a factor in GALV and amphotropic vector containing medium. Efficient transduction of CB $CD34^+$ cells was achieved by preloading of vector to Retronectin surface; further addition of vector in the medium reduced transduction efficiency. The results suggested that the inhibitory effect could be explained by interference phenomena at the level of the retroviral receptor on the target cell. Using a simplified transduction protocol with only a single exposure to vector, high efficiency gene transfer to CB derived SRC could be achieved.

Overexpression of GALV receptor on hematopoietic cells **(Paper III)**

Having described the inhibitory effect of GALV vector VCM on oncoretroviral vector transduction of human hematopoietic progenitor, we wanted to study the underlying mechanisms in more detail. The results from *Paper II* led us to the hypothesis, that the inhibition was caused by interference on the GLVR1 receptor level between functional and non-functional viral particles and that this effect could be overcome by increasing expression of the receptor, which was done in two different ways. First, target cells were stimulated by the phorbol ester PMA (phorbol 12-myristate 13-acetate), which is known to increase the expression of GLVR1 and MLVAR in hematopoietic cell lines through stimulation of protein kinase C (PKC) (Sabatino, et al., 1997). Second, in order to more specifically analyze the effect of increased GLVR1 expression, we generated an MSCV based vector, MGLIG, coding for GLVR1 followed by IRES and GFP, which was produced with amphotropic envelope. For the final readout we made another MSCV vector, MYIN with the gene for the yellow fluorescent protein (YFP), which was produced by PG13 cells to yield vector particles with GALV envelopes. FACS analysis separating the signals from GFP and YFP was performed as described (Stull, et al., 2000).

Initially, we demonstrated that the inhibitory effect from high-titer GALV VCM seen with MGIN preparations in transduction of CB was also seen when transducing K562 cells, and when using the new MYIN vector to transduce cord blood cells. Interestingly, when a vector pseudotyped with feline endogenous virus envelope RD114 was used, which utilizes a

different receptor for cell entry, no inhibitory effect was seen, similar to what was shown for VSV-G pseudotyped vectors in *Paper II*. This may be explained by relative abundance of the RD114 receptor (RDR) on hematopoietic progenitors compared to GLVR1 and MLVAR or, alternatively, by a lower level of defective vector particles in these vector preparations.

Receptor interference blocks gene transfer in multiple hit protocols

In order to study to what extent the inhibitory effect from VCM persisted over time, we performed PL transduction of CB cells suspended in VCM, which had been incubated at 37°C for different periods of time. We found a virtually unchanged inhibitory activity from VCM, showing that the components responsible for inhibition of transduction were not degraded during the 48 hours studied, despite loss of transduction-competent vector particles. We thereafter showed that exposure of CB cells to vector partially blocked a second hit 24 hours later with vector with the same envelope in a two-hit gene transfer protocol. No interference was seen if vectors with different envelopes were used for the two transductions. These findings have important implications for the design of clinical gene transfer protocols, routinely involving multiple rounds of exposure to a single type of vector, which may not be the optimal way to transduce cells. For example, Hennemann *et al.* reported no increase of transduction efficiency if human BM cells were exposed to GALV vector three times instead of one (Hennemann, et al., 2000). Receptor interference has been well described in the context of wild-type, replication competent retroviruses (Rein, et al., 1982), but was also demonstrated by MacNeill *et al.* to persist for up to 24 hours using amphotropic vector preparations (MacNeill, et al., 1999). In our experiments, interference was seen although cells were transduced under optimal (preload) conditions.

Overexpression of GLVR1

In order to overexpress GLVR1, we first studied CB cells stimulated with PMA for different periods of time, thereby increasing GLVR1 mRNA up to 21-fold over baseline levels after 6 hours of PMA stimulation as determined by Q-RT-PCR. When PMA was added to CB cells at transduction, no inhibitory effect from GALV VCM was seen and, in fact, a slight but consistent increase of transduction was observed also in the group subjected to PL transduction.

Next, to overexpress GLVR1 in a more specific way and to study the consequences of this overexpression, K562 or CB $CD34^+$ cells underwent transduction with amphotropic MGLIG

vector followed by exposure to GALV MYIN in a 96-hour protocol. FACS analysis determining expression of GFP and YFP separately revealed that the inhibitory effect from GALV VCM was completely abolished in cells overexpressing GLVR1 (and GFP), but persisted in GFP^- cells as well as in control vector transduced cells.

Thus, in support of our hypothesis, PMA or retrovirally mediated overexpression of GLVR1 increased gene transfer to hematopoietic cells and, most notably, the inhibitory effect from VCM could be overcome.

What paper III adds:

The results obtained from increasing the GALV receptor on hematopoietic cells by PMA treatment or retroviral overexpression show, that low expression of GLVR1 limits GALV vector mediated transduction on Retronectin. The inhibitory effect from vector containing medium could be completely abolished by GLVR1 overexpression. Our results strongly suggest that interference at the receptor level between functional and non-functional vector particles, in combination with low levels of receptor expression on the target cells, underlie the observed inhibitory effect from VCM. Furthermore, we demonstrated that interference phenomena can reduce the effects of repeatedly exposing target cells to vector in multiple hit transduction protocols, which may have important practical implications.

Comparison between retroviral vectors with three different envelopes **(Paper IV)**

Transduction of primitive hematopoietic progenitors

There is no report comparing retroviral vectors with GALV, amphotropic and VSV-G envelopes with respect to their ability to mediate gene transfer to human hematopoietic cells with repopulating ability. We used a GFP expressing vector (MGIN) and generated vector producing cells to get preparations with all three envelopes. First, CB $CD34^+$ cells were sorted based on their expression of CD38 (high or low/negative) and transduced at 0 to 96 hours to study the time course of transduction. Efficient transduction of the less primitive $CD34^+CD38^+$ cells was seen already after a prestimulation period of 24 hours, whereas the primitive $CD34^+CD38^{low}$ cells needed 48-72 hours of cytokine stimulation to become efficiently transduced. These results are in agreement with the report by Hennemann *et al.*, who found that that the first cell divisions occurred after 24-48 hours and that approximately 90 % of the clones to be recruited into proliferation were activated by day 4 (Hennemann, et

al., 1999). We found that the GALV pseudotyped vector was the most efficient in transducing $CD34^+CD38^{low}$ CB cells (62 % GFP^+ cells), followed by the amphotropic vector (39 %) and VSV-G pseudotyped vector (27 %).

Transduction of NOD/SCID repopulating cells – comparison of vector envelopes

Based on the results above, our protocol for transduction of SRCs from CB was designed to include a first exposure to vector preloaded to Retronectin after 48 hours of prestimulation, followed by a second hit merely by adding $1/10^{th}$ volume of vector to the cells, and harvest of transduced cells for transplantation and clonogenic assays after 96 hours of culture. The technique performing the second hit with vector was based on our findings of optimal transduction at 1:10 dilution of GALV and amphotropic vectors as shown in *Paper II*, Fig 1. The expanded equivalent of $2.5x10^5$ $CD34^+$ CB cells was injected intravenously into sublethally irradiated NOD/SCID mice, which were analyzed by FACS 6-8 weeks following transplantation for human cell engraftment, lineage distribution and transgene expression.

In addition to comparing vectors with the three envelopes separately in their ability to transfer genes to human cells with NOD/SCID repopulating ability, we wanted to test transduction using all three envelopes simultaneously. This could have theoretical benefits, and has been suggested (MacNeill, et al., 1999), as the vectors use different receptors to gain entry into the target cells. Furthermore, as FCS may negatively affect expansion of primitive cells (ELTC-IC) (Ramsfjell, et al., 1999) and reduce their long-term repopulating activity in vivo (Bryder and Jacobsen, 2000), it would be desirable to perform transductions under serum-free conditions. However, amphotropic vector preparations could not be harvested at high titer, as reported previously by others (Conneally, et al., 1998), and transduction using VSV-G pseudotyped vector without FCS was inefficient yielding transduction rates of only 3.9 % GFP^+ $CD34^+$ CB cells. Therefore, only the GALV vector was used in the absence of serum.

Interestingly, despite markedly different gene marking efficiency to $CD34^+$ cells and primary CFU-GM, vectors with all three envelopes were equally efficient in mediating gene transfer to human CB cells with NOD/SCID repopulating ability when transductions were carried out in the presence of serum (25-33 % GFP^+ human cells). The levels of human cell engraftment in the BM of NOD/SCID mice were also equal among the three groups, but significantly lower than obtained with freshly thawed cells.

As expected, gene transfer efficiency to $CD34^+$ cells, CFU-GM and SRCs was very high in the Mix group, in which vectors with all three envelopes were used simultaneously. However, a dramatic loss of CD34 expression was seen, which was paralleled by a significant loss of

engraftment capacity. The reasons for this could not be fully explained. One reason could be toxic effects from high levels of GFP expression in the cell, which seems unlikely as hematopoietic cells subjected to adenoviral transduction tolerate higher GFP levels. Toxic effects from multiple vector integration events could potentially contribute. It seems, however, more likely that simultaneous occupancy of the retroviral receptors GLVR1 and MLVAR negatively affects the cells by disturbing sodium dependent phosphate transport, as some cell types are known to be highly sensitive to this type of manipulation (L Pedersen, Aarhus, personal communication).

Transduction using the GALV pseudotyped vector was highly efficient with limited differences in gene transfer rates *in vitro* between results obtained with and without serum, respectively. In the serum-free group, 46.2 ± 16.6 % of the human cells in the NOD/SCID BM were GFP^+ and gene transfer efficiency to secondary human CFU-GM cultured from mouse bone marrow was 55.5 ± 22.7 %. There was a clear trend towards higher level of engraftment, GFP^+ human cells and GFP^+ CFU from the BM of NOD/SCID mice in the serum-free group, although the differences did not reach statistical significance. Moreover, the percentage of gene marked cells in the BM of NOD/SCID mice, which is affected by the gene transfer rates and the levels of engraftment, were the highest in the group subjected to serum-free GALV vector transduction, reaching levels two-fold higher than in the corresponding group with FCS, but this difference was not statistically significant. To study whether transduction and culture with the three cytokines used in our studies affected the frequency of SRC, transplantation experiments comparing freshly thawed and transduced $CD34^+$ CB cells were carried out at limiting dilution. The frequencies of SRC, calculated using Poisson statistics (Taswell, 1981), were found to be very similar, 1 in 39700 $CD34^+$ CB cells in the transduced group and 1 in 43600 in the group with freshly thawed cells and are in agreement with previous reports (Ueda, et al., 2000). The mean levels of engraftment were somewhat lower for the transduced cells, reflecting a slightly reduced proliferative capacity among these cells. These data show that there was no loss of SRC during four-day culture and transduction, a very important feature, which is frequently overlooked in gene transfer studies.

What paper IV adds:

In a direct comparison of identical vectors with GALV, wild-type amphotropic and VSV-G envelopes, efficient gene transfer to human CB derived NOD/SCID repopulating cells could be demonstrated. In the presence of serum, vectors with all three envelopes were equally efficient in mediating gene transfer to SRCs, but engraftment of gene modified cells in the bone marrow of NOD/SCID mice was reduced compared to uncultured cells. Using a GALV pseudotyped vector under serum-free conditions, highly efficient gene marking of SRCs could be achieved. Moreover, as determined by transplantation experiments at limiting dilution, the serum-free transduction procedure was not associated with any loss of SRCs.

General discussion

Receptor interference, viral titer and transduction of primary hematopoietic cells

Quantification of transduction competent vector particles in retroviral preparations usually relies on determination of end-point titer. To determine the titer of a vector preparation, cell lines are transduced at a low concentration of vector, where the relationship between vector particle number and their ability to transduce cell lines is believed to be linear, i.e. every vector particle is assumed to transduce one cell. The vector titer calculations in our experiments are based on the frequency of GFP expressing cells following transduction of a human cell line, HT1080, at a certain dilution of the vector preparation. A classical way to determine vector titer relies on the transfer of the neomycin resistance gene (*neo*) to cell lines. When cultured in the presence of G418, only cells expressing the *neo* gene will grow and the vector concentration can be calculated based on the number of colonies and the dilution of vector. An alternative way to analyze vector in a preparation is to perform RNA dot blot analysis contents (used in *Paper I* in addition to titration), which may be a method of choice to semiquantitatively screen vector producer clones lacking selectable markers (Onodera, et al., 1997). In general, efforts have focused on achieving a maximal vector titer, usually 10^5-10^7 infectious particles per ml depending on the type of vector and on the type of cell line used for titration. Vectors with stable envelopes, e.g. VSV-G and RD114, can be concentrated 100-fold by ultracentrifugation, but this method is not compatible with preserved function of amphotropic vectors.

Vector titers can reproducibly be increased 10-30-fold by ultrafiltration with preserved biological activity, but this will not necessarily lead to a proportional increase of

transduction, not even of cell lines (Paul, et al., 1993). Forestell and co-workers showed that end-point titers did not correlate with gene transfer efficiency when amphotropic vectors were used (Forestell, et al., 1995). In contrast to what might have been expected, when testing filtration concentrated vectors with proven high titer, gene transfer rates were clearly inferior to what was obtained using unmanipulated vector, but could be improved by dilution of the vector preparation. Apparently, considering our results, the obstacles when producing amphotropic and GALV vectors seem very similar. In retroviral vector preparations harvested from packaging cell lines, only a small minority of the produced particles are transduction competent (0.5-1 %) (Andersen and Nexo, 1983). Non-functioning particles, as well as shed envelope proteins from degraded vector particles (Bolognesi, et al., 1975) are thus present in great excess of functional particles and compete for receptor occupancy, termed receptor interference. These factors, together with the low expression levels of both amphotropic and GALV receptors on primitive human progenitors, most likely account for the inhibitory effect seen in our experiments in *Papers II* and *III*. This may also explain why transduction using GALV and amphotropic vectors is not optimal at high vector concentrations, but rather at a lower MOI if cells are suspended in diluted VCM as shown in *Paper II* and by others (Arai, et al., 1999). This view is strongly supported by the results presented by Slingsby *et al.*, who compared different amphotropic packaging cell lines, which produced vector preparations with similar titers but with remarkable differences in their ability to transduce cell lines (Slingsby, et al., 2000). They found that the differences could be explained by an imbalance between production of envelope particles and core proteins and that their poorly transducing producer cell line had an eight-fold higher ratio between envelope and gag/pol proteins compared to the better cell line. This inhibition is highly reminiscent of the results from our experiments, particularly with GALV vectors (*Papers II+III*). The somewhat provocative conclusion was, that when vector producing clones giving maximal titer vectors are chosen, one may be mislead, as end point titration does not reveal the inhibitory effects from overexpression of envelope relative to gag/pol components, although these factors may be relevant when transducing cells at high MOI. To get vector-producing cells with optimal ability to transduce cells at high MOI, a balance between the production of envelope and gag/pol is highly desirable but only rarely taken into consideration. The effects from varying the proportions of the different vector components in producer cells were studied in a publication by the same group (Yap, et al., 2000). When all three components (vector genome, envelope, gag/pol) were present in equal amounts, a large number of empty vector

particles lacking genome were produced, which did not affect the titer negatively. In situations with relative lack of envelope components, vector titer was reduced, as expected. Unfortunately, the authors did not test these vector preparations for their ability to transduce cells, which would have seemed logical in view of their previous publication on the subject.
The proportion of non-functional to functional vector in a harvest may also be affected by culture conditions. At 37°C vector production is higher, but the breakdown is also more rapid as compared to 32°C, reflected by a 4-fold increase of half-life and leading to a 5 to 10 times higher titer at the lower temperature (Kaptein, et al., 1997). Interestingly, Forestell *et al.* saw no difference in vector titer from harvest at different temperatures, but could show that supernatants produced at 37°C had lower transduction efficiency on cell lines following concentration, and that this effect could be overcome by diluting the vector (Forestell, et al., 1995). They also demonstrated that the proportion of inactivated vector was higher at 37°C and found a vector half-life of approximately 4 hrs. Thus, the duration of medium incubation on vector producing cells as well as the incubation temperature are important parameters, and may have impact on the transduction of primitive hematopoietic cells. If vector harvest is performed following 24 hours of incubation, at least some vector particles will be non-functional due to spontaneous degradation, but given the high proportion of non-functional particles produced, this fraction may not be of major significance. In summary, the problems with receptor interference and presence of transduction incompetent particles can not be addressed by conventional measurements of end-point titer or by RNA dot blot assays. Standard titration is carried out at a very low concentration of vector, at which there is an excess of cells and available retroviral receptors to vector particles, which makes interference phenomena less likely to occur. In contrast, when undiluted or even concentrated vector is used to transduce cells, vector particles, mostly non-functional, are present in great excess of available receptors making interference phenomena important. Therefore, in order to predict transduction efficiency of retroviral vector preparations, these should be tested functionally at the concentration at which they will later be used and on a relevant cell population, for example $CD34^+$ cells, instead of using cell lines.

Perspectives on retroviral gene transfer to CB derived SRCs

The human-NOD/SCID xenograft model has proven to be an invaluable tool for developing conditions allowing effective gene transfer to primitive human hematopoietic cells with in vivo repopulating capacity. Early studies showed inefficient gene transfer to NOD/SCID

repopulating cells (Larochelle, et al., 1996), but more recently, several groups have reported gene transfer to CB derived SRC, usually marking approximately 20 % of the cells as shown in ***Table 2*** (Bjorgvinsdottir, et al., 2002, Conneally, et al., 1998, Demaison, et al., 2000, Dorrell, et al., 2000, Hennemann, et al., 1999, Kelly, et al., 2000, Marandin, et al., 1998, Novelli, et al., 1999, Rebel, et al., 1999, Schilz, et al., 1998, van Hennik, et al., 1998). A limitation of many of the studies is the lack of quantitative analysis of the repopulating ability of the gene-modified cells, although this is an issue of very high relevance. In the setting of a human clinical trial, particularly if performed without myeloablative conditioning of the recipient, the transduced cells will have to compete with endogenous hematopoietic cells and may easily be extinguished if they do not possess some kind of competitive advantage, as is the case when X-SCID is treated with gene therapy (Cavazzana-Calvo, et al., 2000). Where this issue was addressed, SRC frequency was, at best, maintained (Dorrell, et al., 2000), but in most studies, the repopulating activity of the cultured cells declined considerably over time. In the study by Rebel *et al.* (Rebel, et al., 1999), very rapid loss of SRC was seen following culture, but they were able to transduce repopulating cells already after 1 day of prestimulation. This may appear surprising, considering that the cells had to pass mitosis to be susceptible to transduction, but could be explained provided that the cells contained unintegrated provirus at transplantation and were immediately stimulated to proliferation *in vivo*.

Table 2. Oncoretroviral vector mediated gene transfer to NOD/SCID repopulating cells from CB

Publication	*Vector envelope*	*Transduction of SRC, average % (range)*	*Culture time*	*Repopulating ability of transduced cells compared to non-transduced*	*Comments*
Conneally, 1998	Ampho	17 % neores. 32 % PCR	5 days	NA	SF: 8 % TE
Marandin, 1998	GALV Ampho	15-33 % (PCR) 3 % (FACS)	7 days	NA	Few mice. Cell-free or co-culture transduction
Schilz, 1998	GALV	24 % (5.6-56)	5 days	NA	Spinoculation. SF transduction
Van Hennik, 1998	Ampho GALV	2 % (0-18) 23 % (2-41)	4 days	NA	Different vector backbones. SF transduction.
Hennemannn, 1999	GALV	26.6 % (0.3-72)	6 days	NA	
Novelli, 1999	Ampho	13 % 23 %	4 days or 7 days	Reduced level of engraftment	
Rebel, 1999	VSV-G	25 %	1 or 3 days	↓ SRC frequency after culture	SF transduction
Demaison, 2000	GALV	44 %	4 days	Reduced after 24 h	Spinoculation. SF transduction
Dorrell, 2000	GALV	13 % (0-59)	4 days	Maintenance of SRC	
Kelly, 2000	RD114 Ampho	20 % (0.6-71) 40% (1.8-92) 0.2 % (0-0.6)	4 days	NA	Transduction at 24 or 48 hrs. SF
Gatlin, 2001	RD114	24 % (7-70) by PCR	4 days	NA	Concentrated vector
Björgvinsdóttir, 2002	GALV	10-25 % 5-25 %	5 days 12 days	NA	Engraftment 12 d higher than 5 d
Present investigation, *Paper IV*	GALV	46 % (21-75) (55 % (5-92) of CFU-GM)	4 days	Maintenance of SRC	SF transduction.
	GALV Ampho VSV-G	33% (9-84) 27% (1 -61) 25 % (3-66)	4 days	Reduced level of engraftment.	With serum.

Comparison of reports on oncoretroviral vector mediated gene transfer to human CB cells analyzed using the NOD/SCID xenograft model. NA = not assessed; SF= serum-free; TE = transduction efficiency.

Which envelope is optimal for transduction of human hematopoietic stem cells?

Vectors with the three envelopes used in our *Papers II* and *IV* have not been compared side by side in their ability to transfer genes to primitive human hematopoietic cells. In an early comparison, GALV pseudotyped vectors were found to be superior to amphotropic particles in transducing hematopoietic progenitors from BM or MPB (von Kalle, et al., 1994). Amphotropic and VSV-G pseudotyped vectors have been reported to be equally efficient in transducing hematopoietic progenitors (von Laer, et al., 2000, Yam, et al., 1998). However, as transduction of repopulating hematopoietic cells was not evaluated, only limited conclusions can be drawn from these studies.

Based on promising results using GALV pseudotyped vectors to transduce hematopoietic cells, GALV vectors have been widely introduced as alternatives to amphotropic vectors for human gene transfer purposes. Kiem *et al.* could demonstrate that vectors with a GALV envelope were more efficient than amphotropic vectors in transducing repopulating cells from baboon BM in a competitive repopulation model (Kiem, et al., 1997). In a study by van Hennik *et al.* (van Hennik, et al., 1998), gene transfer rates obtained with GALV and amphotropic vectors were directly compared regarding gene transfer to SRC. In these experiments, the GALV pseudotyped vector was clearly superior in vitro and in vivo, but as not only the envelopes but also the vector backbones differed, the results must be interpreted with some caution.

The feline endogenous virus envelope protein RD114 has been presented as a novel and promising alternative to pseudotype retroviral vectors for gene transfer to human hematopoietic cells. In a report by Kelly *et al.*, retroviral vectors with RD114 or amphotropic envelope were compared regarding their ability to transfer genes to primitive hematopoietic progenitors including SRCs (Kelly, et al., 2000). The RD114 vector could efficiently transduce repopulating cells, although with high variability, and was clearly superior to the amphotropic vector. However, gene transfer efficiency to SRCs using the amphotropic vector was unreasonably low, which makes interpretation of the results difficult. The mean frequency of GFP expression in repopulating human cells was 20-40 % using the RD114 pseudotyped vector, which is comparable to our results with any of the three envelopes tested in our *Paper IV*. RD114 pseudotyped vector mediated significantly higher gene transfer to PB derived SRCs compared to GALV vector in a recent publication; however, although transductions were carried out in the presence of fibronectin, preload transduction was used only with the RD114 vectors, which may have distorted the results (van der Loo, et al.,

2002). Additional support for the use of RD114 pseudotyped vectors comes from a recent presentation in which the receptors for RD114 and feline leukemia virus (FeLV) were found to be expressed at higher levels on $CD34^{+}CD38^{-}$ cells than on unfractionated bone marrow cells (Lee Lucas, et al., 2002). Following transduction and injection of human cells into preimmune fetal sheep, gene modified human cells were found only in animals transduced with RD114 or FeLV vectors, and not detectable in animals transplanted with GALV vector transduced cells. However, it has also been mentioned, without presenting any data, that RD114 pseudotyped retroviral particles transduce SRCs derived from MPB inefficiently (Hanawa, et al., 2002). RD114 and GALV pseudotyped vectors were equally efficient in mediating gene transfer to long-term repopulating BM cells in dogs (Goerner, et al., 2001).

For transduction of hematopoietic cells, lentiviral vectors have mostly been pseudotyped with the VSV-G envelope (Akkina, et al., 1996, Case, et al., 1999, Miyoshi, et al., 1999, Uchida, et al., 1998, Woods, et al., 2000). However, lentiviral vectors can also be generated with alternative envelopes. Recently, lentiviral vectors with the amphotropic, the VSV-G and the RD114 envelopes were compared regarding transduction of human MPB hematopoietic progenitors (Hanawa, et al., 2002). Somewhat surprisingly, vectors with an amphotropic envelope were found to transduce SRCs more efficiently than did vectors with VSV-G or RD114 envelopes. The frequency of GFP expression in human cells in the NOD/SCID BM averaged 11.6 % following transduction with amphotropic vector, 4.7 % if transduced by VSV-G vector but only 1.6 % following RD114 transduction.

Thus, in summary, the data regarding which retroviral envelope is most suitable for mediating efficient retroviral gene transfer to human hematopoietic stem cells are somewhat conflicting. Our data, as presented in *Paper IV,* show equal gene transfer efficiency using vectors with GALV, amphotropic and VSV-G envelope, but suffer from the lack of comparison to RD114 vectors and from the fact that only GALV could be used under serum-free conditions. Data from non-human primates clearly show that GALV vectors can mediate efficient transduction of long-term repopulating hematopoietic progenitors (Kiem, et al., 1998), and that the GALV envelope seems superior to the amphotropic one in that context (Kiem, et al., 1997). GALV vectors can be used to efficiently transduce NOD/SCID repopulating cells under serum-free conditions as shown by us in *Paper IV* and by others (Demaison, et al., 2000, Kelly, et al., 2000, Schilz, et al., 1998, van Hennik, et al., 1998).

However, despite limited success in early clinical trials, amphotropic vectors have been used to transduce repopulating hematopoietic cells from PB with high efficiency in rhesus monkeys (Wu, et al., 2000). These results were obtained with transduction in the presence of serum, but, more recently, equally efficient gene transfer with amphotropic vectors to rhesus monkey repopulating cells was obtained regardless of whether cells were transduced in the presence or absence of serum (Kluge, et al., 2002). Moreover, the successful results in the French and Italian gene therapy trials in SCID were obtained using retroviral vectors with amphotropic envelopes (Aiuti, et al., 2002a, Cavazzana-Calvo, et al., 2000). It remains to be established whether RD114 pseudotyped vectors offer any advantages over the envelopes used this far.

Conclusions

Conclusions that can be drawn from *Papers I-IV*:

- Long-term bone marrow culture transduction is inappropriate for retroviral gene transfer to hematopoietic cells, as the method is inefficient for and leads to loss of repopulating cells *(Paper I)*.

- Retroviral gene transfer to primitive human hematopoietic progenitors on fibronectin is inhibited by factors in GALV and amphotropic vector-containing medium *(Paper II)*.

- The inhibitory effect from GALV vector medium is most likely caused by a combination of non-transducing vector particles present in the medium and low levels of the GLVR1 receptor, i.e. interference phenomena *(Papers II+III)*.

- Due to interference phenomena following exposure of hematopoietic cells to retroviral vector, the contribution to overall gene transfer from subsequent hits may be blocked *(Paper III)*.

- Human umbilical cord blood cells with capacity to establish hematopoiesis in the bone marrow of NOD/SCID mice can be transduced with equal efficiency using retroviral vectors with GALV, amphotropic and VSV-G envelope *(Paper IV)*.

- Highly efficient retroviral transduction of human umbilical cord blood cells repopulating the bone marrow of NOD/SCID mice can be achieved using GALV pseudotyped vector under serum-free conditions. Importantly, this procedure can be carried out without loss of cells with NOD/SCID repopulating capacity *(Paper IV)*.

Future perspectives

As a consequence of the two serious adverse events in the French trial enrolling children with X-linked SCID, safety issues are now of highest priority for gene therapy based on integrating vectors. It remains to be elucidated, whether the apparently high risk for treatment-induced leukemia is in some way specific for X-linked SCID or not. What is the role of the delivered transgene (γ-chain) and the very strong selective proliferation of gene-modified T-cells mediated by restoration of cytokine signaling? Why was the vector found inserted into and driving expression of the LMO2 gene in the leukemic cells? This gene is known to be associated with T cell leukemia. Are there insertions into other potential oncogenes, which are not expressed in this context but might be problematic in treatment of other diseases? Further molecular analyzes and long-term follow up will be needed to answer these questions. It will be difficult to start new clinical trials using integrating vectors before at least some of these questions are answered.

Although possibly problematic in X-linked SCID, some selective advantage of the gene modified cells is likely to be necessary for success in future gene therapy trials. Considering that the fraction of cells subjected to gene transfer in vitro is very small compared to the remaining pool of hematopoietic cells, a technique to expand gene-modified cells in vivo in a safe way is highly desirable, and might obviate the need for myeloablation. In this context, the issues of homing and engraftment of cultured and gene-modified cells are also essential. Cell cycle inhibitors, such as SDF-1, and manipulations of adhesion molecules on the surface of graft cells to improve homing may be interesting to explore.

Furthermore, methods to expand HSC *in vitro* without depletion of any stem cell properties would be highly useful for the field of oncoretroviral based gene transfer. Novel approaches such as signaling via Notch receptors have been shown to promote expansion and inhibit differentiation of primitive hematopoietic cells (Stier, et al., 2002, Varnum-Finney, et al., 2002, Varnum-Finney, et al., 2000). Bone morphogenetic proteins (BMPs), which belong to the transforming growth factor-β superfamily (TGF-β), are involved in the regulation of proliferation in primitive hematopoiesis. Treatment of human hematopoietic cells with BMP-4 has been shown to affect differentiation and proliferation of cells capable of repopulating the bone marrow of NOD/SCID mice (Bhatia, et al., 1999). Similarly, also involving BMP signaling, proliferation of primitive hematopoietic cells has been shown following treatment

with Sonic Hedgehog protein, suggested to be potentially useful for stem cell expansion (Bhardwaj, et al., 2001).

Lentiviral vectors seem promising for transduction of resting cells; however, safety issues, not only related to the risks of insertional mutagenesis, need to be addressed, and there is currently no experience from gene therapy trials in humans with this vector system. If stem cell expansion can be achieved in some way, the obvious advantages of lentiviral compared to oncoretroviral vectors may be lost. It also remains to be settled which viral envelope is optimal for transduction of human HSCs, especially using lentiviral vectors. For oncoretroviral vectors, some data suggest that RD114 pseudotyped vectors offer advantages over GALV and amphotropic vectors, but this remains to be confirmed.

For many disorders, tissue restricted expression of the transgene is sufficient, for example expression limited to the erythroid lineage in treatment of hemoglobinopathies. This can already be achieved with some success using tissue specific promoters driving the transgene. Targeted insertion of the provirus into "safe" parts of the genome to avoid integration-related side effects such as insertional mutagenesis would be highly advantageous, but is currently not possible.

Populärvetenskaplig sammanfattning på svenska

Blodbildande stamceller förekommer i mänsklig benmärg i en frekvens på ca 1/100 000 celler. Med hjälp av cellytemarkörer, t.ex. CD34, kan man rena fram cellpopulationer som är anrikade på stamceller, men det saknas exakt kunskap om hur enskilda stamceller ser ut. De blodbildande stamcellerna har förmåga att vid celldelning antingen förnya sig själva eller att mogna ut. Stamcellerna kan mogna ut och föröka sig till alla typer av blodceller: röda blodkroppar, olika typer av vita blodkroppar och blodplättar (trombocyter).

Genterapi är en experimentell behandlingsform som bygger på att nytt genetiskt material förs in i celler. Man har kommit längst inom genterapi riktad mot ärftliga sjukdomar som drabbar blodbildande stamceller. Teknikerna har prövats i djurförsök och de senaste 10 åren också i försök att behandla sjukdomar hos människor. År 2000 rapporterades det första stora genombrottet inom genterapi vid behandlig av en ovanlig immunbristsjukdom (SCID). Man utnyttjar s.k. retrovirus, som har förmåga att införa sitt genmaterial i celler som infekteras. Dessa virus har modifierats till sk. vektorer, som innehåller en önskad gen, och kan infektera t.ex. stamceller och då föra över genmaterial en gång, men sedan inte föra infektionen vidare. Fördelen med retrovirala vektorer är att de permanent kan föra in nytt genetiskt material i celler som infekteras (transduceras), men detta är också en nackdel eftersom det medför en risk för oönskade genförändringar i mottagarcellerna. En annan nackdel är att dessa virus kräver att mottagarcellen genomgår celldelning för att kunna transduceras. Eftersom blodstamceller i vanliga fall sällan delar sig, måste de odlas och stimuleras med olika tillväxtfaktorer för att bli mottagliga för retrovirus. Odlingsproceduren medför risk att cellerna mognar ut och förlorar sina unika stamcellsegenskaper, eller att deras förmåga att slå an i benmärg efter transplantation försämras.

Målet med denna avhandling har varit att vidareutveckla metoder för retrovirusbaserad genöverföring till mänskliga blodstamceller i syfte att i framtiden kunna erbjuda genterapibehandling till patienter med olika blodsjukdomar. Vi har använt vektorer som innehåller en markörgen, GFP, green fluorescent protein, som blir självlysande i ultraviolett ljus och kan påvisas med hjälp av mikroskop, flödescytometri eller molekylära metoder.

I ***arbete I***, som utfördes med musceller, utvärderades en genöverföringsmetod som bygger på långtidsodling av blodbildande celler på en matta av bindvävsceller från benmärg med

tillförsel av vektor vid två tillfällen. Efter 3 veckor skördades cellerna och transplanterades till mottagarmöss. Våra resultat visade att genöverföringen var föga effektiv och att de genmodifierade cellerna förlorade mycket av sin förmåga att slå an och växa ut i mottagardjurens benmärg.

I ***arbetena II-IV*** undersöktes genöverföring till mänskliga blodbildande celler, vilka efter genöverföring tranplanterades till immundefekta möss (NOD/SCID-stam), vilka har förmåga att understödja mänsklig blodbildning och som anses vara den bästa tillgängliga metoden att analysera blodstamceller. I ***arbete II*** studerades vektorer som försetts med virushöljen från tre olika virus avseende förmågan att föra över gener till blodbildande celler. Vi fann då att genöverföringen med ett virushölje (från Gibbon Ape Leukemia virus) hämmades under vissa förhållanden. Sannolikt kunde hämningen orsakas av förekomst av icke-fungerande viruspartiklar samt låga nivåer av receptorer för retrovirus på cellytan, och vi utarbetade en effektiv metod för genöverföring. I ***arbete III*** studerades denna hämning vidare och vi undersökte effekterna av att på olika sätt öka förekomsten av receptorer för GALV-virus på de mänskliga blodcellernas yta. En slutsats som kunde dras var att låga nivåer av GALV-receptorer på de humana blodstamcellerna var en väsentlig faktor som bidrog till att förhindra effektiv genöverföring med retrovirala vektorer.
I ***arbete IV*** jämfördes slutligen tre retrovirala vektorer som försetts med olika höljeproteiner avseende förmågan att föra över en markörgen till humana blodstamceller transplanterade till NOD/SCID-möss. Trots betydande skillnader i genöverföring vid analys av celler omedelbart efter odling var genöverföringsfrekvensen till primitiva (stam-) celler i NOD/SCID benmärg lika för alla tre vektortyperna. Dock kunde endast GALV-vektorn användas under odlingsbetingelser utan serum, vilket är eftersträvansvärt. Genöverföring till ca 50 % av mänskliga blodbildande stamceller kunde uppnås med GALV-vektor utan serum, då man också fann välbevarad förmåga hos de genmodifierade cellerna att transplanteras och ge upphov till ny blodbildning.

Sammanfattningsvis har vi utvecklat metoder för effektiv retroviral genöverföring till blodbildande stamceller hos människa. Dock hämmas behandlingsmetodens praktiska tillämpning av två nyligen rapporterade fall av behandlingsutlöst leukemi hos patienter behandlade med genterapi pga. en ärftlig immunbristsjukdom.

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Appendix: Papers I-IV