Immunodeficient mouse models for the quantitative evaluation of normal, genetically marked, and malignant human hematopoietic stem cells

Monique Maria Andrea Verstegen

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IMMUNODEFICIENT MOUSE MODELS FOR THE QUANTITATIVE EVALUATION OF NORMAL, GENETICALLY MARKED, AND MALIGNANT HUMAN HEMATOPOIETIC STEM CELLS

Immunodeficiënte muismodellen voor quantitatieve evaluatie van normale, genetisch gemarkeerde en maligne hematopoietische stam cellen

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Front cover designed by Ellen Nijs
'Solitary trees, if they grow at all, grow strong' *Sir W. Churchill*

Het heurt allemaol biej het laeve.
Auch het kruuts op diene rök.
Waat dich net zo good waert gegaeve,
As dien dageliks gelök
Toos Benjamin

Voor pap en mam

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I

GENERAL INTRODUCTION

Hematopoiesis

Introduction

Mature blood cells have a finite life span and are continuously replenished by the proliferation and differentiation of lineage-specific progenitor cells derived from rare pluripotent hematopoietic stem cells.[1, 2] Stem cells are capable of reproducing them-selves (self-renewal) and of producing cells belonging to all lineages of blood (hematopoietic) cell differentiation (Figure 1.). Definitions of stem cells vary on the model of hematopoiesis used but, in general, these cells are referred to by the functional property of being capable of long-term reconstitution of the hematopoietic system of recipients.

Bone marrow transplantation (BMT) is potentially used for the treatment of hematopoietic disease such as leukemia, autoimmune diseases and to improve hematopoietic recovery and prevent mortality after intensive treatment of cancer patients. The purpose of BMT is to provide the recipient with a new, permanently functioning hematopoietic system. As has been shown in transplantation assays in mice, hematopoietic recovery after BMT develops through 2 distinct phases with the initial wave of hematopoiesis being brought about by stem cells with short term repopulating ability (STRA) with transient and restricted potential and the second phase of sustained multilineage hematopoiesis being established by stem cells with long-term repopulating ability (LTRA).[3, 4]

The majority of hematopoietic stem cells *in vivo* are quiescent and in the G0 phase of the cell cycle (as demonstrated by S-phase specific or cycle-dependent agents), only a small number of hematopoietic stem cells are actively proliferating.[5] Candidate stem cells possess a range of phenotypic properties that can be exploited in stem cell purification and enrichment procedures. These include the expression of the cell surface antigens CD34[6] and Thy-1[7], the absence of lineage-related markers, transferrin receptors[8] and the high molecular weight form of the human leukocyte antigen CD45RA[9], a low expression of HLA-DR[10] and a low uptake of

Rhodamine[123].[11] Physical features such as size and light scatter properties add to distinguish cells of different stages of hematopoietic development.

CD34-expression on stem cells

The CD34 antigen (a 90-120 kD integral membrane glycoprotein) is expressed on developmentally early lympho-hematopoietic stem and progenitor cells[6], small-vessel endothelial cells[12] and embryonic fibroblasts.[13] Human BM cells expressing the CD34 antigen comprise ~1.5% of marrow nuclear cells and are enriched for progenitor cells.[6] However, this population is heterogeneous, with most cells already committed to either erythroid, lymphoid, or monomyeloid cell lineages.[14] The function of CD34 is not clear yet.

$CD34^+$ cells co-express L-selectin (CD62L; a surface adhesion glycoprotein) which is involved in redistribution and homing of hematopoietic progenitor cells to the bone marrow (BM) following cytotoxic damage.[15] In addition, recent experiments indicate that CD34 expressed on endothelial cells may play a role in leukocyte adhesion and "homing" during the inflammatory process acting as a ligand for L-selectin[16, 17], and it has been hypothesised that CD34 plays a role in stem/progenitor cell localisation/adhesion in the BM.[6] It may also be involved in maintenance of the stem cell/progenitor phenotype.[18] CD34 is widely used to enrich for progenitor cells to use in transplantation and gene therapy studies, including studies to expand hematopoietic stem/progenitor cells *ex vivo*.

Long-term culture of human hematopoietic stem cells[19], the use of immunodeficient mouse models as *in vivo* assay for human hematopoiesis[20], and successful transplantation of CD34-selected BM cells into baboons[21, 22] and humans[23] have demonstrated that $CD34^+$ cells are responsible for short-term repopulation and probably for long-term repopulation as well.

Multidimensional flow cytometry of $CD34^+$ cells showed that expression of the CD38 antigen (a 45 kD glycoprotein) is an early event in the differentiation of cells into erythroid, myeloid, B-lymphoid and T-lymphoid lineages.[24, 25] Candidate stem cells do not express the CD38 antigen (Figure 2.). Cells with the $CD34^+CD38^-$ phenotype embrace a small population of ~5% of the $CD34^+$ subset and ~0.05% of the total nucleated cell population). Long-term cell culture assays[25] and transplantation of purified $CD34^+CD38^-$ cells into immunodeficient non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice[26, 27] have shown that this subset contains cells involved in hematopoietic repopulation.

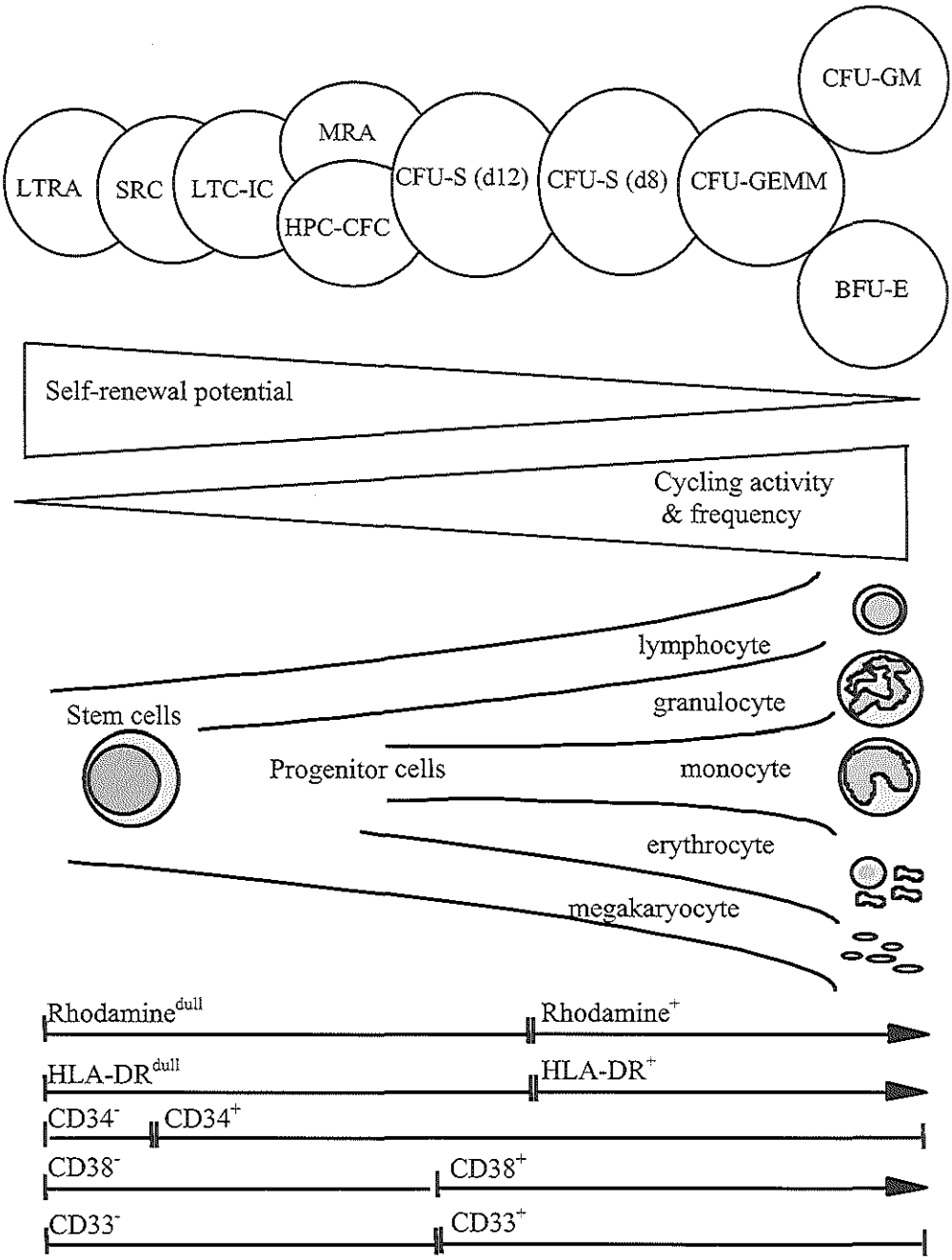

Figure 1. Schematic representation of the hematopoietic cell hierarchy.

Studies performed in murine models support the presence of a $CD34^-$ cell population that is enriched for long-term repopulating cells and does not express detectable levels of cell-surface CD34.[28, 29] Single murine Lin^-CD34^- cells transplanted into lethally irradiated mice can sustain long-term multilineage engraftment. In the human hematopoietic system Lin^-CD34^- cells also seem to be able to repopulate the BM of NOD/SCID mice, but show limited biological function *in vitro*. These repopulating Lin^-CD34^- cells were found in all hematopoietic tissues, with the highest frequency found in foetal liver (3.25%) and foetal blood (2.65%). The lowest levels were found in BM (0.28%), suggesting that Lin^-CD34^- cells are produced at the highest levels early in human ontogeny.[30] Human BM $CD34^-$ cells also repopulate the foetal sheep xenograft model.[31] The results of this study provide evidence for the existence of a primitive $CD34^-$ (human) cell population with repopulating potential. Transplantation assays in mice have revealed that the expression of CD34 on immature sub-populations of stem cells is reversible and associated with activation stage.[32] The majority of the stem cells with long-term repopulating ability in normal adult mice are $CD34^-$, but *in vitro* and *in vivo* activation of stem cells results in reversible expression of CD34.[32] These findings presently serve well in resolving a growing controversy on the CD34 state of repopulating cells.

Stem cell commitment

Hematopoiesis is a tightly regulated process that ensures that blood cell turnover is balanced by production of new blood cells and in which blood cell production can be rapidly adapted to increased demands due to blood loss, infection or other emergencies. Different models have been developed in which stem cell commitment and differentiation is described as either a stochastic/random process or in which stem cell fate is regulated by external stimuli.[33-36] Whatever the precise mechanism by which stem cell commitment, proliferation or differentiation occurs, the regulation of hematopoiesis relies on complex interactions between hematopoietic cells and stromal cells in the BM, growth factors and their receptors, extracellular matrix molecules and cell-cell interactions through specialised cell adhesion molecules.

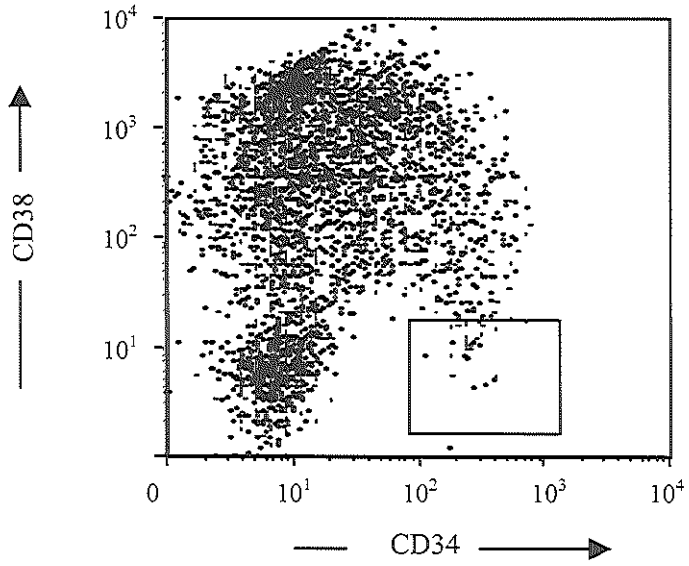

Figure 2. Flow cytometric analysis of human $CD34^+$ enriched human hematopoietic cells stained with CD34 versus CD38. $CD34^-CD38^-$ cells cover ~5% of the $CD34^+$ population and ~0.05% of the total nucleated cell population.

Hematopoietic growth factors

Hematopoietic growth factors (GFs) or cytokines are involved in the proliferation and differentiation of the hematopoietic compartment and thus play a fundamental role in the regulation of blood cell production. Although GFs act mainly directly on the hematopoietic cells, GFs also have indirect effects through accessory cells releasing other cytokines.[2] Many GFs display overlapping biological activity (either in synergy or as antagonists) on a variety of cell types. Some GFs stimulate primitive cells as well as committed progenitors or end stage cells of specific lineages. Cytokines involved in stem cell proliferation/differentiation are stem cell factor (SCF)[37], leukemia inhibitory factor (LIF)[38], interleukin 3 (IL-3)[39], interleukin 6 (IL-6)[40] and the more recently cloned fms-like tyrosine kinase-3 ligand (Flt3-L)[41] and thrombopoietin (TPO).[42-45] Lineage specific factors (late acting), such as granulocyte-colony stimulating factor (G-CSF)[46], monocyte-colony stimulating factor (M-CSF)[47], erythropoietin (EPO)[48], interleukin 1 (IL-1)[49] and interleukin 2 (IL-2)[50], but also IL-3, IL-6 and TPO act on the specific

differentiation into blood cell lineages, i.e., granulocyte (G-CSF) and monocyte (M-CSF) differentiation, erythropoiesis (EPO) or megakaryocytopoiesis (TPO). Hematopoietic growth factors are produced locally by stromal cells or cells in the hematopoietic organs, by mature blood cells, endothelial cells, and by specialised cells in various organs, such as lungs, brain, pancreas, kidneys and liver. Apart from stimulatory GFs, also negative regulators influence the differentiation and proliferation of stem and progenitor cells, i.e., TGF-β[51], macrophage inflammatory protein-1α (MIP-1α)[52], interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α).[53]

Analysis of expression of GF-receptors (GF-R) on hematopoietic cells showed that peripheral blood (PB) and BM $CD34^+$ cells express diverse high affinity hematopoietic GF-R, mainly IL-3-R, IL-6-R, GM-CSF-R, c-kit (SCF-R) and c-mpl (TPO-R).[54-57] Small size $CD34^+$ cells are characterised by more intense expression of the GF-R than large ones.[58] EPO-R, G-CSF-R and M-CSF-R are expressed at barely detectable levels.[59-61] Receptors for late acting GFs are almost exclusively expressed with high levels on cells of specific subsets, i.e., the EPO-R is expressed on more mature $CD34^+CD38^+CD71^{++}$ erythroid progenitors but not on $CD34^+CD38^+CD64^+$ GM-CFU.[62, 63] Hematopoietic GFs, which are all glycoproteins, mostly belong to two GF-receptor families, the tyrosine kinase receptor family[64, 65] and the hematopoietin or cytokine receptor super family.[66] The tyrosine kinase receptor family consists of cell-surface receptors that are classified into three subclasses based on structural differences. Subclass I receptors include the receptor for epidermal growth factor (EGF)[67], Subclass II receptors include receptors for insulin and insulin-like growth factor-1[68, 69], and subclass III contain the SCF-receptor, the M-CSF-receptor, Flt3 (receptor for Flt3-L) and platelet-derived growth factor (PDGF) receptor.[70-73] The cytokine receptor superfamily include receptors for most other GFs, such as receptors for IL-2, 3, 5, 6, 7, 9 and 11, GM-CSF, G-CSF, EPO, TPO, LIF.[74-86] Hematopoietic GFs can also be grouped according to their phylogenetic background. TPO, for instance, consists of a highly conserved amino-terminal domain that is related to EPO. Similar to EPO, TPO contains 4 cysteins, of which 3 present in EPO are at similar positions in TPO.[42, 87] SCF, M-CSF and Flt3-L may have been derived from a common ancestral gene as these GFs have similar intron/exon structures[88], and the GM-CSF gene is closely linked with the IL-3 gene, which also might originate from one gene as GM-CSF and IL-3 are structurally similar and share overlapping functions.[89, 90] It is of interest for evolution genetics that the mouse has two closely related IL-3-receptor β-chains, one shared with IL-5 and GM-CSF, one

specific for IL-3[91], whereas primates, including humans, have only the former. Remarkably, IL-3 is one of the fastest evolving proteins known.[92]
The precise actions of individual GFs on stem cell maintenance, proliferation, commitment and differentiation are not well understood. A major challenge is to elucidate these mechanisms and to select the most suitable GFs or GF combinations and culture conditions to maintain or increase stem cell numbers *in vivo* and influence their differentiation. This is, for instance, relevant in studies directed at *ex vivo* expansion of immature cells for transplantation purposes and gene transfer.

Stem cell sources
Until recently, the most frequently used source in clinical stem cell transplantation was BM. However, hematopoietic cells mobilised into the PB by growth factor treatment are increasingly replacing BM for clinical transplantation. Human umbilical cord blood (UCB) is a rich source of hematopoietic stem/progenitor cells[93] and in 1989 experimental and clinical studies were published indicating that human UCB could be used in clinical settings. UCB has a significantly higher number of primitive and committed progenitors than adult PB.[94] Compared to BM, UCB contains a higher proportion of immature CFC (i.e., CFU-GEMM, CFU-GM and CFU-MK)[95, 96], an 8-fold higher frequency of HPP-CFC[97], and similar frequencies of LTC-IC.[98] The frequency of $CD34^+$ cells is about the same in BM and UCB (~1%).[99-102] Additionally, the cloning efficiency of $CD34^+CD38^-$ UCB cells is higher as compared to BM[103] which may imply a more efficient engraftment after transplantation.[104] In contrast to allogeneic BM and PB, UCB has a low risk for acute graft-versus-host disease. However, problems associated with the use of UCB for transplantation are that the collection can only take place once. One cord blood sample may not contain sufficient numbers of stem cells to transplant adults and in case an additional graft is needed there is no possibility to aspirate another.
The first UCB transplant was successfully performed in a 14-year old child with Fanconi's disease.[104] Although more than 500 UCB transplants have taken place world-wide, the biology of cord blood stem cells is not yet fully understood. As the number of stem cells in an average UCB sample is too low to transplant large adults, *ex vivo* expansion of hematopoietic stem cells would seem to be necessary to make UCB stem cell transplantation a feasible option for adults. For the development of procedures to efficiently expand the numbers of transplantable

stem cells in culture, characterisation of stem cell physiology and knowledge about stem cell regulation by GFs is essential.

Progenitor cell assays (in vitro / in vivo)

Many *in vivo* and *in vitro* assays have been developed to study mechanisms controlling cell differentiation at specific stages to identify, quantitate and characterise hematopoietic cells and to manipulate the expansion of the progeny. Furthermore, *in vivo* transplantation assays are used for tracing early representants of the stem cells and for evaluation of the function of BM stroma. Although the various functional definitions may overlap as some cells may show up in multiple assays, none of these assays fully satisfies the criteria for hematopoietic stem cell analysis. The definition of stem cells has changed through the years as result of the development of new techniques and assays to characterise these multipotential cells, resulting in a more precise link between phenotypes and functional properties.

In vitro frequency analysis of hematopoietic stem cells and progenitors is often performed in a semi-solid colony assay (CFU-C), which is, however, neither specific nor predictive for the most immature stem cells with long-term engraftment potential but identifies more committed cells with limited proliferative and differentiation capacity. The first colonies grown from normal mouse bone marrow cells were composed of neutrophilic granulocytes and/or macrophages (GM-CFU)[105-107] and represented a heterogeneous population of hematopoietic progenitor cells. Cells of the erythroid lineage can grow in EPO-stimulated semi-solid cultures forming erythroid colony-forming units (CFU-E).[108] The existence of a cell type of greater erythroid colony-forming capacity was termed erythroid burst-forming unit (BFU-E) because of the disperse appearance of the colonies containing abundant numbers of erythroid cells.[109] BFU-E are developmentally early, EPO-independent progenitor cells, and CFU-E late, EPO-dependent progenitors.[109] Also megakaryocyte progenitors can be cultured in semi-solid medium to form megakaryocyte colonies (Meg-CFU).[110, 111] The CFU-GEMM are multipotential progenitor cells that can form colonies comprising mixtures of granulocytes, erythroblasts, megakaryocytes and macrophages. Another clonogenic assay for the analysis of primitive cells is the high proliferative potential colony-forming cell assay (HPP-CFC). HPP-CFC contain progenitors of granulocytes, erythrocytes and megakaryocytes, and cells from these colonies are able to repopulate the BM of mice.[112]

In vitro hematopoiesis can be sustained for weeks or even months in culture systems in which hematopoietic cells are maintained using a layer of marrow-derived stromal cells. At first, these long-term bone marrow cultures (LTBMC) were developed to support replication of mouse pluripotent stem cells[113, 114], and were adapted to sustain long-term hematopoiesis with bone marrow of primates, including humans.[115, 116] Changes in hematopoietic activity are usually monitored by periodic measurement of the production of committed progenitors, assayed in colony assays (long-term culture initiating cell assay, LTC-IC assay)[19, 117] or by detection of characteristic areas of hematopoietic cells which are in close contact with the stroma, so called cobblestone areas (cobblestone area forming cell assay, CAFC assay).[118-120] The CAFC assay can be used to measure the frequency of different hematopoietic stem cell subsets under limiting dilution conditions using Poisson statistics. In addition, the colony forming cell production in the LTC-IC assay is used to estimate the total graft quality, and in combination, these assays can be applied to calculate the average quality of individual stem cells.[121] Studies focussing on the hierarchy of stem cell subsets revealed that early appearing (week 2) CAFC represents a more mature subset, than the late appearing (week 6) CAFC.[122] The former correlates with the number of spleen colony forming cells day 12 (CFU-S_{12}) and cells with short-term repopulating ability, whereas the latter correlates with cells with long-term repopulating ability.

Definitive information about functional primitiveness of cells and the ability to repopulate the BM of recipients can only be gained by transplantation assays. The spleen colony forming (CFU-S) assay[123] was previously considered the only true stem cell assay available. In this assay, irradiated mice are transplanted with BM cells. The number of macroscopic colonies that appear on the surface of the spleen of the recipient, 7 to 13 days after injection is directly proportional to the number of BM cells transplanted. The cells in the CFU-S compartment are structured in a hierarchic system; early appearing day 8 CFU-S lack stem cell properties and overlap with the committed progenitor populations as measured by the *in vitro* erythrocyte burst-forming unit (BFU-E) assays.[124] Late-appearing day 12 CFU-S are capable of some self-renewal as demonstrated by serial transplant studies in mice.[125] Other studies show no overlap of long-term reconstituting cells and day 12 CFU-S.[3] The methodology involved in spleen colonies for a stem cell assay has been published in detail.[126-128]

In 1985, an *in vivo* assay was developed to determine the marrow-repopulating ability (MRA) of transplanted bone marrow cells in the mouse. The MRA assay[129] measures a more primitive cell population than the CFU-S. In the MRA assay, BM

from irradiated mice is collected and injected into lethally irradiated recipient mice. After 13 days colony-forming cells are analysed in the BM of the recipient mice. The number of secondary *in vitro* clonogenic progenitors in the BM of such recipients is a measure of the MRA of the graft which are cells closely associated with those that provide sustained hematopoiesis after BM transplantation. In contrast to the MRA assay, which is analysed at one time point of the repopulation in the BM after 13 days, the long-term repopulating ability (LTRA) assay[130] measures the dynamics and the level of multilineage reconstitution over extended periods of time, thus giving insight into hematopoietic stem cell development *in vivo*. The LTRA assay is performed in mouse strains that enable a discrimination of donor-derived and recipient-type cells, such as α-thallassemic $Hba^{th/+}$ mice which are genetically anaemic. Infusion of normal mouse BM cells into sublethally irradiated α-thallassemic mice results in chimeric mice in which differences in size between α-thallassemic red blood cells and healthy (donor-type) red blood cells are detected using flow cytometry.[131] This red blood cell chimerism is used at different time intervals as a measure for chimerism at the level of the hematopoietic stem cell. A similar approach, using unirradiated W^{41}/W^{41} anaemic mouse mutants, was used to study lymphoid and erythroid repopulation.[132] Recent work on the mechanism of the radioprotective effect of TPO further supported the functional hierarchy of LTRA, MRA, and CFU-S.[133]
These *in vivo* assays (CFU-S, MRA, LTRA) are used for quantitative analysis of murine hematopoiesis. *In vivo* studies for primate hematopoietic stem cells can only be performed in models such as developed in rhesus monkeys and baboons. The use of immunodeficient mice as a transplantation assay for human repopulating cells is a helpful alternative assay for human repopulating stem cells. The inter-relationship of the different assays for hematopoietic stem cells is presented schematically in Figure 1.

Immunodeficient mouse models for human cell transplantation
Congenital immunodeficiency in mice has shown to be useful to assess human stem cell capacity in a transplantation assay.[134-136] Mouse strains originally used for these studies include beige athymic nude X-linked (bnx) immunodeficient mice[136], and C.B-17 scid/scid severe combined immunodeficiency (SCID) which are superior in human cell engraftment as compared to the bnx mice.[137] The SCID mouse has an autosomal recessive mutation mapping to mouse chromosome 16[138] that causes Severe Combined Immunodeficiency (SCID).[139] Characteristic for

SCID mice is the lymphocytopenia, a rudimentary thymic medulla, and relative empty splenic follicles and lymph nodes.[140] The low numbers of functional B and T cells is due to a defective V(D)J recombination[141] and a defect in the repair of DNA double-strand breaks results in hypersensitivity to ionising radiation.[142] To overcome the non-specific immunity which is still present in the mouse, SCID mice have been treated with injections of the polyclonal rabbit antibody anti-asialo GM1[143, 144] or liposomes capturing di-chloromethylene-di-phosphonate (CL_2MDP)[145] as a conditioning regimen for transplantation with human hematopoietic cells. In order to facilitate the selective elimination of NK cell activity, the *scid* mutation was back-crossed in the non-obese-diabetic (NOD)/Lt background, which results in an immunodeficient stock (NOD/LtSz-*scid/scid*; NOD/SCID) with multiple defects in adaptive as well as non-adaptive immune function[146] and, provides a more suitable microenvironment for reproducible reconstitution with human hematopoietic cells.[27, 147] NOD/SCID mice support approximately five-fold higher levels of human stem cell marrow engraftment than do SCID mice.[147]

Use of immunodeficient mouse models

To study the human immune system, humanised SCID mice (SCID-hu) have been developed by co-implantation of small fragments of human foetal liver, thymus and lymph node beneath the kidney capsule.[134] These mice showed reproducible and long-term reconstitution of human T and B-cell activity.[148, 149] Also mice transplanted with human foetal bone, spleen and thymus (SCID-hu-BTS) showed a multilineage graft able to produce hematopoietic cells for at least 36 weeks.[150, 151] Also subcutaneous implantation of human foetal bone fragments in non-irradiated SCID mice (SCID-hu-bone)[152] has been used to study the effects of various recombinant human hematopoietic growth factors on the development of different hematopoietic lineages.[153] However, the SCID-hu-bone model was not able to maintain progenitors after 12 weeks post-transplant.[58, 152, 153] Functional studies such as the injection of Staphylococcal enterotoxins in the SCID-hu mice, showed effect on the development and responsiveness of human T cell populations.[154] Hu-PBL-SCID mice (SCID mice transplanted with peripheral blood lymphocytes) were developed as models to study xenogeneic graft-versus-host-disease (XGVHD) and for evaluation of immunosuppressive agents and transplantation protocols for XGVHD.[155]

Apart from studies on the immune system, immunodeficient mice are used for stem cell assays. Intravenous (iv.) transplantation of unfractionated human BM cells resulted in reconstitution of (3 to 4 Gy) irradiated SCID mice BM with myeloid, erythroid and B cell lineages.[156] Human hematopoiesis was spurred in these animals by recombinant human cytokines injected intraperitoneally (ip.) at 48 hour intervals during the engraftment period.[137] Transgenic SCID mice expressing the genes for human IL-3, GM-CSF and SCF have also been developed and, when compared to non-transgenic SCID mice, these transgenic mice engrafted $CD34^+$ selected umbilical cord blood cells for periods twice as long (for up to 6 months).[157] In contrast to studies with human BM, treatment with human cytokines was not required for unseparated or Ficoll-fractionated human UCB or peripheral blood stem cells to establish high-level engraftment in SCID mice, suggesting that neonatal cells either respond differently to the murine micro environment, provide their own cytokines in a paracrine fashion[158], or that UCB is more enriched for primitive progenitors.[159] However, some studies indicate that, while UCB cells may not require cytokine supplementation to achieve short-term engraftment, more durable engraftment is dependent on cytokine support.[157]

Early studies in SCID mice, showed that primitive human cells are responsible for the multilineage engraftment in immunodeficient mice. One of the first experiments to suggest this involved growth factor treatment of the SCID mice after transplantation of human BM cells. Injection of GFs immediately after transplantation resulted in increased numbers of human cells as compared to untreated mice. When these GFs were administered one month after BM transplantation, a 10 to 100-fold cell increase was found as compared to animals that did not receive GFs, indicating that resting primitive cells that were still present in the mouse BM can engraft and survive in the mouse microenvironment.[137]

More evidence was found in that engrafted mice contained significant numbers of $CD34^+Thy\text{-}1^{lo}$ human cells (a phenotype very immature in *in vitro* assays)[25] and human LTC-IC, which is the earliest cell type that can be assayed *in vitro*. Initial studies showed that only $CD34^+$ and not $CD34^-$ BM and UCB cells are responsible for SCID mouse and NOD/SCID mouse[160] engraftment. In subsequent experiments, when NOD/SCID mice were more commonly used for stem cell characterisation, it was found that a small subpopulation of $CD34^+$, i.e., $CD34^+CD38^-$ cells were exclusively able to repopulate the mouse BM.[20, 27] Cells of this immature phenotype were able to reconstitute the mouse BM with lymphoid and myeloid cells as well as with $CD34^+CD38^-$ cells.

Through limiting dilution analysis using Poisson statistics (assuming a single-hit action) a frequency of 1 (NOD)SCID repopulating cell (SRC) in 617[20] or in 3,500[27] $CD34^+CD38^-$ UCB cells was estimated. Using a similar approach, differences in SRC frequencies have been observed between the frequencies in UCB of 1 in $9{,}3x10^5$ unfractionated cells, which was significantly higher than the frequency of 1 SRC in $3{,}0x10^6$ adult BM cells or 1 in $6{,}0x10^6$ mobilised peripheral blood cells from normal donors.[161]

Administration of combinations of SCF, Flt3-L, IL-3, IL-7 and GM-CSF to NOD/SCID mice transplanted with $CD34^+Lin^-$ UCB cells can modulate the lineage distribution of the human graft *in vivo*.[162] Flt3-L and IL-7 promote stromal-independent expansion differentiation of human foetal pro-B cells *in vitro*.[163] In contrast, *in vivo*, Flt3-L and IL-7 (which work in synergy) did not induce T and B cell development, but instead a markedly reduced B cell development with a concomitant shift in the lineage distribution towards the myeloid lineage.[162]

The (NOD)/SCID mouse assay has provided a useful tool in the *in vivo* analysis of human hematopoiesis, although only the short-term repopulating ability of stem cells can be analysed due to the development of lymphomas and leakiness in ageing NOD/SCID mice. Furthermore, for quantitative analysis of transplanted human cells, NOD/SCID mouse assays are not entirely satisfactory due to large variations between individual mice.

(NOD/)SCID mice are also suitable hosts for hematopoietic cells from human proliferative diseases such as leukemia.[156, 161, 164] Cells from patients with acute lymphoblastic leukemia (ALL) are known to grow in a pattern similar to the growth of the leukemic cells in the patients.[165, 166] This feature makes the assay a useful tool to study the development of the disease but also to test treatment regimens meant to counteract disease progression. In contrast to ALL, some acute myeloid leukemias (AML) grow less well in immunodeficient mice[167, 168] unless the mice are treated with cytokines (IL-3) to stimulate leukemic proliferation.[169] Similar to healthy hematopoietic cells, $CD34^+CD38^-$ as well as $CD34^+CD38^+$ AML cells were able to give rise to colonies *in vitro*, but only the $CD34^+CD38^-$ cells could re-establish leukemia in transplanted SCID mice, suggesting a hierarchy within the leukemic cell clone and the existence of a leukemic stem cell.[169] However, other studies show that $CD34^-$ AML of some patients have a similar capacity to initiate and maintain long-term growth of AML *in vivo* (SCID mice) and *in vitro* (CAFC assay) as has the $CD34^+$ fraction.[170] This indicated that, at least in some patients, the immunophenotype of engrafting AML cells does not correspond with that of their normal counterparts and/or that the leukemogenic

potential of leukemic stem cells is not always lost during differentiation. Alternatively, the finding may support the contention that CD34 is expressed reversibly, dependent on activation state and independent on differentiation stage.[32] Philadelphia-chromosome positive (Ph^+) leukemic cell lines and primary blast crisis cells from chronic myeloid leukemia (CML) patients also grow predominantly in immunodeficient mice, with exclusive production of Ph^+ cells.[171] Engraftment of low density or purified $CD34^+$ cells from chronic phase CML patients after injection in immunodeficient mice is low[172, 173] which may be due to low numbers of stem cells in the grafts.

Ex vivo expansion

Ex vivo stem cell manipulation might be necessary to expand cells to gain enough immature (UCB) cells for transplanting large adults or for gene therapy purposes. One of the current problems in stem cell expansion is that the stem cell GF receptor phenotype of repopulating stem cells is not sufficiently well known. Hence, stimulation of suspensions of hematopoietic cells with GFs or GF combinations may result predominantly in activation and expansion of progenitor cells rather than stem cells. Stem cells may either be insufficiently stimulated, or when stimulated, lose repopulating activity through differentiation. Furthermore, GF stimulation may have an effect on the homing of stem cells as several hematopoietic GFs, such as IL-3 and GM-CSF, are involved in the interaction between stromal cells and hematopoietic stem cells.[174-176] Many studies have focussed on culture conditions in which immature cells proliferate without differentiation and maturation.[20, 177-185] To date, approximately 25 cytokines with prominent actions on the hematopoietic system have been identified. In early *ex vivo* expansion studies and gene transduction studies the most commonly used GF combination included SCF, IL-3 and IL-6, but with the cloning of new GFs such as TPO and Flt3-L, these combinations have become obsolete and replaced by GF combinations with SCF, TPO and Flt3-L.[186, 187] Results of transplantation studies in mice have shown that Flt3-L induces proliferation of highly purified hematopoietic progenitor cells in synergy with other GF such as IL-6, IL-3, G-CSF or SCF.[188] Using human hematopoietic cells, Flt3-L appeared to act in synergy with IL-3, IL-6 and SCF to induce proliferation of $CD34^+CD38^-$ BM cells and expansion of colony-forming units (CFU) from LTC-IC.[189] The effect of Flt3-L on $CD34^+CD38^+$ cells and on immature $CD34^+CD38^-$ UCB cells was less pronounced.[189] However, other studies show that Flt3-L does not expand the stem

cell pool by itself but rather acts as a costimulatory factor in the recruitment and proliferation of myeloid and lymphoid progenitors.[190] Next to the effect of SCF on stem cells, specific activities of SCF include promotion of erythroid cell formation[191] and mast cell regulation.[192] TPO is, apart from regulating the platelet production involved in the reconstitution of immature multilineage repopulating stem cells.[193] Certain stromal cell lines also have the capacity to support stem cell proliferation.[179, 194-196] Cocultured adult BM or UCB cells with allogeneic human stroma were assayed in CFC, LTC-IC and transplanted in irradiated NOD/SCID mice. The SRC frequency declined 6-fold after one week of culture. In contrast to this loss, the transplanted inocula of cultured cells frequently contain equal or higher numbers of CFC and LTC-IC as compared to the inocula of fresh cells. The mechanisms that underlie the loss of SRC during culture on stroma is not known and could be due to differentiation of primitive cells during culture or changes in the expression and/or function of adhesion receptors that could affect the engraftment potential of the immature repopulating cells.[98]

Gene transfer

Strategies for efficient gene transfer into hematopoietic cells

The hematopoietic stem cell has been an obvious target for gene-transfer because of its ability to permanently reconstitute the hematopoietic and immune systems after transplantation. A relatively small number of genetically modified hematopoietic stem cells could potentially generate large numbers of differentiated hematopoietic cells carrying and expressing an exogenous gene for long periods of time, possibly the life span of the recipient.

Effective stem cell gene transduction can be used as somatic gene therapy for the correction of mutations or deletions as treatment of hereditary diseases only affecting (part of) the hematopoietic system such as adenosine deaminase (ADA) deficiency[181, 197-199], chronic granulomatous disease[200] or diseases affecting both the hematopoietic system and other organs such as Gaucher's disease.[201] In addition, genes can be introduced as a vehicle for products that are deficient in other organs, as an adjuvant to cancer therapy or to create resistance against infectious disease. Another aspect of introducing genes into primitive hematopoietic cells is its use in stem cell characterisation. By stable integration of marker genes into the DNA of purified immature hematopoietic subsets, the mechanisms involved in *in vivo* repopulation of the BM after transplantation can be elucidated by tracing the transduced cells and their progeny, which in turn may yield useful information on the option of *ex vivo* stem cell expansion.

At this time point, only two of the currently available gene transfer systems can possibly accomplish this. The systems include viral vectors based on retroviruses and adeno-associated viruses (AAV). Vectors based on, for instance, adenoviruses and liposome-based DNA delivery methods have no potential for stable gene transfer at high frequencies and are therefore less useful for studying long-term hematopoiesis.

Adeno-associated virus mediated gene transduction

Adeno-associated viruses (AAV) are non-pathogenic and replication-defective human parvoviruses with a single-stranded DNA genome. AAV needs co-infection with helper virus (for instance herpes simplex virus or adenovirus) for stable integration into a wide variety of hosts and are used in studies for treatment of hereditary diseases[202, 203], anti-viral approaches to AIDS[204], anti-oncogenic strategies[205] and stem cell characterisation.[206]

Retrovirus mediated gene transduction

Retroviral vectors, the prototype based on Moloney's murine leukemia virus (MoMLV), are most commonly used for transduction of hematopoietic cells. The genomes of these viruses contain the 3 genes *gag*, *pol*, and *env,* encoding proteins involved in virion encapsidation and assembly, reverse transcription of viral RNA and integration of viral DNA into host chromosomal DNA, and virus adsorption to the target cells, respectively.[207] Upstream of the *gag*, *pol* and *env* genes the packaging signal (φ) is located (Figure 3). This signal directs specific packaging of full-length viral RNA into virions. The protein coding sequences (*gag*, *pol* and *env*) are not included in the retroviral vector, but are provided by packaging cell lines, transfected with the viral genes. Packaging cell lines that by themselves can only produce virus particles without viral genome or soluble viral proteins, can package retroviral genomes into virions.[208] Because the packaging sequence is devoid of the packaging signal φ and the coding sequences for viral proteins in the retrovirus vector are replaced by a gene of interest, the virus particles can only go through one round of replication and are therefore replication defective (Figure 4). Retrovirus genomes contain long untranslated regions at both their 5' and 3' ends, the so-called long-terminal repeats (LTR) which include signals needed for the initiation of transcription by RNA polymerase and for 3' cleavage and polyadenylation of transcripts.

Depending on the specific envelope gene (*env*) expressed in individual packaging cell lines, replication-defective retroviruses can be generated with an ecotropic (infectious to rodent cells) or an amphotropic (infectious to cells of several mammalian species including humans) host range. Ecotropic packaging cell lines synthesise specific envelope proteins different from amphotropic cell lines, which bind to specific cell surface receptors on the target cells. The amphotropic (MoMLV) receptor (sodium-dependent phosphate transporter-2; Pit-2 or receptor for amphotropic murine retrovirus-1; Ram-1) shows significant sequence similarity

with the gibbon ape leukemia virus (GALV) receptor (sodium-dependent phosphate transporter-1; Pit-1 or gibbon ape leukemia virus receptor-1; GLVR-1), although the binding to these receptors by virions occurs without cross-interference.[209]

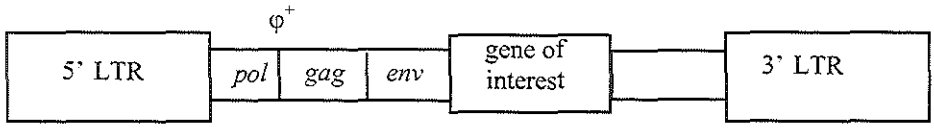

Figure 3. Schematic presentation of the structure of a retrovirus vector containing gag, pol and env sequences, the packaging signal (φ) and sequences of the gene of interest between the 5' and 3' long-terminal repeats (LTR).

The level of expression of the amphotropic receptor, as measured by mRNA levels, appears low in human and primate $CD34^+$ hematopoietic cells which has led to speculation that the low level of expression of the receptor protein is among the causes for the low level transduction using amphotropic-packaged vectors.[210-212] Most human hematopoietic cells analysed express higher levels of the gibbon ape leukemia virus (GALV) receptor GLVR-1[213, 214] and this might explain the higher gene transfer into human progenitors using viruses packaged into the pseudotyped GALV envelope protein as compared to viruses that express the amphotropic envelope.[215]

Vectors based on the onco-retroviruses, including MoMLV depend on cell proliferation for integrating genes in the genome of the target cells. Stem cells are not cycling and consequently resistant to gene transfer if not appropriately stimulated.[216] Lentiviruses (a subgroup of retroviruses, an example being the acquired immune deficiency syndrome virus HIV) differ in that they are relatively independent of cell division and therefore might facilitate the transduction of quiescent human hematopoietic stem cells.[217] Unlike murine retroviruses, lentiviruses have a complex genome that, in addition to the essential structural genes, *env*, *gag* and *pol*, also contains regulatory (*tat* and *rev*) and accessory genes (*vpr*, *vif*, *vpu* and *nef*), with which a pre-integration complex can be made to traverse an intact membrane of the nucleus of a target cell.[218] However,

lentiviruses can not transduce G0 cells because they need reverse transcriptase activity (present in G1 phase) to translate the viral RNA genome into DNA.[219, 220] Thus, lentiviruses can infect nondividing cells, but they need a basal activity (reverse transcriptase) for integration into host DNA. The lentiviral vector is speculated to be superior to the retroviral vector for the transduction of non-dividing, primitive hematopoietic progenitors.[221] Recent studies reveal a high efficiency transduction using a lentiviral vector based on HIV, in the absence of cytokine stimulation which resulted in transgene expression in multiple lineages of human hematopoietic UCB cells transplanted into NOD/SCID mice.[222, 223] However, the transduction efficiency of the retroviral vector used in this study for comparison was exceedingly low compared to that found in other studies[224] and the transduction levels of repopulating hematopoietic cells with the lentiviral vectors were similar to those found with retrovirus vectors in previous studies.[224] In addition, transduction of primitive $CD34^{+}CD38^{-}$ human hematopoietic cells resulted in transduction levels of 12% in contrast to an amphotropic retroviral vector which could not transduce this cell subset at all.[221] Although high levels of transduction can be reached, the possibility of recombination between transfer and packaging vectors, which may give rise to replication-competent virus with pathogenic potential, gives rise to safety concerns.[225] To eliminate this potential risk, vectors derived from nonhuman lentiviruses such as simian immunodeficiency virus, bovine immunodeficiency virus, or equine infectious anemia virus could be developed.[226, 227] Also the construction of self-inactivating HIV vectors (by replacement of part of the upstream LTR in the vector construct by constitutively active promoter sequences which abolishes the LTR promoter activity but does not affect the vector titers or the transgene expression *in vitro*) may improve the biosafety of HIV-derived vectors.[226, 228, 229]

Selectable reporter molecules

Retroviral vectors encoding selectable reporter molecules have been used to study the efficiency of transduction in immature hematopoietic cells. The most widely used of these have been intracellular components that confer resistance to toxic compounds such as neomycin[230-232], hygromycin[233, 234], methotrexate[235-238], mycophenolic acid[239] and various chemotherapy agents.[240-243] The neomycin phosphotransferase gene (neo) has proven to be particularly useful in tracking retrovirus transduced human hematopoietic cells, both *in vitro* as *in vivo*, and has, for instance, been used for the identification of cells capable of causing leukemic

relapse after autologous BM transplantation.[244-246] These intracellular markers are less suitable for the enrichment of immature and transduced hematopoietic cells, mostly because selection requires time-consuming exposure to toxic drug concentrations under *in vitro* culture conditions that stimulate cell division and terminal differentiation. The bacterial β-galactosidose gene (LacZ) has also been used as a selectable reporter molecule for hematopoietic cells.[247-250] The expression of the gene is measured by a colorimetric test for its product, the enzyme β-galactosidose. However, the relatively high endogenous β-galactosidose activity in some cell types and the requirement for transporting fluorogenic substrates across the cell membrane while maintaining cell viability have limited its application.

Transduction of hematopoietic stem cells with retroviral vectors encoding marker molecules expressed at the cell surface offers several advantages, such as rapid, non-toxic and quantitative analysis and selection of the transduced cells by flow cytometry. Genes encoding for molecules that are expressed on the cell surface of various cell types such as the human multi-drug resistance (MDR1) gene[251], mouse CD24[252, 253], CD9[254] or nerve growth factor[255], are incorporated into vectors. Transduced cells can be identified after staining with a specific antibody conjugated to a fluorochrome by flow cytometry. Disadvantages of this type of cell surface markers are the low-level expression of the reporter molecule in the target cells, the requirement for specific antibodies to detect the marker and relatively large genes used as a selectable marker, leaving limited space for other genes of interest. In contrast to these markers, use of the autofluorescent green fluorescent protein (GFP) or its derivatives allows rapid identification of transduced cells by fluorescence microscopy or flow cytometry in living cells in real time without additional staining steps.

The Enhanced Green Fluorescent Protein (EGFP) marker gene

In nature GFP is produced by the jellyfish *Aequorea victoria* found in the Pacific Ocean. Light is emitted when calcium binds to the photoprotein aequorin in response to shaking or attack. In 1994 Chalfie *et al.* demonstrated that GFP, expressed in prokaryotic cells (*Escherichia coli*) and eukaryotic cells (*Caenorhabditis elegans*) is capable of producing a strong green fluorescence when excited by blue light.[256] Purified GFP (a protein of 238 amino acids[257]) absorbs blue light (maximally at 395 nm) and emits green light (peak emission at 509 nm).[258, 259] The fluorescence is stable with virtually no photobleaching.[256]

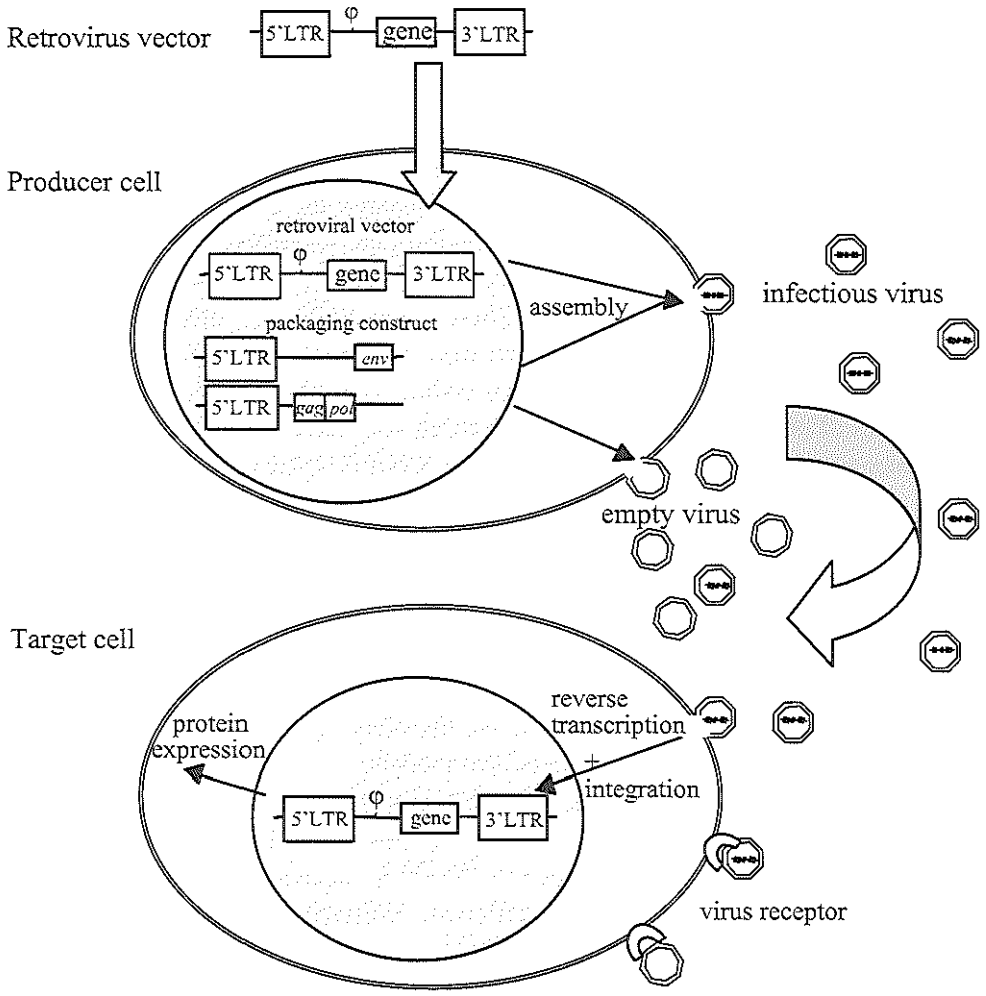

Figure 4. Retroviral mediated transduction. The retroviral vector, without sequences for viral proteins, is introduced into the packaging cell line that by itself can only produce empty virus particles without the viral genome. Within the packaging cell line, the retroviral genomes are packaged into virions and released outside the cell. These virus particles are replication-incompetent as they lack the essential *gag*, *pol* and *env* sequences. After infection of a target cell via specific receptors, the virus RNA will be reverse transcribed into DNA and integrated into the target cell DNA after which it can be expressed.

Because wt-GFP produces a weak (but stable) green fluorescence signal, several GFP variants have been created which shift the maximal excitation peak to approximately 490 nm and are better suited for detection of expression by fluorescence microscopy and flow cytometry. The GFP (S65T) variant and its "humanised" form, hGFP (S65T) contain a Ser to Thr substitution at amino acid position 65[260]; in the red-shifted GFP variant RSGFP the amino acids Phe-64, Ser-65 and Gln-69 are substituted by Met, Gly and Leu, respectively[261]; the enhanced GFP (EGFP) contains the double amino acid substitution of Phe-64 to Leu and Ser-65 to Thr.[262, 263] In addition, hGFP (S65T) and EGFP are optimised to obtain higher expression levels in mammalian cells by silent substitution of *Aequorea victoria* codons for the preferred human codons (i.e. humanised).[263] The relative intensities of peak green fluorescence observed with different GFP mutants were in the order EGFP>hGFP(S65T)>GFP-PTS1 or RSGFP>wtGFP.[264]

Gene transfer of hematopoietic cells in transplantation assays

Early experiments using retroviral gene transfer showed that while CFU and LTC-IC are easily transduced, these gene-marked cells do not contribute significantly to the repopulation of engrafted NOD/SCID mice, suggesting that the efficiency of gene transfer into SRC is low.[265] It is, therefore, of importance to study the characteristics of transduced cells in *in vivo* models.

In rodent models, efficient and reproducible gene transfer to a high percentage of long-term repopulating stem cells has been achieved and long-term expression of introduced genes in several lineages.[243, 266-268] In large animal models, retroviral gene transfer has been much less efficient, although long-term expression of transferred genes has been shown.[238, 269-274]

Gene transduction in large animals (canine, nonhuman primates) has shown disappointing results on the long-term repopulation of the transduced hematopoietic precursor cells, possibly caused by the lack of receptors expressed for the retroviral vectors used in most studies. Also the low number of hematopoietic stem cells in most target populations might lead to low efficiencies. Another explanation could be that hematopoietic stem cells might be inherently resistant to transduction or (transduced) stem cells may be unlikely to proliferate due to their quiescent nature. The problem might be of a more technical nature in that the conditions during the transduction procedure may not be optimal.[275]

To stimulate hematopoietic stem cells to enter the cell cycle, donors have been conditioned *in vivo* with growth factors (G-CSF / SCF)[276] and/or non-specific

killing of differentiated blood cells (5-fluorouracil[277-279] and hydroxy-urea[280]). Also *ex vivo* incubation of bone marrow cells with growth factors[177-184] with or without stromal layers[179, 195] has been used to stimulate stem cells into cycle to increase the gene transduction levels. For instance, the transduction efficiency of purified $CD34^{+}CD38^{-}$ UCB cells in the presence of combinations with Flt3-L, SCF, IL-6, TPO, IL-3, IL-6, G-CSF was tested[281], and resulted in the largest transduction levels (after transplantation into NOD/SCID mice and in LTC-IC assays) with Flt3-L, SCF, IL-6, TPO or Flt3-L, SCF, IL-3, IL-6, G-CSF.

Optimisation of gene transfer protocols

Stimulation with different growth factor combinations and culturing on stromal layers has been shown to result in increased efficiency of gene transfer into primitive cells. Physical methods such as adherence to human fibronectin have also been shown to increase the likelihood of target cell-vector interaction. Fibronectin has been successful in improving gene transfer efficiency into hematopoietic *cells in vitro* by colocalising the virus particles and the target cells. Its use eliminates the need for cocultivation, the use of polycations such as polybrene (hexadimethrine bromide) or protamine sulphate, and extended *in vitro* exposure to growth factors and/or stromal cells[282, 283], thus improving the chance that cells with repopulating ability are maintained. Polycations have essentially the same effect as fibronectin; they neutralise the opposing charges on virus particles and the target to facilitate transduction. Fibronectin is an extracellular matrix molecule which is abundantly expressed in the BM microenvironment.[284, 285] It participates in cell adhesion through at least 3 cell binding sites (Figure 5), including a cell binding domain (CBD) which mediates adhesion via the integrin VLA-5, the heparin binding domain which interacts via cell surface proteoglycan molecules, and the CS1 sequence which mediates adhesion via the integrin VLA-4. Adhesion of primitive hematopoietic stem cells to fibronectin takes place at the C-terminal cell adhesion-promoting sequence which is formed by the combination of the CS1 peptide and the adjoining sequences in the heparin binding domain (type III12-14) of fibronectin.[286-288]

Retrovirus particles bind directly and with high efficiency to sequences within the type III12-14 repeats of fibronectin. A recombinant human fibronectin fragment (CH-296, also termed retronectin®) which consists of the 3 binding sites (Figure 5B), was most efficient in transducing hematopoietic progenitor cells with efficiencies comparable to co-culture of the target cells directly on the producer

cells.[282] The fibronectin fragment is thought to bring retroviruses and target cells into close proximity, thus improving the likelihood that these cells will interact.[282] Subsequently, short-range-specific binding is mediated by the envelope protein on the retrovirus and the cognate receptor on the cell (for instance GALV-receptor or MoMLV-receptor), a process called adhesion strengthening.[289] Another parameter which can be expected to determine the efficiency of retroviral supernatant transduction is the virus-to-target cell ratio (multiplicity of infection, or m.o.i.).[290]

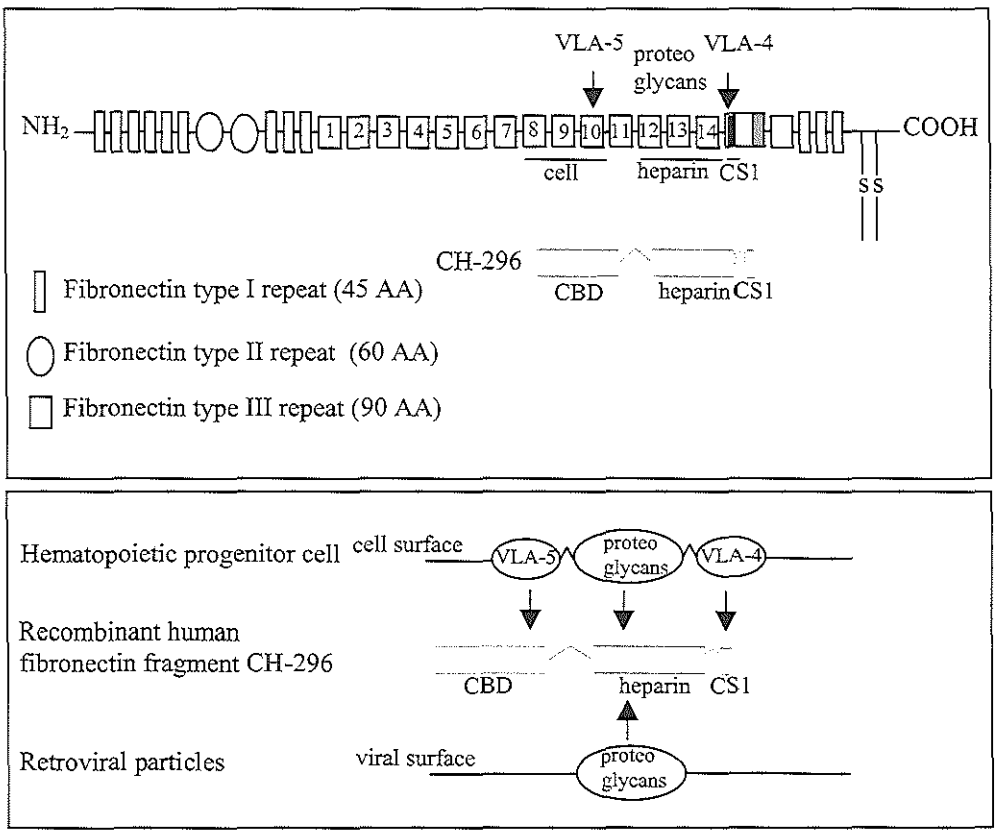

Figure 5. Schematic representation of human fibronectin α-chain and the recombinant human fibronectin fragment (CH-296). The fibronectin fragment contains three domains at which target cells and virus particles can bind. The cell binding domain (cell, CBD) binds hematopoietic cells via the integrin VLA-5. The CS1 sequence is known to bind cells via the integrin VLA-4 and the heparin binding domain binds cells and virus particles via proteoglycans. *Adapted from Williams DA, et al*, 1991[286]

A linear relation exists between amphotropic retrovirus supernatant-dilution and gene transfer efficiency to NIH/3T3 cells suggesting that a high virus titer is of importance for increasing transduction levels. In co-cultivation assays the direct relation between virus titer and transduction efficiency was not found.[291]
Additional techniques to improve the transduction efficiency include centrifugation of the retrovirus and the target cells[292, 293], increasing the viral titer via filtration, the use of roller bottles or other cell culture systems[292, 294, 295] or 'flow through' technology.[296] Cryopreservation of target cells before transduction with an amphotropic retroviral vector upregulates the amphotropic receptor (amphoR) levels[297-299], which has been reported to result in higher transduction efficiency.[300] It is possible that other retroviral receptors, such as the GALV receptor (Pit-1 or GLVR-1) which is present on hematopoietic progenitor cells, is upregulated as well following cryopreservation.
In addition to approaches to optimise gene transfer *in vitro*, *in vivo* approaches to increase the transduction efficiency may be relevant. Hematopoietic stem cells could be manipulated *in vivo* to trigger the cells into cycle, for instance, using 5-fluorouracil (5-FU)[277-279] which has extensively been used in mouse experiments but not in large animal models, or hydroxy-urea as an S-phase killer[280] to synchronise the cell cycle. Also an increase of the viral-receptor density on the target cells might be of importance to reach high transduction efficiencies in long-term repopulating cells. Growth factor stimulation is known to alter receptor expression[182] and it is conceivable that receptor expression of certain retroviral receptors can be affected by administration of various growth factors. Bone marrow or PB obtained from rhesus monkeys after treatment with hematopoietic GF (G-CSF and SCF) were more efficiently marked than cells obtained from untreated animals.[276]

Rationale and outline of this thesis

This thesis is firstly based on the hypothesis that hematopoietic stem cell characterisation requires the (further) development of transplantation assays. For human hematopoietic stem cells, such assays are not feasible other than the use of specifically adapted experimental animals as recipients or the use of a nonhuman primate species as a model. Hence, the use of SCID mice as recipients of human

normal and malignant stem cells was examined in detail with emphasis on its quantitative use, and advantage was taken of the existing quantitative model in rhesus monkeys.[301-304] Second, it was hypothesised that 1) the development of retrovirus mediated gene transfer required elucidation of stem cell biology rather than the development of novel vectors[305] and 2) gene marking of *in vivo* repopulating stem cells serves stem cell characterisation and the development of gene therapy equally well.

Based on these hypothesis the studies summarised in this thesis are directed at gaining insight into the characteristics of cells capable of multilineage *in vivo* repopulation. The approach chosen involved the further development of transplantation assays in immunodeficient (SCID) mice[137, 145, 158, 306] for normal and leukemic human stem cells (chapters 2, 3 and 6). In addition, gene marking of purified hematopoietic cell subsets using a fluorescent marker was developed to be able to track the transplanted cells after transplantation in the mouse assay (chapter 4). Marking and transplantation of distinct hematopoietic cell subsets, for instance purified Philadelphia-chromosome$^+$ (Ph^+) and Ph^- cells from chronic myeloid leukemia patients, could be used to study the possible selective outgrowth of such cells *in vivo*. For genetic marking the gene encoding the enhanced version of the green fluorescent protein (EGFP) was selected for the transduction of human umbilical cord blood immature cells and rhesus monkey BM cells, based on previous studies in the laboratory.[264, 307, 308] The use of the autofluorescent EGFP gene as a marker allows visualisation of the transduction efficiency and tracking of the fate and progeny of the transplanted cells in real time, without additional steps. The EGFP is transduced using retroviral vectors which were expressed using amphotropic and pseudotyped virus producer cell lines (Am12 and PG13, respectively). Although studies with mice have confirmed that retrovirus vectors are efficient vehicles to integrate exogenous genes into the DNA of pluripotent repopulating stem cells, it has been difficult to achieve a similarly high efficiency of gene transfer into human stem cells. Therefore, analysis of the variables influencing transduction efficiency and optimisation of the transduction protocol was of major importance to gain high transduction levels in stem cells (chapter 5), making use of co-localisation of virus vector and target cells[282, 283], and novel growth factors to stimulate immature cells, in particular thrombopoietin, c-kit ligand and Flt3 ligand. Subsequent transplantation of transduced UCB cells into NOD/SCID mice and rhesus BM cells into rhesus monkeys enabled tracking of the short- and long-term repopulating cells. The thesis is concluded by a general

discussion (chapter 7) relating the progress made to concurrent other studies and developments.

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CHAPTER

II

Transplantation of human umbilical cord blood cells in macrophage-depleted SCID mice: evidence for accessory cell involvement in expansion of immature $CD34^{+}CD38^{-}$ cells

Co-authors: Paula B. van Hennik, Wim Terpstra, Cor van den Bos, Jenne J. Wielenga, Nico van Rooijen, Rob E. Ploemacher, Gerard Wagemaker and Albertus W. Wognum

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Abstract

In vivo expansion and multilineage outgrowth of human immature hematopoietic cell subsets from umbilical cord blood (UCB) were studied by transplantation into hereditary immunodeficient (SCID) mice. The mice were preconditioned with Cl_2MDP-liposomes to deplete macrophages and 3.5 Gy total body irradiation (TBI). As measured by immunophenotyping, this procedure resulted in high levels of human $CD45^+$ cells in SCID mouse bone marrow (BM) 5 weeks after transplantation, similar to the levels of human cells observed in non-obese diabetic (NOD) /SCID mice preconditioned with TBI. Grafts containing ~10^7 unfractionated cells, ~10^5 purified $CD34^+$ cells or $5x10^3$ purified $CD34^+CD38^-$ cells yielded equivalent numbers of human $CD45^+$ cells in the SCID mouse BM, which contained human $CD34^+$ cells, monocytes, granulocytes, erythroid cells and B-lymphocytes at different stages of maturation. Low numbers of human GpA^+ erythroid cells and $CD41^+$ platelets were observed in the peripheral blood (PB) of engrafted mice. $CD34^+CD38^-$ cells ($5x10^4$/mouse) failed to engraft, while $CD34^-$ cells (10^7/mouse) displayed only low levels of chimerism, mainly due to mature T lymphocytes. Transplantation of graded numbers of UCB cells resulted in a proportional increase of the percentages of $CD45^+$ and $CD34^+$ cells produced in SCID mouse BM. In contrast, the number of immature, $CD34^+CD38^-$ cells produced *in vivo* showed a second-order relation to $CD34^+$ graft size, and mice engrafted with purified $CD34^+CD38^-$ grafts produced 10-fold fewer $CD34^+$ cells without detectable $CD34^+CD38^-$ cells than mice transplanted with equivalent numbers of unfractionated or purified $CD34^+$ cells. These results indicate that SCID repopulating $CD34^+CD38^-$ cells require $CD34^+CD38^+$ accessory cell support for survival and expansion of immature cells, but not for production of mature multilineage progeny in SCID mouse BM. These accessory cells are present in the purified, non-repopulating $CD34^+CD38^+$ subset as was directly proven by the ability of this fraction to restore the maintenance and expansion of immature $CD34^+CD38^-$ cells *in vivo* when added to purified $CD34^+CD38^-$ grafts. The possibility to distinguish between maintenance and outgrowth of immature

repopulating cells in SCID mice will facilitate further studies on the regulatory functions of accessory cells, growth factors (GF) and other stimuli. Such information will be essential to design efficient stem cell expansion procedures for clinical use.

Introduction

Traditional sources of hematopoietic stem and progenitor cells for transplantation include autologous and allogeneic bone marrow and mobilised peripheral blood (PB). Recently human umbilical cord blood (UCB) has been shown to be a realistic alternative source of stem cells.[1, 2] UCB contains cells of all hematopoietic lineages including cells that can produce granulocyte-macrophage colony forming unit (GM-CFU) after extended long-term stromal cell supported culture. Most of these long-term culture-initiating cells (LTC-IC) are found in the small subset of $CD34^+CD38^-$ cells.[3] The ability to cryopreserve, select, and expand progenitors without loss of proliferative capacity[4] makes UCB an appropriate model to identify immature hematopoietic cell subsets involved in hematopoiesis *in vivo*, select appropriate growth factor (GF) combinations and culture conditions to maintain and expand stem cells *in vitro*, and design optimal gene transfer conditions aimed at efficient and stable transduction of transplantable stem cells.[5]

Hereditary immunodeficient SCID and NOD/SCID mice are useful recipients to assess human stem cell capacities in a transplantation assay and appear particularly suitable to assess the outgrowth of purified UCB cell subsets and the effects of *ex vivo* manipulation on hematopoietic capacities after transplantation. Several approaches for engrafting immunodeficient mice with normal or leukemic human hematopoietic cells have been described. The most frequently used systems involve injection of mobilised human PB, BM[6] or UCB cells in sublethally irradiated mice[7, 8], electively followed by human growth factor (GF) treatment[9-12] and/or cotransplantation with non-repopulating $CD34^-$ accessory cells[13], human BM long-term culture-derived stromal cells or rodent cell lines that produce human GFs.[14] Transgenic SCID mice expressing the genes for human IL-3, GM-CSF and SCF have also been used to promote human cell engraftment[15], while human fetal liver, thymus[16, 17] and/or bone fragment[18] implantation has been used to create a human micro-environment in the mouse. Injection with human cytokines or other

additional treatment is not required to establish high-level human cell engraftment after transplantation of human UCB cells in immunodeficient mice, which suggested that neonatal cells either respond differently to the murine microenvironment or provide their own cytokines in a paracrine fashion.[7, 8]
However, analysis of the hematopoietic potential of UCB cells in SCID is limited by the large number of cells required to achieve significant engraftment levels, possibly because of low seeding efficiencies of stem cells or elimination of transplanted cells by natural killer (NK) cells or the mononuclear phagocytic system, which are intact in SCID mice. More reproducible and higher levels of engraftment with smaller graft sizes have been achieved with NOD/SCID mice, which has been attributed to the lack of functional macrophages, NK cells and complement activity in this mouse strain.[19] Specific elimination of phagocytic cells in spleen and liver of SCID mice can be achieved within 24 hours after a single iv. injection of liposome-encapsulated dichloromethylene diphosphonate (Cl_2MDP).[20-22] As shown recently for human acute myeloid leukemia (AML) and UCB cells, macrophage-depleted SCID mice supported the production of similar levels of human cells from 10-fold fewer transplanted cells as compared to SCID mice conditioned with TBI alone. For AML cells, preconditioning of SCID mice with liposomes led to similar levels of engraftment as observed in NOD/SCID mice which suggested that macrophages have a prominent role in eliminating injected human cells in SCID mice.[22] The present study was undertaken to quantitatively analyse the maintenance and outgrowth of distinct UCB immature cell subsets in macrophage-depleted SCID mice and to assess the hematopoietic cell lineages produced.

Material and methods

Human umbilical cord blood cells

UCB samples were obtained after informed consent in conformity with legal regulations in The Netherlands from placentas of full-term normal pregnancies. Mononucleated cells were isolated by Ficoll density gradient centrifugation (1.077 g/cm^2, Nycomed Pharma AS, Oslo, Norway), and were cryopreserved in 10% dimethylsulphoxide, 20% heat-inactivated fetal calf serum (FCS) and 70% Hanks Balanced Salt Solution (HBSS, Gibco, Breda, The Netherlands) at -196°C as

described.[23] After thawing by stepwise dilution in HBSS containing 2% FCS, the cells were washed with HBSS containing 1% FCS and used for flow cytometric analysis, transplantation into SCID mice (unfractionated grafts) or subset purification.

Subset purification

Purification of $CD34^+$ cells was performed by positive selection using Variomacs Immunomagnetic Separation System as described[24] (CLB, Amsterdam, The Netherlands). The percentage $CD34^+$ cells in the unseparated population (unfractionated UCB) and in the purified $CD34^+$ and $CD34^-$ fractions was determined by FACS-analysis. For isolation of $CD34^+CD38^+$, $CD34^+CD38^{+/-}$ and $CD34^+CD38^-$ subsets, purified $CD34^+$ cells were stained with fluorescein isothiocyanate (FITC) and R-phycoerythrin (PE) conjugated antibodies against human CD34 and CD38 (CD34-FITC, CD38-PE, Becton Dickinson) for 30', on ice in HFN (HBBS, 2% (wt/vol) FCS, 0.05% (wt/vol) sodium-azide) containing 2% (vol/vol) normal human serum (NHS). After incubation, the cells were washed twice, resuspended in HBSS and sorted using a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA).

Transplantation of UCB cells into immunodeficient mice

Female, specified pathogen-free (SPF) CB-17-scid/scid (SCID) mice, 6 to 9 weeks of age, were obtained from Harlan, CPB, Austerlitz, The Netherlands. NOD/LtSz-scid/scid mice (NOD/SCID) were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). The mice were housed under SPF conditions in a laminar airflow unit and supplied with sterile food and acidified drinking water containing 100 mg/l ciprofloxacine (Bayer AG, Leverkusen, Germany) *ad libitum*. The plasma Ig levels of the mice were determined with an ELISA using a sheep anti-mouse antibody reacting with mouse IgG (Boehringer Mannheim Biochemica, Penzberg, Germany) and animals with plasma Ig levels over 40 μg/ml were excluded.[25] To deplete macrophages, the SCID-mice were injected iv. into a lateral tail vein with 200 μl liposome stock solution containing di-chloromethylene di-phosphonate (Cl_2MDP, a gift of Boehringer Mannheim GmbH, Germany) one day prior to transplantation of hematopoietic cells.[26] In previous studies[22] with human acute leukemia and UCB cells this approach required ten-fold fewer cells for uniform engraftment than in SCID mice conditioned with TBI alone. All mice received total

body irradiation (TBI) at 3.5 Gy, delivered by a ^{137}Cs source adapted for the irradiation of mice (Gammacell, Atomic Energy of Canada, Ottawa, Canada), 2-4 hours before transplantation. The transplants were suspended in 200 µl HBSS containing 0.1% BSA (Sigma) and injected iv. into a lateral tail vein. Transplanted cell numbers were 10^7 (unfractionated and $CD34^-$ cells), 10^5 ($CD34^-$ cells), $5x10^4$ ($CD34^+CD38^+$ cells) and $5x10^3$ ($CD34^+CD38^-$ cells) unless stated otherwise in the results.

In vitro colony assay

Unfractionated and purified $CD34^+$ and $CD34^-$ grafts as well as chimeric mouse BM samples were assayed for the presence of GM-CFU and erythroid burst-forming units (BFU-E) by *in vitro* colony formation in viscous methylcellulose culture medium as previously described.[27-29] Briefly, unfractionated and $CD34^-$ cells were plated at a concentration of 25,000 per 35 mm Petri dish (Becton Dickinson), $CD34^+$ purified grafts at 1000 per dish and chimeric mouse BM at 50,000 per dish. Culture medium consisted of 1 ml Dulbecco's medium (Gibco, Gaithersburg, MD), containing 0.8% (wt/vol) methylcellulose, 5% (vol/vol) FCS, and further supplemented with 1.5% (wt/vol) BSA, 10 mg/ml insulin, 0.3 mg/ml human transferrin, 15 mmol/L ß-mercaptoethanol, 0.1 mmol/L sodium selenite, 1 mg/ml nucleosides, 15 µmol/L linoleic acid, 15 µmol/L cholesterol, 100 U/ml penicillin, and 50 mg/ml streptomycin. For BFU-E, cultures were supplemented with 0.2 mmol/L bovine hemin (Sigma, St. Louis, USA), 200 ng/ml human SCF and 4 U/ml (25 µg/ml) human recombinant Epo (Behringwerke AG, Marburg, Germany). For GM-CFU, cultures were supplemented with 5 ng/ml human recombinant GM-CSF (Behringwerke AG, Marburg, Germany), 200 ng/ml SCF and 30 ng/ml human recombinant IL-3. The cultures were maintained in a humidified atmosphere of 10% CO_2 at 37°C for 14 days, after which the colonies were counted. Data of duplicate dishes were expressed as average number of colonies per 10^5 cells plated.

Tissue collections and analysis

Mice were examined at a single time point, 35 days after transplantation, to enable meaningful comparisons between experiments, because individual hematopoietic subsets show differences in engraftment kinetics in immunodeficient mice.[12] Mice were killed by CO_2 inhalation followed by cervical dislocation in accordance with

institutional animal research regulations. From each mouse, both femurs were collected and BM cell suspensions were prepared by flushing. After counting, the cells were cultured in colony assays and analysed by flow cytometry to determine the percentage of human cells in the mouse bone marrow. Cells were suspended in HBSS containing 2% (vol/vol) FCS, 0.05% (wt/vol) sodium azide, 2% (vol/vol) human serum and 2% (vol/vol) mouse serum and stained for 30' at 4°C with the pan-leukocyte surface marker CD45-FITC antibody and with CD33-PE antibody. Positive samples were further analysed by incubation with FITC and PE labelled mouse monoclonal antibodies to human CD34, CD19, CD16, CD15, CD38, CD33, CD56, CD4, CD8 (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), and glycophorin A (GpA), CD3, CD71 (Dako A/S, Denmark). Parallel samples were incubated with isotype matched control antibodies. Cell samples of non-transplanted mice were stained as negative controls. Fluorescence was measured using a FACScan flow cytometer and Lysis II software (Becton Dickinson, USA). Dead cells were excluded by adding 1 μg/ml propidium iodide (PI) and gating for PI^- cells in the FL3 (PI) channel. For all samples 10,000 events were collected in a gate for PI^- cells. To quantitate $CD34^+$ subsets in selected samples, 1,000 - 10,000 events were also collected in a gate that included all viable human $CD34^+$ cells. $CD34^+$ and $CD34^+CD38^-$ expansion were calculated on the assumption that one femur contains 8.5% of all bone marrow cells.[30]

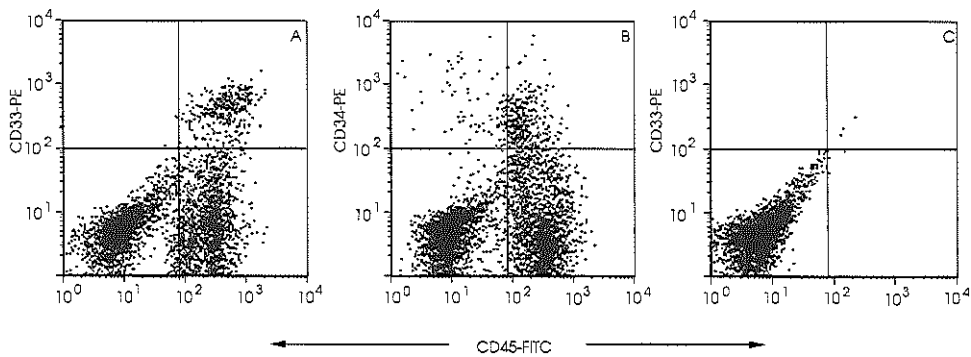

Figure 1. Flow cytometric analysis of chimeric mouse bone marrow stained with CD45-FITC vs. CD33-PE (A), and CD45-FITC vs. CD34-PE (B). BM of non-transplanted mice showed no staining with the CD45-FITC (not shown) or CD33-PE antibody (C).

In a number of experiments, peripheral blood was collected weekly from the tail vein and analysed for the presence of human GpA^+ erythrocytes and $CD41^+$ platelets, by flow cytometry. Blood samples were collected in EDTA coated tubes and stained with CD41-FITC (PharMingen, San Diego, USA) and GpA-FITC, respectively (Dako A/S, Denmark) in HBSS with 2% (vol/vol) FCS, 0.05% (wt/vol) sodium azide, 2% (vol/vol) human serum 2% (vol/vol) mouse serum and 2 g/L EDTA for 30' at 4°C. Cell samples of non-transplanted mice and human blood cells were stained as controls.

Statistical and regression analysis

Results are expressed as individual data or as arithmetic mean ± standard deviation. The regression analysis of the percentage of human $CD45^+$, $CD34^+$ and $CD34^+CD38^-$ cells in the chimeric BM as a function of the number of $CD34^+$ cells transplanted was done by plotting the data on a double logarithmic scale and calculating the regression using the general formula $y = axb$. By this method, an exponent b=1 proves first order (single-hit) kinetics, i.e., direct proportionality (linearity) of chimeric cell numbers and cells transplanted, while an exponent b=2 demonstrates second order (two-hit) kinetics. The frequency of repopulating cells in the SCID mice was approximated using Poisson statistics.

Results

Chimeric bone marrow analysis

Chimerism in SCID mouse bone marrow was assessed by flow cytometric analysis 35 days after UCB transplantation. Typical results of chimeric bone marrow stained with CD45-FITC vs. CD33-PE and CD45-FITC vs. CD34-PE are shown in Figure 1A and 1B, respectively. The percentage of $CD45^+$ cells was used as a measure for engraftment levels of human cells in the mouse BM. Only mice with percentages larger than 1% $CD45^+$ cells were considered to be engrafted. Positive staining for any of these markers was not found in non-transplanted mice (Figure 1C and data not shown), demonstrating the specificity of the antibodies for human cells. As shown in Figure 1A and 1B, the $CD45^+$ cells were heterogeneous with respect to CD33 and CD34 expression.

Parallel groups of mice were injected with unfractionated mononucleated UCB cells or with purified $CD34^+$ or $CD34^-$ cells (Figure 2) in SCID mice conditioned with either TBI or TBI and macrophage depletion, or in TBI conditioned NOD/SCID mice. Transplantation of unfractionated mononucleated UCB cells into macrophage-depleted SCID mice resulted in more prominent engraftment levels compared to SCID mice conditioned with TBI alone. After transplantation with 10^7 unfractionated or 10^5 purified $CD34^+$ cells, the macrophage-depleted SCID mice showed similar levels of chimerism as NOD/SCID mice preconditioned with TBI. $CD34^-$ cells (10^7 cells transplanted) did not result in high levels of chimerism in either mouse strain (Figure 2).

Table 1. Engraftment of UCB cells in pre-conditioned SCID mice

Graft	Graft Size*	Chimeric† mice/Injected	% Chimerism ($CD45^+$cells)	$CD34^+$ cells		$CD34^+CD38^-$ cells	
				Cells/ Mouse BM	Expansion factor	Cells/ mouse BM	Expansion factor
				x10^6		x10^6	
unfract.	10^7	22/22	34.5±19.3‡	1.1±1.4§	12.9±19.2	1.84±2.7	2.19±4.92
$CD34^-$	10^7	5/18	7.8±8.6	0.1±0.1	4.0±3.7	0	0
$CD34^+$	10^5	35/38	20.4±16.3	1.1±0.9	14.1±18.6	6.9±13.2	10.8±16.6¶
$CD34^+CD38^+$	5x10^4	1/4	1.7	0	0	0	0
$CD34^+CD38^-$	5x10^3	4/6	18.4±8.7	0.1±0.1	18.6±5.2	0	0¶

* % $CD34^+$ cells in graft: unfract. UCB 0.7 - 3.1%, $CD34^+$ 77 - 83% and $CD34^-$ 0.1 - 0.6%

† mice are considered chimeric at >1% $CD45^+$ cells

‡ mean ± SD of chimeric mice, results of 5 UCB

§ $CD34^-$ BM cells, calculated on the assumption that 1 femur represents 8.5% of all BM cells[30]

¶ significantly different from unfractionated graft (p=0.03, Fisher's exact test)

As shown in Table 1, transplantation of 10^7 unfractionated cells from 5 different UCB samples resulted in high levels of chimerism in all mice (n=22) transplanted. Transplantation of 10^5 purified $CD34^+$ cells also resulted in high levels of human cells in 35 out of 38 mice, whereas mice transplanted with 10^7 $CD34^-$ cells showed

only low levels of engraftment in 5 out of 18 mice transplanted. These results demonstrate that relatively low numbers of purified $CD34^+$ UCB cells are capable of proliferation in the macrophage-depleted SCID mouse microenvironment without the support of accessory cells or addition of hematopoietic growth factors. The $CD34^+$ cells were further separated into a $CD38^+$ subset (~50% of $CD34^+$ cells containing >90% of clonogenic progenitors) and a $CD38^-$ subset (~5% of the $CD34^+$ population, enriched for immature, multipotent progenitors[31]) and transplanted into preconditioned SCID mice; cell numbers were $5x10^4$ and $5x10^3$, respectively. The $CD34^+CD38^-$ subset showed high levels of engraftment in 4/6 mice with chimerism levels similar to those obtained with 20-fold larger numbers of $CD34^+$ cells and 200-fold larger numbers of unfractionated UCB (Table 1). Despite the 10-fold larger cell numbers, only 1 of 4 mice engrafted with sorted $CD34^+CD38^+$ cells at the low level of 1.7%. These results show that the ability to repopulate SCID mice resides exclusively in the $CD34^+CD38^-$ immature population.

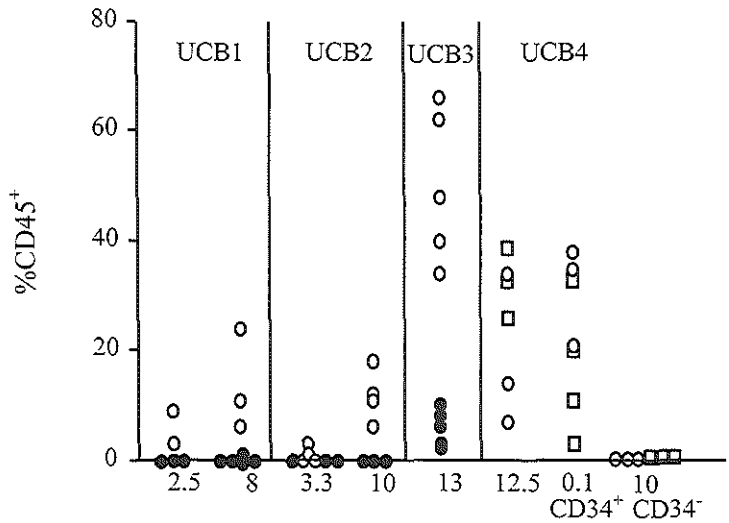

Figure 2. Engraftment levels of 4 different UCB samples in SCID and SCID/NOD mice. The percentages $CD45^+$ cells are shown for individual SCID mice preconditioned with TBI and Cl_2MDP (○), SCID mice preconditioned by TBI alone (●) and NOD/SCID mice preconditioned by TBI alone (□).

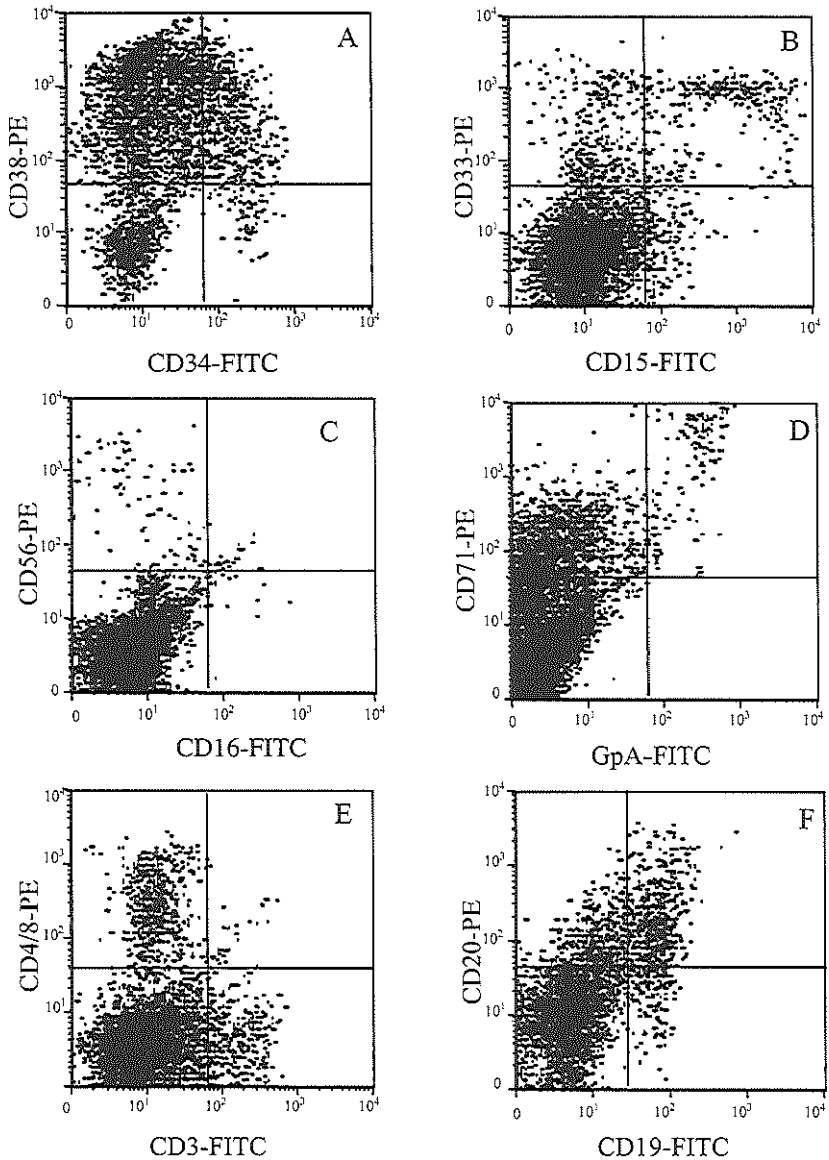

Figure 3. Immunophenotyping of chimeric mouse BM. BM (>10% $CD45^+$) was stained with a panel of antibodies specific against different human blood cell lineages. FACS profiles of a representative mouse are shown for CD34 vs. CD38 (A), CD15 vs. CD33 (B), CD16 vs. CD56 (C), GpA vs. CD71 (D), CD3 vs. CD4 and CD8 (E) and CD19 vs. CD20 (F) expression, respectively.

Multilineage outgrowth of UCB cells

BM cells of chimeric mice were cultured in standard methylcellulose culture under conditions of stimulation with recombinant human GF which selectively favour the outgrowth of human monomyeloid and erythroid progenitors and fail to stimulate mouse progenitors. Comparison of clonogenic cell numbers in 15 chimeric mice with the numbers of colony-forming cells in the grafts showed a median expansion of 2.7-fold (range : 0 - 11) and 1.7-fold (range: 0 - 13) for GM-CFU and BFU-E numbers, respectively, as measured 35 days after transplantation. Since these progenitor cell populations have a high turnover rate, this observation demonstrates that monomyelocytic and erythroid progenitors are produced from more immature progenitors in the mouse hematopoietic environment. The composition of the human cell population in the BM of chimeric mice was assessed by flow cytometry using a panel of lineage specific markers (Figure 3). The percentage of cells in each subset identified was expressed relative to the percentage cells stained with the panleukocyte marker CD45 (Figure 4). Mice transplanted with 10^7 unfractionated UCB cells showed multilineage outgrowth (Figure 4A). The most prominent population (25-50% of the human $CD45^+$ cells) consisted of B lymphoid cells, which contained immature $CD19^+CD20^-$ as well as mature $CD19^+CD20^+$ cells (Figures 3F, 4). $CD15^+CD33^+$ monocytes, $CD15^+CD33^{+/-}$ granulocytes, and $CD15^-CD33^+$ immature myelomonocytic cells were present at percentages ranging between 6 and 16% of the human cells (Figure 3B, 4). GpA^+CD71^{++} erythroblasts and, occasionally, GpA^+CD71^- mature red blood cells (not visible in Figure 3D) were present in low numbers. In keeping with the presence of CD71 on activated non-erythroid cells, the large population of $CD71^+GpA^-$ cells (Figure 3D) contained cells of multiple lineages.[32] The composition of the BM of mice transplanted with $CD34^+$ (Figure 4B) or $CD34^+CD38^-$ cells (Figure 4C) was similar to that of mice transplanted with unfractionated UCB. The few mice that showed detectable chimerism after transplantation of $CD34^-$ cells also had outgrowth of low numbers of myeloid, erythroid and B lymphoid cells, which were possibly derived from the low numbers of $CD34^+$ cells (0.1-0.6%) still present in the fraction. However, >50% of the cells growing in these mice consisted of mature $CD3^+$ T lymphocytes, which also expressed CD4 or CD8. $CD3^+$ cells were also identified in mice transplanted with unfractionated, $CD34^-$ or $CD34^+CD38^-$ cell subsets, but these $CD3^+$ cells neither expressed CD4 nor D8 (Figures 3, 4A-C). These cells may represent a subset of NK cells as CD3 is expressed on some $CD56^+$ cells[33] and $CD56^+$ cells were also identified in low numbers in chimeric mice, including those transplanted with purified $CD34^+CD38^-$ cells (Figures 3C,

4C). The large population of $CD3^-$ cells which expressed CD4 or CD8 (Fig 3E), most likely consisted of $CD4^+$ monocytes. In spite of large numbers of human cells in the BM of SCID mice, very few human cells were detected in the leukocyte fraction of peripheral blood, spleen and thymus (data not shown). Whole tail vein blood samples of $CD34^+$ transplanted mice collected at various time points after transplantation, contained human GpA positive erythrocytes at very low levels (~0.1%) which could only be detected if very large cell numbers ($>10^5$) were analysed (Figure 5C).

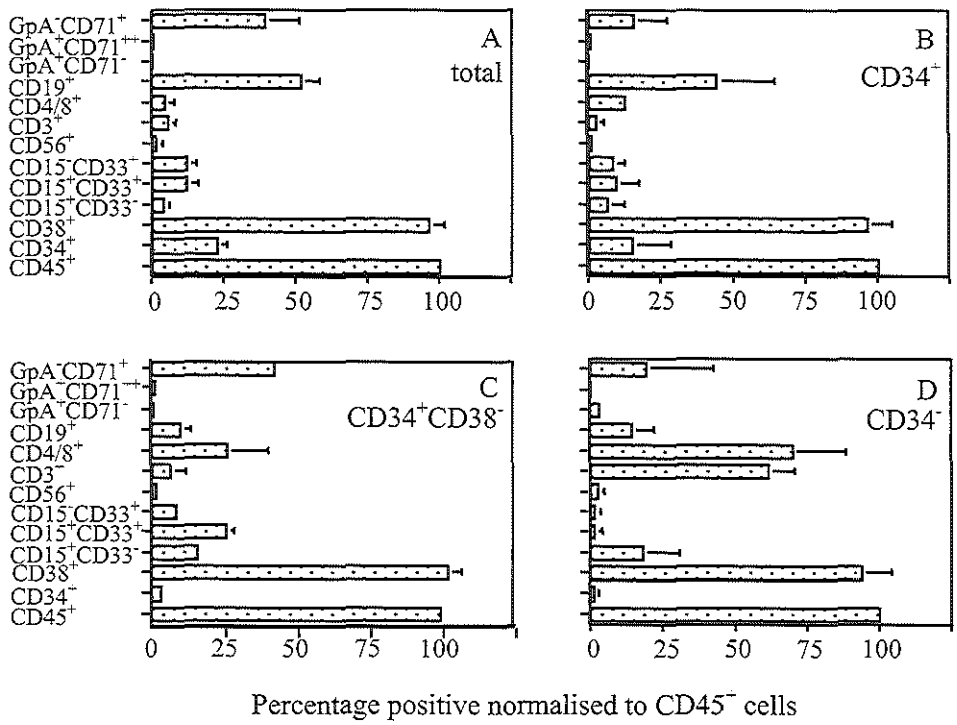

Figure 4. Composition of the human $CD45^+$ cell population in chimeric SCID mice stained for the human markers, shown in Figure 3. Results (average ± SD) of 23 mice in total, transplanted with unfractionated UCB (A), purified $CD34^+$ (B), $CD34^+CD38^-$ (C) and $CD34^-$grafts (D) derived from 5 UCB samples. Percentage of cells in each subset was expressed relative to the $\%CD45^-$ cells present in the BM of each mouse. The % chimerism ranged between 10 and 40% for the data shown in A-C, and between 1 and 15% for the data shown in D.

The largest quantities (0.1 - 0.2%) were found 2 weeks after transplantation. From the third week on the level decreased and became undetectable in the fifth week. Human $CD41^+$ platelets could also be detected in the mouse peripheral blood and followed a similar time course as the erythroid cells, with peak levels of 0.5% in the second week (Figure 5B).

Evidence for accessory cell requirement for immature cell expansion but not for outgrowth of human UCB cells in SCID mice

The UCB cell number required for engraftment was analysed by injection of graded numbers of unfractionated or $CD34^+$ cells. Transplantation of $2x10^3$ $CD34^+$ cells resulted in a low, but measurable level of chimerism of 1.4% $CD45^+$ cells (Figure 6). The level of chimerism increased proportionally with cell dose, reaching ~60% human $CD45^+$ cells after injection of $2x10^5$ purified $CD34^-$ cells. Engraftment after transplantation of unfractionated mononuclear UCB cells and purified $CD34^+$ cells followed similar proportional patterns with exponents of 0.8 and 1, respectively (Figure 6). Also the percentage of human $CD34^+$ cells detected in SCID mouse BM after 35 days showed a linear relation with graft size (Figure 6). These results demonstrate that the outgrowth of human UCB cells in the SCID mouse BM does not require the support from accessory cells present in either the $CD34^+$ or $CD34^-$ UCB fractions. Figure 3A shows that the $CD34^+$ cells produced in SCID mouse BM were heterogeneous with respect to CD38 expression and included cells with low CD38 expression, which suggested that very immature cells were maintained and/or expanded in the mouse micro-environment.

As shown in Figure 6, the production of cells with an immature $CD34^-CD38^-$ phenotype showed a much steeper dependence on the number of $CD34^+$ transplanted, with an exponent of 2, demonstrating second order (two-hit) kinetics. $CD34^+CD38^-$ cells were not detected in BM of mice transplanted with purified $CD34^+CD38^-$ cells (Table 1, Figure 8B) while, in addition, the numbers of $CD34^+$ cells in these mice were 10-fold lower than in mice transplanted with unfractionated or $CD34^+$ grafts, despite similar levels of $CD45^+$ cells (Table 1). Taken together, the non linear relation between graft size and % $CD34^+CD38^-$ cells after 35 days, the lower number of $CD34^+$ cells and absence of $CD34^-CD38^-$ cells in mice transplanted with purified $CD34^+CD38^-$ grafts demonstrate that immature $CD34^+CD38^-$ cells can be maintained in the mouse micro-environment, but only with the support of accessory cells.

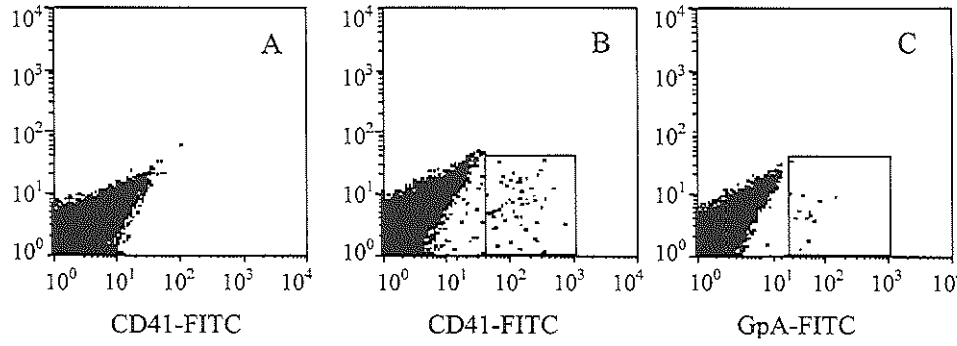

Figure 5. Circulating $CD41^+$ platelets and GpA^- erythrocytes in the peripheral blood of $CD34^+$ transplanted SCID mice. Blood was collected in the presence of 2 g/L EDTA and stained immediately with CD41-FITC (B) and GpA-FITC (C). Figure 5A shows blood of a non-transplanted mouse stained with CD41-FITC.

Figure 7 shows the actual expansion of $CD34^+CD38^-$ cells in BM of the 32 (from 69) chimeric mice in which such cells were detectable. The expansion ranged between 0.2 and 22.1 with a median expansion of 3-fold for unfractionated mononucleated UCB cells and between 1.6 and 63.1 with a median value of 7-fold after transplantation of $CD34^+$ grafts. This difference is statistically not significant. Direct proof of an accessory role of $CD34^+CD38^+$ cells in the maintenance of the transplanted $CD34^+CD38^-$ population *in vivo* was obtained by transplantation of $CD34^+CD38^-$ cells, supplemented with $CD34^+CD38^+$ cells. Dot plots of CD38 vs. CD34 expression, collected in a gate for $CD34^+$ cells, show that transplantation of $5x10^3$ $CD34^+CD38^-$ cells results in production of $CD34^+$ cells, which are all $CD38^+$ (Figure 8B). After transplantation of $5x10^3$ $CD34^+CD38^-$ cells to which $25x10^3$ $CD34^+CD38^+$ cells were added, $CD34^+CD38^-$ cells were clearly produced in the mouse BM (Figure 8C) at frequencies similar to those observed in mice transplanted with 10^5 $CD34^+$ cells (Figure 8D). Also in this experiment, transplantation of $5x10^4$ $CD34^+CD38^+$ cells alone did not result in human cell engraftment (similar to the data presented in Table 1). Sorted $CD34^+CD38^{+/-}$ cells (corresponding to 20% of the $CD34^+$ cells) also repopulated transplanted SCID mice with propagation of immature $CD34^+CD38^-$ cells, which can be explained by the presence of repopulating and accessory cells in this subset (Figure 8E).

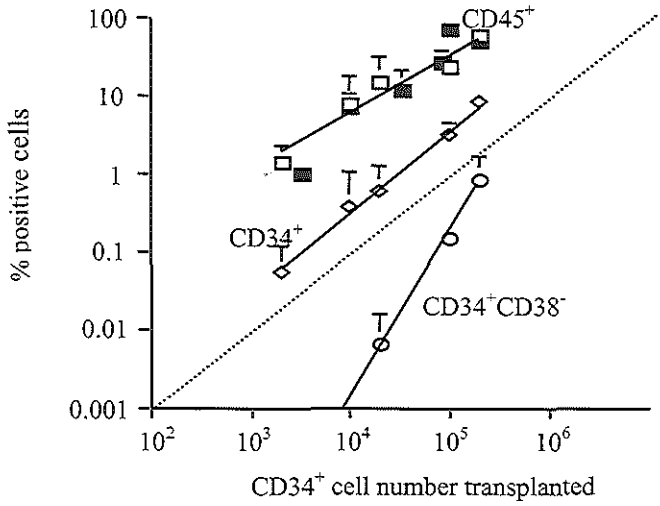

Figure 6. Relation between the number of $CD34^+$ cells transplanted and percentage of human $CD45^+$ (□), $CD34^+$ (◇) and immature $CD34^+CD38^-$ (○) cells detected in SCID mouse BM after 5 weeks. Results show the mean ± SD for 3 mice per data point. For comparison, the numbers of $CD45^+$ cells detected in BM of mice transplanted with graded numbers of unfractionated cells are also shown (■).

Repopulating cell frequency

The maintenance or expansion of $CD34^+CD38^-$ cells in SCID mice might be considered as a more significant characteristic of the capacity of repopulating stem cells than the ability to produce mature progeny. Taking into account that the seeding efficiency of repopulating cells in transplanted SCID mice is unknown and the support provided by accessory cells may be suboptimal, a lower limit for the frequency of cells with the ability to maintain or expand the numbers of $CD34^+CD38^-$ cells was estimated using the pooled data of 69 mice engrafted with graded doses of unfractionated or purified $CD34^+$ cells from 5 different UCB samples (Table 2). By using Poisson statistics, a value of 1 repopulating cell per 70,000 $CD34^+$ cells was estimated (95% confidence limits: 54,000 - 102,000). This would correspond to 1 repopulating cell per ~$7x10^6$ unfractionated UCB cells and 1 per 3500 $CD34^+CD38^-$ cells.

Discussion

Engraftment of UCB in SCID mice preconditioned by 3.5 Gy TBI and injection of CL_2MDP liposomes was more prominent than in SCID mice conditioned with TBI alone and similar to that observed in NOD/SCID mice. The macrophage-depleted SCID mice supported the multilineage outgrowth of unfractionated UCB, purified $CD34^+$ cells and the immature subset of $CD34^+CD38^-$ UCB cells, with production of B lymphocytes, monocytes, granulocytes, erythroid cells, NK cells and platelets as well as production of $CD34^+$ cells, including phenotypically immature $CD34^+CD38^-$ cells. Small numbers of purified $CD34^+CD38^-$ cells also engrafted efficiently with chimerism levels similar to those observed in accessory cell and/or GF supported NOD/SCID mice[13], whereas $CD34^+CD38^-$ cells did not engraft, indicating that the SCID repopulating potential resides exclusively in the $CD34^+CD38^-$ subset.

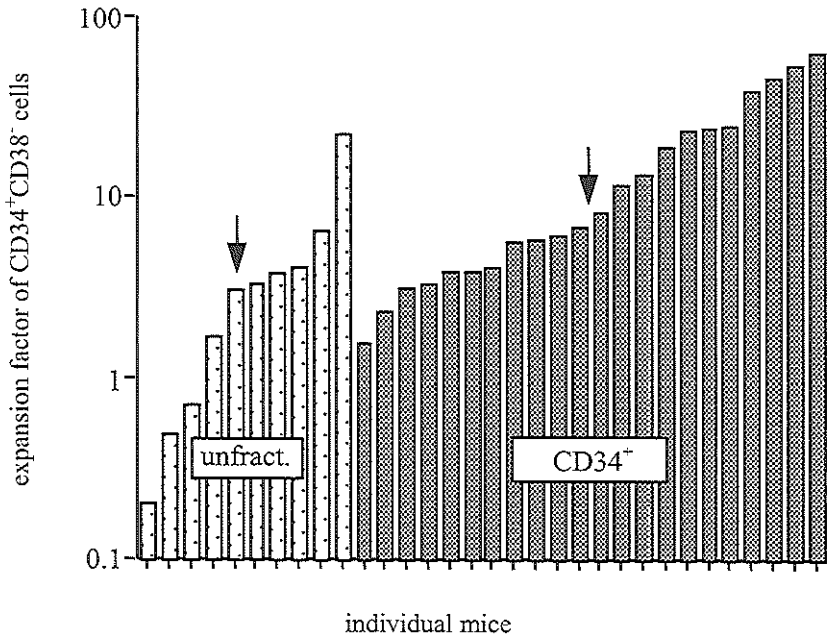

Figure 7. Expansion of $CD34^+CD38^-$ cells after transplantation of unfractionated or $CD34^+$ cells. Data for 32 mice which showed detectable $CD34^+CD38^-$ cells from a group of 69 chimeric mice (>1% $CD45^+$ cells) 28 mice were transplanted with unfractionated (unfract.) cells and 41 mice with $CD34^+$ grafts from 5 different UCB samples. The arrow shows the median expansion factor of $CD34^+CD38^-$ cells in each group.

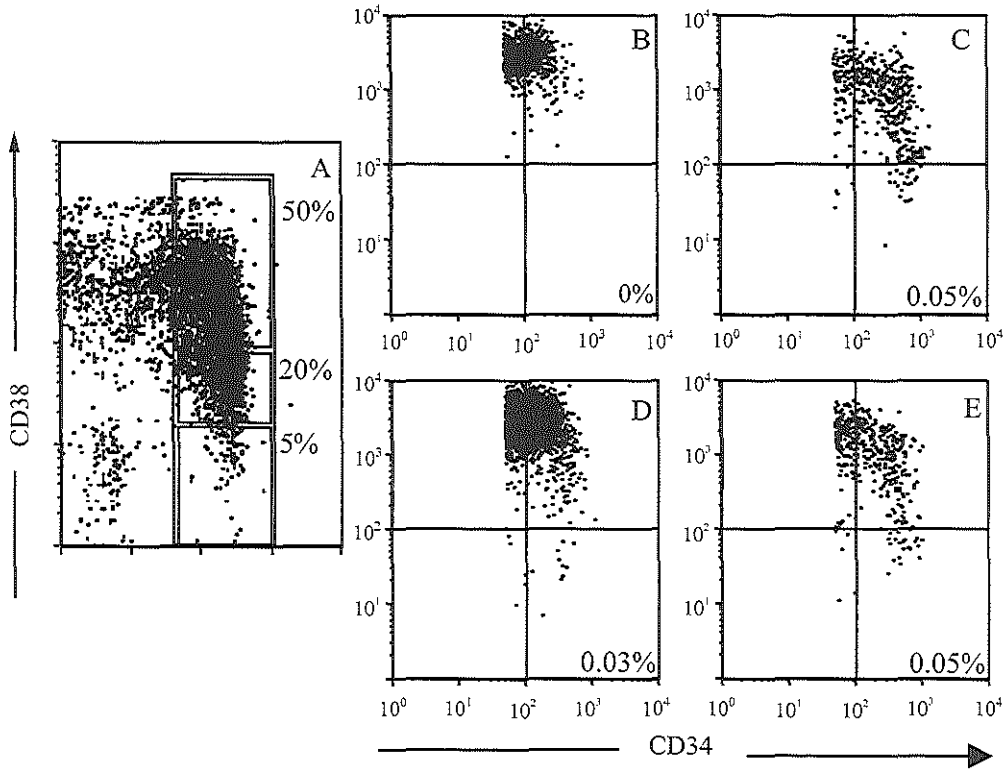

Figure 8. Distribution of human CD34 and CD38 in chimeric mouse BM after transplantation of $CD34^+$ subsets, sorted as defined by the windows in (A). (B) through (E) provide the results 35 days after transplantation of $5x10^3$ $CD34^-CD38^-$ cells (B), $5x10^3$ $CD34^-CD38^-$ + $25x10^3$ $CD34^+CD38^+$ cells (C), 10^5 $CD34^+$ cells (D), or $25x10^3$ $CD34^+CD38^{+/-}$ (E). One thousand to 10,000 events were collected in a window containing $CD34^+$ cells only. Quadrants were set to indicate $CD34^+CD38^-$ and $CD34^-CD38^+$ cells. The percentages indicate the frequency of human $CD34^+CD38^-$ cells in mouse BM. $CD34^+CD38^+$ cells did not engraft (data not shown). The dissociation of outgrowth of $CD45^+$ cells and maintenance or expansion of $CD34^+CD38^-$ cells is also in this experiment indicated by the CD45 percentages, ie, 25.1% for (B) without $CD34^+CD38^-$ cells and 5.5% for (C), 46.7% for (D), and 2.9% for (E) with similar frequencies of $CD34^+CD38^-$ cells.

The detection of $CD34^+CD38^-$ cells in SCID mouse BM is consistent with the finding that $CD34^+CD38^-$ cells recovered from the BM of engrafted SCID and NOD/SCID mice have retained the capacity to produce clonogenic progeny in long-term culture and to differentiate into myeloid and lymphoid cells in single

cell/well cultures.[34, 35] Results showing that purified human cells from NOD/SCID mouse BM may engraft secondary recipients, also suggest that repopulating stem cells are maintained in the BM of immunodeficient mice.[36] Taken together, these data demonstrate that immature $CD34^+CD38^-$ UCB cells can survive and expand in transplanted immunodeficient mice.

Table 2. Frequency analysis of repopulating cells

Graft*	$CD34^+$ cells transplanted†	Mice positive for $CD34^+CD38^-$	Mice negative for $CD34^+CD38^-$	% negative
		(n)	(n)	
$CD34^+$	1,660	0	2	100
	8,300	0	2	100
	16,600	1	1	50
	80,000	10	12	55
	104,097	8	2	20
	166,000	3	0	0
total	3,200	0	2	100
	9,600	0	3	100
	32,000	0	4	100
	60,000	2	3	60
	70,000	3	3	50
	111,000	2	1	33
	198,900	3	2	40

* Only chimeric SCID mice (>1% $CD45^+$ cells by flow cytometry)

† n=5 UCB, 69 mice transplanted

The level of expansion of the immature $CD34^+CD38^-$ subset in chimeric mouse bone marrow, but not the multilineage production of more differentiated progeny, appeared to be dependent on accessory cells. This is most clearly demonstrated by the second order (two-hit) kinetics of the relation between graft size and the numbers of immature $CD34^+CD38^-$ cells produced in the SCID mouse BM in contrast to the directly proportional relation between graft size and the numbers of mature $CD45^-$ cells and the $CD34^+$ population as a whole (Figure 6). Additional data show that engraftment levels and types of human cells produced in the bone

marrow of mice transplanted with $5x10^3$ $CD34^+CD38^-$ cell were similar to those obtained with 20-fold more $CD34^+$ cells or 2000-fold larger numbers of unfractionated mononucleated UCB cells, which also demonstrated that accessory cells or exogenous GFs are not needed for multilineage outgrowth of immature human cells in immunodeficient mice. In contrast, the observation that SCID mice transplanted with $CD34^+CD38^-$ grafts produced 10-fold fewer $CD34^+$ cells and no detectable $CD34^+CD38^-$ cells, despite equal numbers of $CD45^+$ cells, than mice transplanted with unfractionated or $CD34^+$ grafts with equivalent numbers of $CD34^+CD38^-$ cells (Table 1), provides additional evidence for an involvement of accessory cells in the maintenance and expansion of immature UCB cells in the SCID mouse microenvironment. Because mice transplanted with unfractionated mononucleated UCB cells did not show larger numbers of $CD34^+$ cells (Table 1) or more extensive expansion of $CD34^+CD38^-$ cells (Figure 7) than mice transplanted with purified $CD34^+$ cells, we postulate that the accessory cells needed for the support of immature UCB cells are present in the $CD34^+$ population. Formal proof was obtained by injection of $CD34^+CD38^-$ cells supplemented with $CD34^-CD38^-$ cells (Figure 8). Whereas transplantation of $CD34^+CD38^-$ cells alone did not result in the maintenance of these cells, the addition of 50% $CD34^+CD38^+$, a fraction that by itself did not result in substantial chimerism, restored the propagation of $CD34^-CD38^-$ cells in engrafted mice (Figure 8).

One possible function of the accessory cells UCB cells might be to prevent elimination of the small numbers of $CD34^+CD38^-$ cells by residual immune-reactivity in the SCID mouse by providing an excess of human cells. However, because small numbers of $CD34^+CD38^-$ cells produced equal numbers of mature progeny in the macrophage-depleted SCID mice than much larger unfractionated or $CD34^+$ grafts (Table 1), it is unlikely that such a mechanism plays a prominent role in promoting $CD34^+CD38^-$ cell engraftment. It is more likely that accessory cells provide essential GFs or other stimuli needed for the self-renewal of immature human cells which are not provided by the mouse microenvironment. $CD34^+$ UCB cells and their immediate progeny have been demonstrated to produce various GFs, including IL-3, G-CSF and GM-CSF, which stimulate *in vitro* colony formation by UCB in an autocrine or paracrine fashion.[37] A role of accessory cell derived GF in the maintenance of immature cells is also suggested by the supportive role of a cocktail of Epo, SF, IL-3 and GM-CSF for expansion of human cells in NOD/SCID mice transplanted with high numbers of unfractionated human BM cells. This was only observed late after transplantation, when the number of human cells was reduced.[12] Further studies are required to examine to what extent optimal

combinations of these or other GFs can replace accessory cells in maintaining and expanding $CD34^+CD38^-$ cells in immunodeficient mice.

Estimation, by Poisson statistics, of the frequency of original UCB cells that can maintain or expand $CD34^+CD38^-$ cell numbers during the five weeks engraftment period yielded a value of 1 in 70,000 $CD34^+$ cells (corresponding to 1 in 3500 $CD34^+CD38^-$ cells). This value is lower, but in the same order of magnitude than the 1 in 600 SCID repopulating $CD34^+CD38^-$ cells which has been calculated on the basis of the frequency of transplanted NOD/SCID mice with detectable numbers of human cells in the BM as assessed by Southern blots.[38] The difference is most likely due to the criteria chosen in that the ability to expand $CD34^+CD38^-$ cells is a more stringent parameter for engraftment of immature cells than the production of mature progeny at a level of as low as 0.05% human cells detected by DNA blotting analysis.[38] Such low engraftment levels can in principle be derived from contaminating mature cells, as demonstrated in our study by the low, but detectable (>0.5% of mouse BM), engraftment with mature T cells in some mice transplanted with purified $CD34^-$ cells. The ability to maintain or expand $CD34^+CD38^-$ cell numbers in SCID mouse BM is probably more characteristic for repopulating stem cells than production of mature progeny per se, because it may reflect an essential hematopoietic stem cells feature, i.e., the ability to maintain its own numbers *in vivo*.

The differences in repopulating cell frequencies might also be due to the co-transplantation of accessory cells and/or administration of growth factors in the NOD/SCID mouse model which may have promoted human cell engraftment.[38] Although it is clear that $CD34^+CD38^-$ cells still represent a heterogeneous cell population with only a minority of cells capable of hematopoietic reconstitution, all frequency-estimates of the SCID mouse repopulating human cells likely underestimate the frequency of human repopulating cells and should be treated with caution. In particular, the seeding efficiency of these cells has not been assessed yet, while the efficacy of the growth stimuli provided by the xenogeneic environment, accessory cells in the transplant or exogenous growth factor administration might very well be suboptimal. Studies into the kinetics of human BM cell engraftment in immunodeficient mice have shown that the number of immature, $CD34^+Thy\text{-}1^+$ cells that can be detected in the mouse BM two days after transplantation is at least two logs lower than input numbers, suggesting that only a very small fraction of the immature human cells develop in these mice.[12]

The present study provides evidence for differential regulation of the expansion as opposed to multilineage outgrowth of immature human hematopoietic stem cells in

transplanted SCID mice. The possibility to distinguish experimentally between these essential functions in the SCID mouse transplantation assay now opens an experimental approach to examine the effects of various GFs, cell subsets and other agonists on the self renewal of human immature stem cell subsets. This information will be essential to design and test conditions for ex vivo activation and expansion of immature hematopoietic cells and for various experimental purposes, such as required for the development of efficient gene transfer protocols into hematopoietic cells with retention of repopulating ability.

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CHAPTER

III

Multilineage outgrowth of both malignant and normal hematopoietic progenitor cells from individual chronic myeloid leukemia patients in immunodeficient mice

Co-authors: Jan J. Cornelissen, Wim Terpstra, Gerard Wagemaker and Albertus W. Wognum

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Abstract

In this study the ability of malignant and normal progenitors in peripheral blood (PB) and bone marrow (BM) of chronic myeloid leukemia (CML) patients in chronic phase to proliferate and produce mature progeny after transplantation into hereditary immunodeficient (SCID and NOD/SCID) mice was examined. Engraftment in NOD/SCID mice preconditioned by total body irradiation (TBI) alone was 10-fold higher than in SCID mice preconditioned by macrophage depletion and TBI, demonstrating that NOD/SCID mice are more suitable for engraftment of chronic phase CML cells. Low-density cells at cell doses of 10-30x10^6 and purified $CD34^+$ cells at doses of ~0.2x10^6 engrafted NOD/SCID mice, with levels of 2 to 20% $CD45^+$ cells with production of monocytes, granulocytes, erythroid cells, B-lymphocytes, $CD34^-$ cells and variable frequencies of erythroid and myeloid colony forming cells. As demonstrated by fluorescent *in-situ* hybridisation (FISH) analysis, purified human myeloid, B-lymphoid, erythroid and $CD34^+$ cells from chimeric mouse BM contained Philadelphia-chromosome (Ph)-positive cells and Ph^- cells in similar frequencies as primary cells from the CML patients. These results demonstrate that production of mature normal as well as malignant cells of multiple lineages were supported with similar efficiency. In contrast, all human erythroid and myeloid clonogenic cells detected in the mice were Ph^-, which can be attributed to less efficient maintenance or more rapid differentiation of immature Ph^+ cells in the mouse microenvironment. CML blast crisis cells also grew well in NOD/SCID mice, with 80-90% of human cells produced containing the Ph-chromosome. The availability of an *in vivo* assay that supports outgrowth of normal and malignant stem cells from chronic phase and blast crisis CML patients will facilitate examination of differential effects of growth factors, inhibitory cytokines and cytotoxic drugs on survival of normal and malignant stem cells *in vivo* and on progression of chronic phase CML towards blast crisis.

Introduction

Chronic myeloid leukemia (CML) is a clonal cancer arising from neoplastic transformation of the hematopoietic stem cell (HSC). The hallmark of CML is the so-called Philadelphia (Ph) chromosome, resulting from a reciprocal translocation between chromosomes 9 and 22. The translocation results in the formation of a chimeric oncogene based on the c-abl gene from chromosome 9 and the bcr gene on chromosome 22. The bcr/abl oncogene encodes a protein with enhanced tyrosine kinase activity, which perturbs the proliferation and differentiation of the neoplastic stem cell and its progeny, eventually resulting in suppression of normal hematopoiesis and overgrowth by malignant cells. All myeloid lineages and often the B cell lineage as well are involved in CML, but expansion of the granulocyte lineage is usually most prominent. Bone marrow (BM) and peripheral blood (PB) of newly diagnosed as well as treated chronic phase CML patients contains normal and malignant stem cells at variable frequencies.[1, 2] Thus, improved treatment strategies may be aimed at purification of normal stem cells for autografting or at selectively inhibiting the survival and outgrowth of Ph^+ cells, by exploiting differences in phenotype and/or growth properties between normal and malignant stem cells.[3-7]

The development and successful implementation of more selective anti-neoplastic approaches in CML will require the availability of functional assays in which the effects of growth factors (GF), inhibitory cytokines and cytotoxic drugs on survival and outgrowth of normal and malignant stem cells can directly be compared in a quantitative manner. Long-term nonstromal cultures in defined serum-free medium have provided information about the GF requirements of immature normal and malignant progenitors.[8] Long-term culture and cobble-stone area forming cell (CAFC) assays supported by the murine stromal cell line FBMD-1 have been developed to examine the frequency of immature normal and malignant cells in purified subsets, as normal and malignant stem cells are maintained in this assay with similar efficiency[9] and these assays have been used to examine the differential responses to Interferon-α of nonresponding vs. responding CML patients at the stem cell level.[10]

The regulatory mechanisms of proliferation and differentiation of human hematopoietic cells with *in vivo* hematopoietic capacity can also be studied in animal models using immunodeficient mouse strains. Severe combined immunodeficient (SCID) mice and non-obese diabetic SCID (NOD/SCID) mice

have been shown to be suitable hosts for normal human hematopoietic cells from various sources as well as for different types of human leukemias.[11-15] Cell lines and primary leukemic cells from acute (pre) B cell, T cell or myeloid leukemias proliferate extensively in SCID mice and show a highly reproducible pattern of infiltration reminiscent of that observed in patients.[16-23] Ph^+ leukemic cell lines and primary blast crisis cells from CML patients also grow predominantly in immunodeficient mice, with exclusive production of Ph^+ cells.[24]

Preferentially a reliable transplantation assay for chronic phase CML cells in immunodeficient mice will require that the mouse-microenvironment can support the outgrowth of normal and malignant cells with *in vivo* reconstituting ability with similar efficiency. Only in case such conditions are met, a mouse transplantation assay for human CML cells will be valuable to study the different effects of cytokines, cytotoxic drugs or other agents on the survival and outgrowth of normal vs. leukemic stem cells *in vivo* and to assess the feasibility of stem cell purification strategies to eliminate malignant cells from autologous grafts. In a recent study, BM and PB cells from chronic phase CML patients repopulated SCID mouse BM with levels of chimerism between 5 and 10% but only after injection of high cell numbers (8 to $14x10^7$ low density cells). In other studies, higher engraftment levels were obtained in NOD/SCID mice.[25, 26] In the latter studies, purified $CD34^+$ cells also engrafted if very large cell numbers were transplanted, similar to those used with unfractionated grafts, suggesting the $CD34^+$ cells engrafted suboptimally. The majority of human colony forming cells produced in SCID mice were Ph^-[24], while in another study, in which NOD/SCID mice were used, both normal and malignant progenitor cells were detected in the mice.[26]

In this study the degree of mosaicism of malignant and normal cells before transplantation and after engraftment in immunodeficient mice was evaluated. Specifically, we set out to compare the outgrowth of malignant and normal cells from individual CML patients in SCID and NOD/SCID mice and to assess the relative contribution of normal and leukemic progenitors to the various subsets of human hematopoietic cells produced in the NOD/SCID mouse.

Material and methods

CML progenitor cells

Hematopoietic progenitor cells were obtained from 10 patients after informed consent. Two patients were diagnosed as CML blast crisis and progenitor cells were recovered directly from the peripheral blood (PB). Eight patients were diagnosed in first chronic phase CML. Progenitor cells were recovered directly either from BM or from PB in the recovery phase following high-dose chemotherapy using Cytarabin and Idarubicin. These progenitor cells were indicated as 'mobilised PB' (Table 1). At the time of harvest no blast cells were present in the chronic phase CML samples used in this study. Mononucleated cells were isolated by Ficoll density gradient centrifugation (1.077 g/cm^2, Nycomed Pharma AS, Oslo, Norway), and were then cryopreserved in 10% dimethylsulphoxide, 20% heat-inactivated fetal calf serum (FCS) and 70% Hanks Balanced Salt Solution (HBSS, Gibco, Breda, The Netherlands) at -196°C as described.[27] After thawing by stepwise dilution in HBSS containing 2% FCS, cells were washed with HBSS containing 1% FCS and used for flow cytometric analysis or subset purification and inoculation into NOD/SCID mice. The viability of the thawed cells was determined by staining with trypan blue.

Subset purification

Purification of CD34$^+$ cells was performed by positive selection using Variomacs Immunomagnetic Separation System as described[28] (CLB, Amsterdam, The Netherlands). The percentage of CD34$^+$ cells in the unseparated pool and in the selected CD34$^+$ and CD34$^-$ cell fractions was determined by FACS-analysis.

Transplantation of CML cells in immunodeficient mice

Female, specific pathogen-free (SPF) CB-17 scid/scid mice (SCID), 6 to 9 weeks of age, were obtained from Harlan, CPB, Austerlitz, The Netherlands. Non obese diabetic SCID NOD/LtSz-scid/scid (NOD/SCID) mice were purchased from Jackson Laboratories, Bar Harbour, MA. The mice were housed under SPF conditions in a laminar air flow unit and supplied with sterile food and acidified drinking water containing 100 mg/l ciprofloxacine (Bayer AG, Leverkusen, Germany) *ad libitum*, in accordance with institutional animal research regulations.

The plasma Ig level of the mice was determined with an ELISA using a sheep anti-mouse antibody reacting with mouse IgG and IgM (Boehringer Mannheim Biochemica, Penzberg, Germany) and animals with plasma Ig levels over 40 µg/ml were excluded. To deplete macrophages in SCID mice, one day prior to transplantation of hematopoietic cells, the SCID-mice were injected iv. into a lateral tail vein with 200 µl liposome stock solution containing di-chloromethylene di-phosphonate (kindly provided by dr J.J. van Rooijen, Department of Biochemistry, Free University Amsterdam, The Netherlands, CL_2MDP was a gift of Boehringer Mannheim GmbH, Germany).[22, 29] In previous studies with human umbilical cord blood (UCB) and human acute leukemia cells, this approach required ten-fold fewer cells for similar levels of engraftment than conditioning with TBI alone.[22] Liposome-treated SCID mice and untreated NOD/SCID mice received a dose of 3.5 Gy total body irradiation (TBI), delivered by a ^{137}Cs source adapted for the irradiation of mice (Gammacell, Atomic Energy of Canada, Ottawa, Canada), 2-4 hours before transplantation. The transplants were suspended in 200 µl HBSS containing 0.1% BSA (Sigma, Zwijndrecht, The Netherlands) and injected iv. into a lateral tail vein.

In vitro colony assay

Mononuclear cells from patients' PB or BM and chimeric mouse BM samples were assayed for the presence of granulocyte-macrophage-colony forming units (GM-CFU) and erythroid burst-forming units (BFU-E) by *in vitro* colony formation in viscous methylcellulose culture medium as previously described.[30-32] Briefly, patients' cells or BM cells of mice 35 days post transplantation were plated at concentrations of 0.5 - $4x10^5$/ml plated in 35 mm Petri dishes (Becton Dickinson). The cultures were maintained in a humidified atmosphere of 10% CO_2 at 37°C for 14 days, after which the colonies were counted. Data of duplicate dishes were expressed as average number of colonies per 10^5 cells plated. After counting, single colonies were plucked, centrifuged on glass slides and stored at -20°C until fluorescent *in situ* hybridisation (FISH) was performed.

Tissue collections and analysis

After 35 days, mice were killed by CO_2 inhalation followed by cervical dislocation. From each mouse, both femurs were collected and BM cell suspensions were prepared by flushing with HBSS. After counting, the cells were cultured in colony

assays and analysed by flow cytometry to determine the percentage of human hematopoietic cells in the mouse bone marrow. Cells were suspended in HBSS containing 2% FCS, 0.05% (wt/vol) sodium azide, 2% human serum and 2% mouse serum and stained with the pan-leukocyte surface marker CD45 antibody conjugated with fluorescein isothiocyanate (FITC) and with CD33 antibody conjugated with R-phycoerythrin (PE), for 30', at 4°C. Positive samples were further analysed by incubation with FITC and PE labelled mouse monoclonal antibodies to human CD34, CD19, CD16, CD15, CD38 (Becton Dickinson, San Jose, CA, USA), glycophorin A (GpA), CD3 and CD71 (Dako A/S, Copenhagen, Denmark). Parallel samples were incubated with isotype matched control antibodies. Cell samples of non-transplanted mice were stained as negative controls. Fluorescence was measured using a FACScan flow cytometer and Lysis II or Cellquest software (Becton Dickinson). Dead cells were excluded from analysis by adding 1 µg/ml propidium iodide (PI) and gating for PI^- cells in the FL3 channel.

For isolation of lineage subsets, isolated BM of chimeric mice transplanted with CML cells and thawed cells of 3 patients were stained with FITC and PE conjugated antibodies against human CD15, CD33, CD19, CD34, CD71, CD45 (Becton Dickinson) and GpA for 30' on ice in HBSS containing 2% FCS, 0.05% (wt/vol) sodium azide, 2% human serum and 2% mouse serum. After incubation, the cells were washed twice, resuspended in HBSS and sorted using a FACS Vantage or FACS Calibur flow cytometer (Becton Dickinson). Sorted subsets were centrifuged on slides (500 rpm, 5'), fixed in methanol for 15' and stored at -20°C until FISH was performed.

Fluorescent in situ hybridisation (FISH)

FISH was performed as described previously.[9, 33, 34] In short, the BCR and ABL specific DNA probe were labelled with biotin-16-dUTP and digoxigenin-11-dUTP (Boehringer), respectively, by nick translation. Cells were pre-treated in RNAse solution (200 µg/ml in 2xSSC buffer pH 7.0, 60' at 37°C; 1xSSC is 0.15 mol/L NaCL, 0.015 mol/L sodium citrate). Pepsin treatment was performed by incubating the slides for 10' at 37°C in a HCl-Pepsin solution (50 µl saturated pepsin in 100 ml 0.01 N HCl). The slides were post-fixated in a 1% formaldehyde solution in PBS containing 50 mM $MgCL_2$ for 10' at room temperature and dehydrated in an ethanol series. The BCR and ABL probes (5 µg/ml) were denaturated for 4' at 72°C. After denaturation and dehydration the cells were hybridised overnight at

37°C in a humidified atmosphere. The slides were incubated for 30' at 37°C with avidin-FITC (2 μg/ml, Vector Laboratories, Burlingame CA, USA) in 4xSSC containing 5% non-fat dry milk (NFD-milk) (protifar, Nutricia, Zoetermeer, The Netherlands). The fluorescence intensity was amplified using subsequent layers of sheep anti-Dig-Rhodamine (Boehringer), diluted to 1 μg/ml in Boehringer milk to block unspecific binding, anti-avidin (Brunschwig chemie; 2.5 μg/ml in Boehringer milk), for 30' at 37°C in a humidified atmosphere. After washing, the cells were incubated with donkey anti-sheep Texas Red (Jackson Immunoresearch Lab; 6.8 μg/ml in Boehringer milk) and avidin-FITC (2 μg/ml in Boehringer milk) for 30' at 37°C in a humidified atmosphere. After washing, the cells were stained for total DNA with 4, 6-diamidino-2-phenyl-indole.2HCl (DAPI; 0.1 μg/ml Sigma, Zwijndrecht, The Netherlands) in 2.3% Vectashield (Vector Laboratory). The slides were analysed using a fluorescent microscope equipped with a triple band pass filter for simultaneous excitation of FITC, Texas Red and DAPI. A random sample of at least 100 complete, non-overlapping nuclei was scored for green and red fluorescent spots and the frequency of Philadelphia chromosomes was determined. A cell was scored Ph^- if two distinct red and two distinct green signals were visible within a nucleus. A cell was scored Ph^+ if it contained one distinct red signal, one distinct green signal and one distinct yellow signal representing the coincidence of a red and green signal or if it contained two distinct red signals and two green signals with one red and one green signal in close juxtaposition. As a positive control cytospin slides prepared from selected $CD34^+$ cells from a patient with established accelerated CML were used. Cytogenetic evaluation of these $CD34^+$ cells yielded 100% Ph^+ metaphases (30/30) without additional cytogenetic abnormalities and evaluation by FISH yielded positivity for bcr/abl of 97±2% (n=8). Normal PB or BM samples were used as negative controls in which 98±1% (n=8) cells were scored as Ph^-. Individual FISH experiments were considered of sufficient quality if positive and negative controls yielded a percentage of more than 95% and less than 5%, respectively.

Results

Chimeric bone marrow analysis

Low density CML cells were injected into parallel groups of SCID mice conditioned with TBI and macrophage depletion and in NOD/SCID mice

conditioned with TBI alone. The level of chimerism in the mouse BM was assessed by flow cytometry 35 days after transplantation. The percentage $CD45^+$ cells was used as a measure for engraftment of human cells. Mice with >1% $CD45^+$ cells were considered to be engrafted. A representative result of chimeric BM stained with CD45-FITC vs. CD33-PE is shown in Figure 1A. The presence of $CD45^+CD33^+$ and $CD45^+CD33^-$ cells demonstrated that human myeloid as well as non-myeloid cells were present.

The ability of the SCID and NOD/SCID mice to support outgrowth of CML cells was compared in 4 separate experiments with samples of 3 patients (Figure 2). After transplantation with 10^7 to $3x10^7$ unfractionated cells, SCID mice preconditioned by macrophage-depletion and 3.5 Gy TBI displayed human cells in only 8 of 24 mice transplanted at average levels of 5.9 ± 8.1%. NOD/SCID mice preconditioned with TBI alone showed higher levels of chimerism in 19 of 23 mice transplanted i.e., 14.0 ± 13.5%. These results demonstrated that NOD/SCID mice provide better support for chronic phase CML cells with fewer graft failures than SCID mice do. All further experiments were therefore performed with NOD/SCID mice.

As shown in Table 1, transplantation of low-density cells (1 - $3x10^7$) from 5 of 8 patients with chronic phase CML resulted in high levels of engraftment in 24 of 27 NOD/SCID mice. The percentage chimerism in the engrafted mice varied among the different patient samples, ranging from 2.5 to 22%. Patients 1 and 2 were also transplanted with purified $CD34^+$ and $CD34^-$ grafts. Engraftment was obtained in 4 of 5 mice transplanted with ~$0.2x10^6$ $CD34^+$ cells. Ten million transplanted $CD34^-$ cells from patient 1 did show low levels of chimerism in 5 of 5 mice, which may be attributed to contaminating $CD34^+$ cells in the graft (3.86% $CD34^+$ cells) and the large number of cells transplanted. Engraftment levels of purified $CD34^+$ cells of patient 1 were significantly lower than those obtained with low density cells which can be attributed to the 6-7-fold lower number of $CD34^+$ cells transplanted as compared to the number of $CD34^+$ cells present in the low density fraction. In patient 2, chimerism obtained with $0.15x10^6$ $CD34^+$ cells was similar to that obtained with 65-fold more low-density cells, which contained an equivalent number of $CD34^+$ cells, whereas none of 4 mice transplanted with 10^7 $CD34^-$ cells from patient 2 engrafted. The results demonstrated, at least for patient 2, that the human cells produced in the NOD/SCID BM were derived exclusively from small numbers of immature progenitors and not from surviving mature cells or from $CD34^-$ cells with *in vivo* hematopoietic ability.

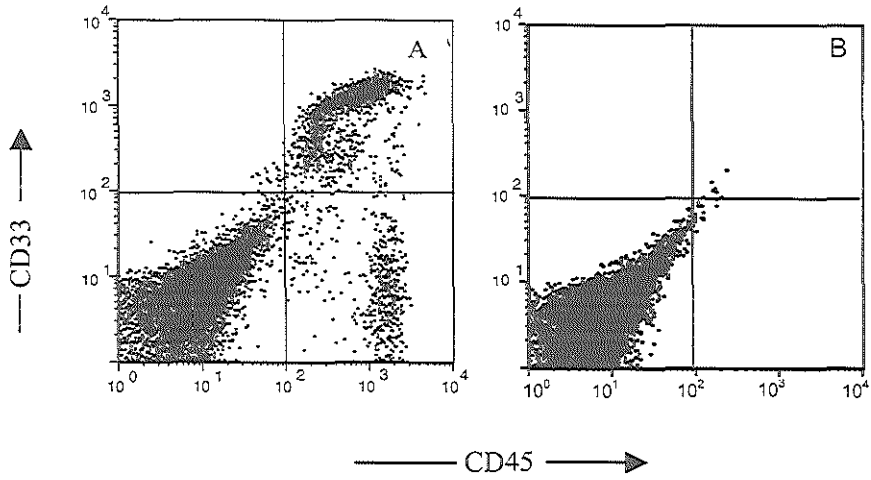

Figure 1. Flow cytometric analysis of chimeric mouse bone marrow stained with CD45-FITC vs. CD33-PE (A). Positive staining for any of these markers was not found in non-transplanted mice (B), demonstrating the specificity of the antibodies for human cells.

Multilineage outgrowth of CML cells

The composition of the human cell population in the BM of mice with sufficiently high levels of chimerism, i.e., those transplanted with cells from patients 1 - 4 in Table 1, was studied by flow cytometry using a panel of lineage specific markers. The percentage of cells in each subset identified was expressed relative to the number of cells that stained with the pan-leukocyte marker CD45 (Figure 3). Mice transplanted with 15 - 20x10^6 CML cells from patients 1 (Figure 3A), 2 (Figure 3B) and 3 (Figure 3C) showed multilineage outgrowth, although the relative frequencies of the various cell types were variable. The most prominent population in mice transplanted with cells from patients 1 to 3 was the myeloid subset (79 ± 24% of the human $CD45^+$ cells, n=15) which consisted of $CD15^-CD33^+$ immature monomyeloid cells, mature $CD15^+CD33^+$ monocytes and $CD15^+CD33^{-/-}$ granulocytes. Cells of the erythroid lineage (15 ± 22%, n=15) consisted of GpA^+CD71^+ erythroblasts and more mature GpA^+CD71^- erythroid cells. The GpA^-CD71^+ subset (27 ± 22%, n=14) probably consisted of early erythroid cells, as well as activated cells of other lineages. $CD20^+$ B cells and, occasionally, $CD56^+$ or $CD16^+$ NK cells and $CD4^+$ or $CD8^+$ cells were present in the mouse BM at low numbers. Most of the $CD4^+$ or $CD8^+$ cells did not express CD3, and probably

consisted of monocytes that express CD4 at low to intermediate levels. Myeloid, erythroid or B-lymphoid cells were not found in BM of mice engrafted with low frequencies of human cells (4%) after transplantation with $20x10^6$ low density cells from patient 4 (Figure 3D). In these mice only $CD38^+$ cells which were $CD4^+$ and/or $CD8^+$ were detected, indicating that these mice were engrafted with activated mature T-lymphocytes. FISH analysis of these cells with BCR and ABL specific probes revealed that virtually all of these T cells were Ph^-, probably derived from expanding mature non malignant T cells (data not shown).

Table 1. Engraftment of CML cells in NOD/SCID mice

Phase	Source	Graft	Cell number	$CD34^+$ cells in graft	Chimeric mice/ injected#	% Chimerism ($CD45^+$ cells)§	$CD34^+$/ mouse BM*	Expansion factor $CD34^+$
			$x10^6$	$x10^6$			$x10^6$	
CP	PB (m)	total^	22	1.0	9/9	21.7±15.8	3.5±3.9	3.5±3.8
		$CD34^+$	0.2	0.16	4/5	1.7±0.5	0.2±0.3	1.4±1.8
		$CD34^-$	10	0.19	5/5	3.9±3.7	0	0
CP	PB (m)	total	10	0.14	5/5	8.1±6.6	0.6±0.4	4.4±2.6
		$CD34^+$	0.15	0.14	4/4	9.0±2.4	0.8±0.4	6.0±3.1
		$CD34^-$	10	0.08	0/4	0	0	0
CP	PB (m)	total	20	0.8	5/5	18.4±9.9	0.8±0.8	1.0±0.9
CP	BM	total	30	0.12	3/4	6.9±6.4	0	0
CP	PB (m)	total	20	1.5	2/4	2.5±0.3	0	0
CP	PB (m)	total	20	0.3	0/4	0	0	0
CP	PB (m)	total	20	0.14	0/5	0	0	0
CP	BM	total	20	0.18	0/3	0	0	0
BC	PB	total	50	13.3	5/5	71.9±6.4	11.3±14.3	1.4±1.7
BC	PB	total	50	15.8	6/6	51.1±8.8	3.9±1.3	0.25±0.1

* $CD34^+$ BM cells, calculated on the assumption that 1 femur represents 8.5% of all BM cells [35]

\# mice are considered chimeric at >1% $CD45^+$ cells

§ mean ± SD of chimeric mice

CP = chronic phase, BC = blast crisis

^ total : low density cells

(m) mobilised PB

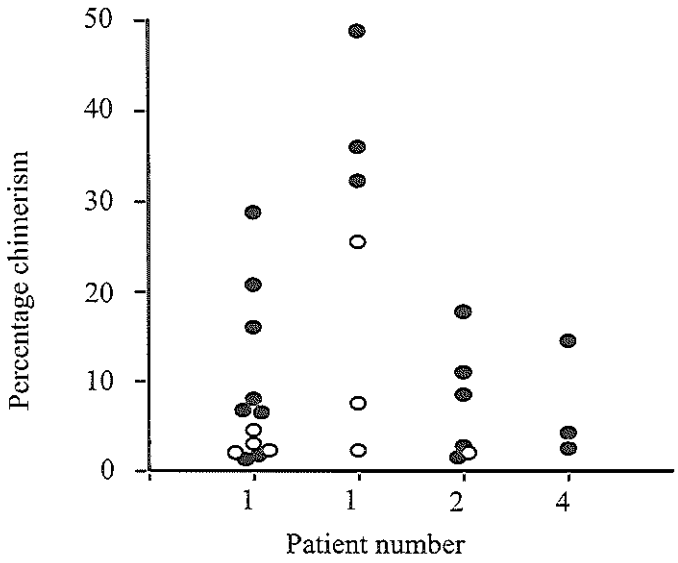

Figure 2. Engraftment levels of 3 different CML samples in SCID and NOD/SCID mice. The percentages human $CD45^+$ cells are shown for individual SCID mice preconditioned with TBI and Cl_2MDP-liposomes (○) and NOD/SCID mice preconditioned with TBI alone (●). Data were obtained in 4 separate experiments; cells of patient 1 were examined twice. Only results for engrafted mice (>1% $CD45^+$) are shown.

FISH analysis on sorted hematopoietic subsets

Human $CD45^+$ cells were purified from chimeric mouse BM by FACS sorting in order to examine the contribution of normal and leukemic cells to engraftment of human cells in NOD/SCID mice. In addition, myeloid, erythroid, B-lymphoid and immature cells were purified from mouse BM, using the markers and sort regions shown in Figure 4. The relative contribution of Ph^+ and Ph^- cells to engraftment of these subsets was evaluated by FISH analysis using BCR and ABL specific probes. To examine to what extent outgrowth of normal and malignant cells in the mice reflects the mosaicism of Ph^+ and Ph^- cells in the patient, the frequencies of Ph^+ cells in the subsets sorted from the chimeric mouse BM were compared to the frequencies in corresponding subsets sorted directly from cryopreserved patient BM cells. (Table 2).

Ph^+ as well as Ph^- cells were present in sorted cell populations for all three patients tested. Sorted $CD45^+$ cells obtained directly from cryopreserved PB samples of these patients contained similar numbers of Ph^- cells as compared to the $CD45^+$ cells recovered from the mice. The frequencies of Ph^+ and Ph^- cells in the immature $CD34^+$ subset and in the monocyte, granulocyte, and erythroid populations did not differ from the cells isolated directly from the patient samples and from the engrafted NOD/SCID mice. Ph^+ as well as Ph^- B-lymphocytes were detectable in primary cells from patient 1 and 2 and in BM of chimeric mice transplanted with cells from these patients. The Ph^- frequency of B-cells isolated from the mouse BM were higher as compared to the Ph^+ frequency in patients 1 and 2 (2-fold and 3-fold, respectively), suggesting selective outgrowth of Ph^+ B-cells in the NOD/SCID mice. All B-cells detected in primary cell samples from patient 3 and in chimeric BM of mice transplanted with cells of this patient were Ph^- (Table 2).
These results demonstrated that both normal and malignant cells obtained from individual CML patients engraft the BM of sublethally irradiated NOD/SCID mice and contribute to multilineage outgrowth of human cells. Moreover, the Ph-distribution pattern of immature cells and cells of the myeloid and erythroid lineages present in the graft is recovered unchanged after transplantation, without selective outgrowth of either normal or malignant cells. The frequency of Ph^+ cells in the PB of all 3 patients examined and in the BM of the engrafted mice was much lower than can be expected for chronic phase CML patients. However, morphological analysis of cells of these patients revealed relatively large frequencies (34%, 9% and 33%, respectively) of immature myeloid precursors and relatively few mature granulocytes (2%, 3% and 5%, respectively). The relative low number of mature Ph^+ granulocytes and predominance of Ph^-CD34^+ cells in the patients' PB samples as well as in chimeric mouse BM were consistent with the relatively strong contribution of Ph^- precursor cells to the circulating cell population after mobilising chemotherapy in chronic phase CML patients.[36-38]

Maintenance of primitive cells in NOD/SCID mice

In addition to mature cells, BM of mice repopulated with cells from patients 1, 2 and 3 also contained immature $CD34^+$ cells, in frequency ranging from 0.2 to 27% (Figure 3). Comparison of the absolute number of $CD34^+$ cells in these mice to the number of $CD34^+$ cells in the grafts showed that $CD34^+$ cell numbers were maintained or increased up to 6-fold during the 35 days engraftment period (Table 1).

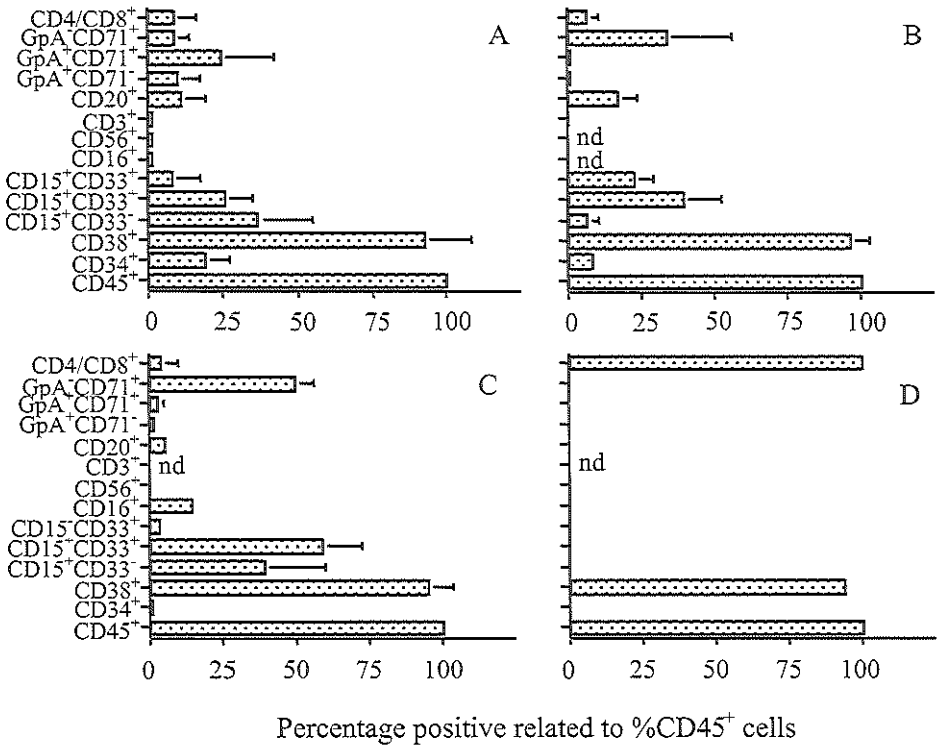

Figure 3. Immunophenotyping of chimeric mouse BM. BM cells were stained with a panel of antibodies specific against different human blood cell lineages to assess the composition of the human cell population. Results for mice transplanted with cells from patients 1 (A), 2 (B), 3 (C) and 4 (D), respectively. The percentage of cells in each subset, relative to the %CD45$^+$ cells present in the BM of each mouse, is expressed as average ± SD for 4, 5, and 5 mice in (A-C), respectively, and as average of 2 mice in (D). The chimerism in individual mice ranged between 8 and 32% for the data shown in A, between 8 and 18% for those shown in (B) and (C), and between 2.4 and 4% for the data shown in (D). Note that CD4 and CD8 expression were assessed simultaneously. nd = not determined

CD34$^+$CD38$^-$ cells were not found in the chimeric BM, suggesting there was no maintenance of this primitive cell population in the engrafted NOD/SCID mice (data not shown). Clonogenic myelomonocytic and erythroid progenitors, i.e., GM-CFU and BFU-E, were identified in engrafted mouse BM but generally in numbers

lower than the GM-CFU numbers injected (Table 3). GM-CFU numbers had increased 1.5 to 5.6-fold relative to input numbers in only 5 of 14 mice transplanted with low density or purified $CD34^+$ cells. BFU-E were also detected in engrafted mice, but in most mice BFU-E numbers were lower than in the graft (Table 3). The low and variable recovery of clonogenic human progenitors could be attributed to the absence of detectable Ph^+ progenitors among the human progenitors cultured from chimeric mouse BM (Table 4). In contrast to the results obtained with the human cell subsets sorted directly from the chimeric BM, BFU-E and GM-CFU colonies grown from chimeric mouse BM were Ph^-, whereas a significant number of colonies grown directly from the patient samples did contain the Ph-chromosome. These results suggested that malignant progenitors with *in vitro* clonogenic ability were maintained less efficiently in mice than their normal counterparts.

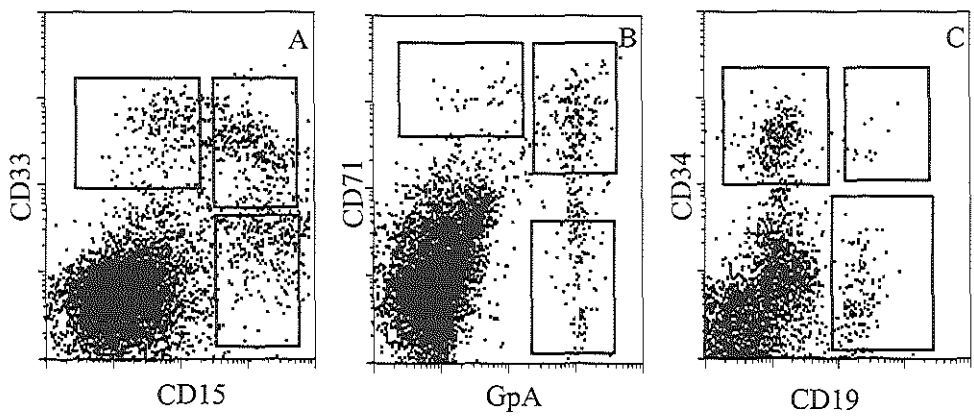

Figure 4. Chimeric mouse BM of patient 1, 2 and 3 were stained with the human myeloid markers CD15-FITC and CD33-PE (A), with the erythroid markers glycophorin A-FITC and CD71-PE (B), with the B cell marker CD19-FITC and with CD34-PE for immature progenitor and precursor cells (C). Cells expressing the different markers were sorted according to the windows shown and centrifuged on glass slides for FISH analysis. Representative dot plots are shown for BM cells of mice transplanted with cells of patient 1.

Engraftment of NOD/SCID mice with cells from CML patients in blast crisis
PB cells of 2 CML patients (no. 9 and 10) in blast crisis were transplanted into NOD/SCID mice in order to compare the engraftment characteristics of these cells to those of chronic phase CML. Transplantation with $5x10^7$ low density cells resulted in high levels of chimerism (patient 9: 72 ± 6%, n=5; patient 10: 51 ± 9, n=6) in all mice injected (Table 1). Immunophenotyping of the human cells produced in mice transplanted with cells from patient 9, showed that most cells co-expressed CD34 and CD19 which was similar to the phenotype of the blast cells in the graft and indicated that the engrafting cells were mainly derived from a leukemic B-cell precursor. CD33 was also expressed on ~40% of the cells (Figure 5A), demonstrating that the mice were engrafted with cells with mixed phenotype, a feature frequently observed with lymphoid blast crisis.[39]

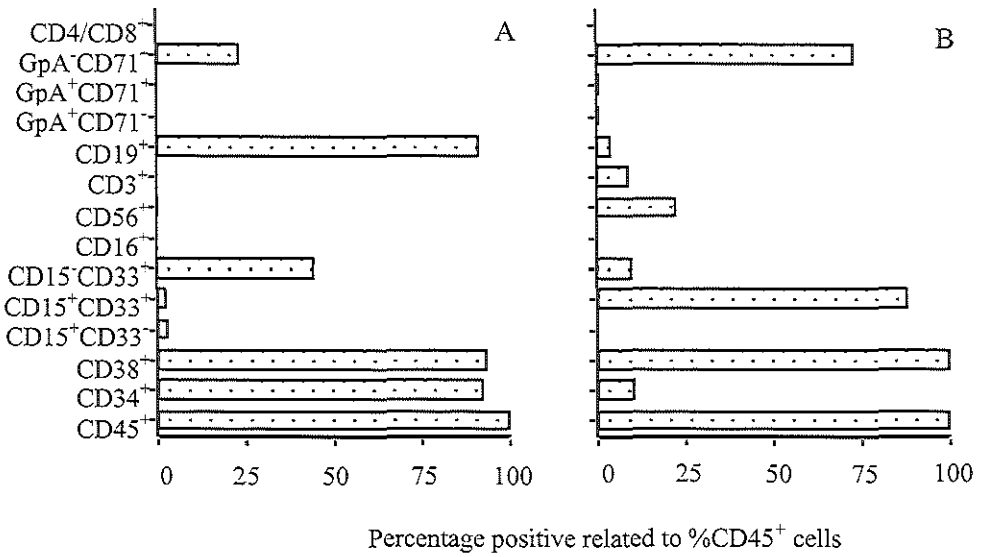

Figure 5. Immunophenotype of BM of a representable chimeric mouse after transplantation of $5x10^7$ blast crisis CML cells of patients 9 (A) and 10 (B). BM of mice transplanted with cells of patient 9 (3 mice) or of patient 10 (2 mice) was stained with a panel of lineage specific markers to assess the composition of the human $CD45^+$ cells present in the mouse BM 35 days post transplantation. The percentage of cells in each subset is expressed relative to the $\%CD45^+$ cells in the mouse. For patient 9 the chimerism ranged between 63 and 78%, and for patient 10 between 41 and 61%.

Cells produced in the mice as well as in the graft of patient 10, mostly expressed myeloid markers (CD15, CD33) (Figure 5B), indicating that the engrafting cells were mainly derived from a leukemic myeloid precursor. A large proportion of engrafted cells were Ph^+, i.e., 82 ± 3% for patient 9 and 87 ± 4% for patient 10. A similar frequency of Ph^+ cells was also detected in the grafts. The frequency of 10-20% Ph^- cells was significantly higher than the frequency of 'false negative' Ph^- cells, i.e. 2 ± 1%, measured by FISH analysis of uniformly Ph^+ cells. These results suggest that residual non-malignant cells with *in vitro* repopulating potential can be detected in NOD/SCID mice transplanted with cells from blast crisis CML patients.

Table 2. Ph-chromosome frequency in sorted subsets

		Patient 1		Patient 2		Patient 3	
Lineage	Subset	Patient	Mouse BM	Patient	Mouse BM	Patient	Mouse BM
Leukocyte	$CD45^+$	32	34	25	10	39	41
Immature	$CD34^+$	38	30	24	3	40	42
Myeloid	$CD15^+CD33^-$	23	18	38	nd	43	58
	$CD15^+CD33^+$	48	37	48	34	61	53
	$CD15^-CD33^+$	25	27	54	56	55	48
Lymphoid	$CD19^+CD34^-$	19	47	3	11	0	0
erythroid	GpA^+CD71^-	36	nd	34	nd	nd	nd
	GpA^+CD71^+	45	50	33	nd	36	37
	GpA^-CD71^+	30	nd	46	nd	38	nd

nd not determined; insufficient cells were available for FISH analysis

Discussion

The present study shows that both normal and malignant progenitor cells from CML patients can be transplanted successfully into NOD/SCID mice with production of granulocytes, monocytes, erythroid cells, B-lymphocytes and immature, $CD34^+$ progenitors. In addition, levels of Ph^+/Ph^- mosaicism of sorted subsets recovered after engraftment reflected the degree of mosaicism before

transplantation. Engraftment of cells from chronic phase CML patients in NOD/SCID mice conditioned with 3.5 Gy TBI was more prominent than in SCID mice preconditioned with TBI supplemented with the injection of CL_2MDP liposomes. This is in contrast to studies with human UCB, in which engraftment levels of unfractionated as well as purified $CD34^+$ and $CD34^+CD38^-$ cells in macrophage-depleted SCID mice and NOD/SCID mice preconditioned with TBI alone were similar[15] and suggests that the additional NK cell and macrophage defects of NOD/SCID mice are necessary to achieve engraftment of normal and malignant cells from CML PB.

Table 3. Number of human GM-CFU and BFU-E in chimeric mouse BM

		Human GM-CFU			Human BFU-E		
Patient ID	Graft	In graft	In mouse BM*	Fold expansion	In graft	In mouse BM*	Fold expansion
		$x10^3$	$x10^3$		$x10^3$	$x10^3$	
1	total	261.6	126.1	0.5	687	73.4	0.1
			83.6	0.3		50.2	0.1
	$CD34^+$	4.1	22.9	5.6	91.6	25.7	0.3
			0	0		4.2	0
2	total	14.4	70.2	4.9	20	21.3	1.1
			24.7	1.7		5.9	0.3
	$CD34^+$	20.5	35.2	1.7	42	8.8	0.2
			31.1	1.5		0.3	0
			2.6	0.1		0	0
			0.6	0		0	0
3	total	276	15.3	0.1	924	13.8	0
			12.4	0		6.2	0
			6.1	0		8.8	0
			10.9	0		2.3	0

* Colony numbers in BM of individual engrafted mice, calculated on the assumption that 1 femur represents 8.5% of all BM cells[35]

Preferably, a useful animal model for chronic phase CML should support and maintain the outgrowth of normal and malignant progenitors, because chronic phase CML is clinically characterised by a mosaicism of Ph^+ and Ph^- progenitors. In addition, only NOD/SCID mice with a stable mosaicism which reflects the relative frequencies of Ph^+ and Ph^- cells in the patients' blood and BM may allow the evaluation of treatment strategies aimed at a selective outgrowth of normal as opposed to malignant cells. It was, therefore, important to evaluate the capacity of normal and leukemic cells from individual patients for engraftment and subsequent outgrowth in NOD/SCID mice. FISH analysis of sorted subsets demonstrated that the distribution of the Ph chromosome in all human cell subsets remained in the same order of magnitude in all human subsets isolated from chimeric mouse BM as compared to the same subsets isolated directly from BM or PB from these patients. These results demonstrated that both normal and malignant progenitor cells proliferate and differentiate in BM of transplanted NOD/SCID mice, resulting in production of human cell populations in mice, which display a similar Ph distribution as the cells in the original graft. The percentages Ph^+ leukocytes as presented in Table 2 appeared low as compared to what can be expected for patients in first chronic phase CML. However, the patients used in our study were treated with intensive chemotherapy including Idarubicin and Cytarabin and the cell samples used for NOD/SCID mouse transplantation were obtained in the recovery phase. It is known that Ph^- precursor cells can effectively be mobilised into the peripheral blood following intensive chemotherapy and that peripheral blood stem cell harvests may even be largely devoid of malignant precursors, as demonstrated by cytogenetic analysis.[37, 38]

In previous studies we have analysed the maintenance of Ph^+ and Ph^- progenitors of purified subsets of $CD34^+$ cells in LTC and CAFC assays, supported by the FBMD-1 stromal cell line.[9, 10] The results of a large group of patients, including one which was tested in NOD/SCID mice in the present study (patient 3), showed that Ph^+ and Ph^- cells are produced with similar efficiency in long-term cultures. The frequency of 41- 42% Ph^+ human $CD45^+$ and $CD34^+$ cells detected in the BM of mice which were engrafted with $CD34^+$ cells of patient 3 (Table 2) was almost identical to the frequency of 42% Ph^+ nucleated human cells produced after 5 weeks of long-term culture of highly purified $CD34^+CD38^-$ cells from this patient (patient 3 in reference 9). Since only the small $CD34^+CD38^-$ population of normal hematopoietic cells has previously been shown to engraft NOD/SCID mice[14, 15, 40-42] and also contains the highest frequencies of both Ph^+ and Ph^- CML PB and BM cells identifiable in long-term cultures.[9, 43, 44], it is very likely that the Ph^+ and

Ph^- human cells detected in engrafted NOD/SCID mice were not derived from relatively mature progenitors, but from very immature cells which are relevant for *in vivo* hematopoiesis.

Table 4. Clonogenic human progenitors from engrafted mouse BM are Ph^-

Colony	patient 1		patient 2	
	graft	mouse BM	graft	mouse BM
GM-CFU	5/13*	0/6	ne	0/6
BFU-E	3/5	0/5	3/4	0/8

* number of Ph^+ colonies / number of colonies analysed

ne slides were not evaluable

The production of Ph^+ and Ph^- myeloid, B-lymphoid and erythroid cells as well as immature $CD34^+$ cells in the NOD/SCID mice clearly demonstrates that multilineage progenitors and not exclusively myeloid progenitors were responsible for *in vivo* engraftment. However, analysis of the clonal origin of the engrafted cells, e.g., using retroviral-mediated gene marking, will be needed to definitively establish the relative contribution of multipotent as compared to committed progenitors to *in vivo* engrafment.

In recent studies, analysis of Ph^+ and Ph^- colonies, grown from human cells under conditions that permit outgrowth of human but not of murine progenitors, recovered from NOD/SCID mice demonstrated production of normal as well as leukemic colony-forming cells in engrafted mice as examined by analysis of metaphase chromosomes[25, 26], whereas in an earlier study in SCID mice normal progenitors made up the majority (70%) of the human hematopoietic cells present.[24] Since in those studies no comparison was made with the relative frequencies of Ph^+ and Ph^- progenitors in the graft, it is difficult to assess whether the different results reflected a more efficient maintenance of Ph^+ progenitors in NOD/SCID as compared to SCID mice, or were caused by variations between individual patients with respect to the relative number of Ph^+ and Ph^- progenitors.[24, 26] In our study, all human monomyeloid and erythroid colonies

grown from chimeric mouse BM were Ph^-. This was in contrast to the mosaicism of Ph^+ and Ph^- cells observed for the noncultured human cell subsets isolated directly from patients' PB and chimeric mouse BM, and for the monomyeloid and erythroid progenitors grown directly from the graft. Explanations for the discrepancy between the Ph^+ cell frequencies of clonogenic progenitors and sorted human cell subsets may include qualitative differences between the ability of Ph^+ and Ph^- NOD/SCID engrafting cells to maintain or produce clonogenic progenitor cells *in vivo*. In particular, the mouse microenvironment may provide less effective support for maintenance or production of immature Ph^+ progenitors with *in vitro* clonogenic ability than for normal immature cells. Alternatively, the malignant progenitors may have a shorter transit time than their normal counterparts in a mouse microenvironment, accounting for a lower Ph^+ progenitor frequency while maintaining relatively large numbers of more mature offspring. In any case, the absolute number of colony-forming cells recovered from the chimeric mouse BM was low, with large variations between individual mice (Table 3). As shown in this study phenotypically identified subsets of human cells can be isolated from the BM of engrafted mice in sufficiently large cell numbers for reproducible FISH analysis, and thus provide more quantitative information about engraftment of normal and leukemic cells than can be obtained by analysis of individual colonies.

In our study, no evidence was found for the maintenance of immature $CD34^+CD38^-$ CML or normal cells in engrafted NOD/SCID mice or for significant amplification of either normal or leukemic $CD34^+$ cells and clonogenic progenitors *in vivo*. This contrasts to our previous study using UCB cells, which also did not require exogenous human growth factor support, but which showed significant expansion of $CD34^+$ cells and clonogenic progenitors.[15] In that study, we noted independent control of immature $CD34^+CD38^-$ cell expansion and mature cell outgrowth, the former requiring support by $CD34^+$ accessory cells present in the graft. Therefore, from the present study we speculate that CML cells and their progeny may similarly require accessory cell and/or growth factor support to display their full potential for immature cell amplification and outgrowth in the NOD/SCID environment. In recent studies, injection of stem cell factor (SCF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and PIXY321 (fusion protein of IL-3 and GM-CSF) failed to improve the overall engraftment levels, suggesting that the stimuli provided by the mouse microenvironment are sufficient for outgrowth of human cells.[24, 25] Treatment with SCF, but not GM-CSF or G-CSF selectively promoted the engraftment and proliferation of leukemic cells *in vivo*[25], suggesting that

exogenous GF support can change the balance between Ph^+ and Ph^- progenitors that are produced *in vivo*. It remains to be established as to whether exogenous GF support can also promote the maintenance and expansion of immature $CD34^+CD38^-$ cells *in vivo*. If such conditions can be developed it will be possible to use the NOD/SCID mouse transplantation assay not only to study outgrowth of normal and malignant cells, but also to test stimuli that can differentially promote the maintenance or expansion of normal cells and elimination of leukemic stem cells *in vivo*.

Cells from BC patients engrafted to much higher levels with less variation of engraftment levels in individual mice than cells from chronic phase CML patients. The engraftment potential of CML BC cells thus resembles that of leukemic blasts from AML patients, which also engraft efficiently in immunodeficient mice[19-21], whereas the much lower and less reproducible engraftment of chronic phase CML cells is more similar to that obtained with normal human BM or mobilised PB cells.[41, 45] The variable engraftment obtained with different human cell types may reflect difference in requirements for cytokines or stromal factors, or in the ability of engrafting cells from different sources to home to the mouse BM.

In conclusion, the present study provides evidence for multilineage outgrowth of chronic phase and blast crisis CML in transplanted NOD/SCID mice. Levels of Ph^+/Ph^- mosaicism did not differ before and after transplantation. Thereby the model will in principle be useful to examine the feasibility of cell fractionation approaches and other *ex vivo* manipulations to eliminate transplantable malignant stem cells from autografts and to evaluate the differential effects of growth factors, inhibitory cytokines or cytotoxic drugs on the survival and differentiation of normal vs. leukemic stem cells. In addition, mice engrafted with chronic phase CML cells may be exploited to study the development of CML from chronic phase towards acceleration and blast crisis and to test treatment strategies to prevent this process.

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CHAPTER

IV

Highly efficient transduction of the green fluorescent protein gene in human umbilical cord blood stem cells capable of cobblestone formation in long-term cultures and multilineage engraftment of immunodeficient mice

Co-authors: Paula B. van Hennik, Marti Bierhuizen, Ana Limon, Albertus W. Wognum, Jose A. Cancelas, Jordi Barquinero, Rob E. Ploemacher and Gerard Wagemaker

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Abstract

Purified $CD34^+$ and $CD34^+CD38^-$ human umbilical cord blood (UCB) cells were transduced with the recombinant variant of Moloney murine leukemia virus (MoMLV) MFG-EGFP or with SF-EGFP in which EGFP expression is driven by a hybrid promoter of the spleen focus-forming virus (SFFV) and the murine embryonic stem cell virus (MESV). Infectious MFG-EGFP virus was produced by an amphotropic virus producer cell line (GP+*env*Am12). SF-EGFP was produced in the PG13 cell line pseudotyped for the gibbon ape leukemia virus (GaLV) envelope proteins. Using a 2 day growth factor prestimulation, followed by a 2 day, fibronectin fragment CH-296 supported, transduction, $CD34^+$ and $CD34^-CD38^-$ UCB subsets were efficiently transduced using either vector. The use of the SF-EGFP/PG13 retroviral packaging cell combination consistently resulted in 2-fold higher levels of EGFP-expressing cells than the MFG-EGFP/Am12 combination. Transplantation of 10^5 input equivalent transduced $CD34^+$ or $5x10^3$ input equivalent $CD34^+CD38^-$ UCB cells in NOD/SCID mice resulted in median engraftment percentages of 8% and 5%, respectively, which showed that the *in vivo* repopulating ability of the cells had been retained. In addition, mice engrafted after transplantation of transduced $CD34^+$ cells using the MFG-EGFP/Am12 or the SF-EGFP/PG13 combination expressed EGFP with median values of 2% and 23% of human $CD45^+$ cells, respectively, which demonstrated that the NOD/SCID repopulating cells were successfully transduced. $EGFP^+$ cells were found in all human hematopoietic lineages produced in NOD/SCID mice including human progenitors with *in vitro* clonogenic ability. EGFP expressing cells were also detected in the human Cobblestone Area Forming Cell (CAFC) assay at 2-6 weeks of culture on the murine stromal cell line FBMD-1. During the transduction procedure the absolute numbers of CAFC week 6 increased 5- to 10-fold. The transduction efficiency of this progenitor cell subset was similar to the fraction of $EGFP^+$ human cells in the bone marrow (BM) of the NOD/SCID mice transplanted with MFG-EGFP/Am12 or SF-EGFP/PG13 transduced $CD34^+$ cells, i.e., 6% and 27%, respectively. The study thus demonstrates that purified $CD34^+$ and highly

purified $CD34^+CD38^-$ UCB cells can be transduced efficiently with preservation of repopulating ability. The SF-EGFP/PG13 vector/packaging cell combination was much more effective in transducing repopulating cells than the MFG-EGFP/Am12 combination.

Introduction

Efficient procedures for gene transfer into human immature hematopoietic cells with repopulating capacities following transplantation may in principle open new avenues for the treatment of a variety of hereditary and acquired diseases. Retroviral mediated gene transfer to such cells, which is attractive by its simplicity and efficiency, has however met with considerable difficulty, which is only partly understood.[1, 2] The availability of a rapid selectable marker, such as the green fluorescent protein (GFP), is thought to be of pivotal importance to study major variables influencing the efficiency of gene transfer, as well as to track the progeny of transduced cells following transplantation. In the present study we evaluated the use of the enhanced (E) recombinant variant of GFP to label immature human umbilical cord blood cells, using outgrowth in NOD/SCID mice[1, 3, 4] as well as cobblestone area forming cells (CAFC)[5, 6] as assays for immature cells with considerable hematopoietic reconstitution capacity.

The CAFC assay and the Long-Term Culture-Initiating Cell (LTC-IC) assay allow for frequency analysis of cells capable of long-term repopulation *in vitro*.[5, 7] Murine studies have shown that the CAFC scored at week 2 are related to CFU-S day 12, while CAFC week 5 strongly correlate with long-term repopulating cells *in vivo*.[6, 8] In human hematopoiesis the rare population with the primitive phenotype of $CD34^+CD38^-$ is highly enriched for CAFC week 6. The primitive nature of CAFC week 6 is further illustrated by enrichment following incubation with 5-Fluorouracil (5-FU), a drug cytotoxic for proliferating cells. The CAFC week 2 however, are absent in the $CD34^+CD38^-$ population and more than 1 log reduced after 5-FU treatment. Based on these results the CAFC week 6 have been proposed to be representative for cells with long-term repopulating ability *in vivo* in the human situation.[9] On this basis, this assay is considered suitable to assess the effect of manipulation of human hematopoietic progenitor cell populations, such as by gene transfer protocols.[10, 11]

The efficiency of gene transfer to stem cells is limited by the inability of most retroviral vectors to integrate DNA into the cellular genome of quiescent cells.[12-15] Stimulation of stem cell cycling with hematopoietic growth factors (HGF) such as IL-3, IL-6, stem cell factor (SCF) or Flt3-L[16] prior to and during virus exposure would seem to be essential to promote transduction[17, 18] but may result in loss of repopulating ability of transduced cells as a result of differentiation.[16, 19] In addition, colocalisation of target cells and virus on dishes coated with the recombinant fibronectin-fragment CH-296 has been shown to further increase gene transfer efficiency.[20, 21]

For transduction of human hematopoietic cells murine retroviruses based on the MoMLV are most commonly used. However, expression of functional receptors for the MoMLV envelope protein is presumably low and pseudotyping the vector with the GaLV envelope protein resulted in higher transduction efficiencies in hematopoietic progenitor cells[22-24], which has been attributed to a higher expression of functional pseudotyped GaLV receptor (Pit-1) by the immature hematopoietic cells[22, 24] than the amphotropic retroviral receptor (Pit-2).[24-28] A study in which $CD34^+$ cells were transduced by the GaLV-pseudotyped retroviral vector revealed that $CD34^+$ cells were efficiently transduced (21-33% transduction) as determined by culture in a colony-forming cell assay.[2] It is not known to what extent the relative transduction inefficiency of the MoMLV type viruses is caused by a low Pit-2 expression on immature stem cells or by inefficient activation and provirus integration in quiescent cells. Transplantation of $CD34^+$ or $CD34^+CD38^-$ transduced cells in immunodeficient beige/nude/xid (bnx) mice showed that 8 of 10 mice transplanted with $CD34^+$ transduced cells contained the retrovirally transduced bacterial neomycin phosphotransferase resistance (neo) gene whereas only 2 of 14 mice that had received $CD34^+CD38^-$ cells contained low levels of transduced cells.[2] The ability to engraft the BM of NOD/SCID mice and provide multilineage outgrowth, which resides exclusively in the $CD34^+CD38^-$ population[3], has been described as unsuccessful, in contrast to the LTC-IC or CAFC week 6 which were transduced with efficiencies ranging between 10 to 70%.[1] These differences led to the suggestion that NOD/SCID repopulating cells are distinct from the LTC-IC or CAFC week 6.[1] However, recent data obtained with vectors that contained the neo-gene show that transplantation of retrovirally transduced $CD34^+$ UCB cells in NOD/SCID mice result in transduced human hematopoiesis in the NOD/SCID BM with transduction levels similar to those obtained for LTC-IC.[29]

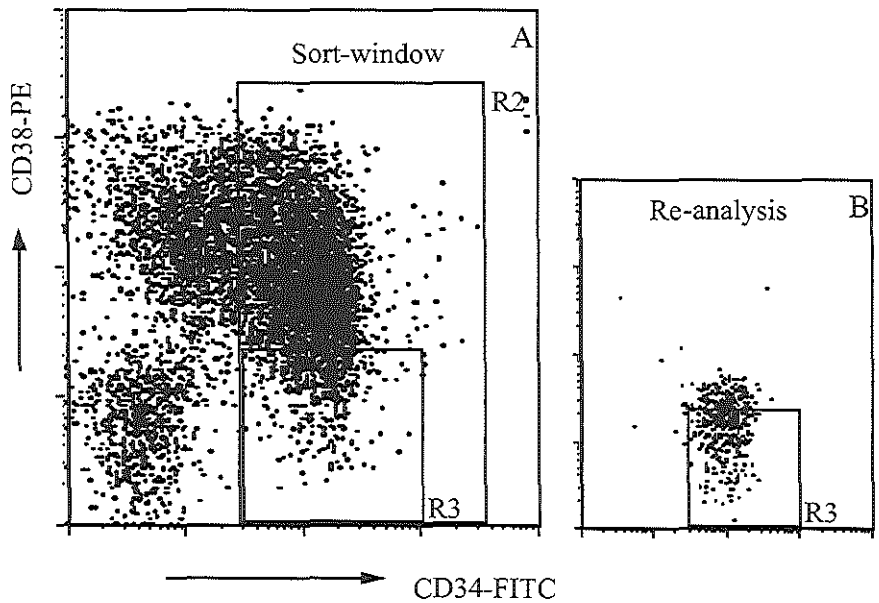

Figure 1. Flow cytometric profile used to define and sort the $CD34^{+}CD38^{-}$ cell population (A). The window R3 was used to define $CD34^{+}CD38^{-}$ cells for sorting and contains 5% of the $CD34^{+}$ population (as defined by window R2) with the lowest CD38 antigen expression. Re-analysis of the sorted cells is shown in (B).

Use of the green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* as a retrovirally transduced marker allows rapid identification of transduced cells by fluorescence microscopy, flow cytometry or culture in real time without additional staining steps in contrast to other genetic markers such as the neo-gene[30-32] and the bacterial ß-galactosidase gene (LacZ).[33-36] As wtGFP produces a weak (but stable) green fluorescence signal, several GFP variants, such as EGFP, have been created which are better suited for detection of expression by fluorescence microscopy and flow cytometry.[37, 38] Studies with murine cells have shown that cells with the ability of *in vivo* reconstitution can be transduced with EGFP.[39] Our ongoing studies show that high expression levels of EGFP could be detected in mouse BM, peripheral blood, spleen and thymus for a current observation period of 6 months after transplantation and were retained in secondary

recipient mice, indicating that long-term repopulating stem cells can be successfully transduced.[40] Human cell lines and purified $CD34^+$ cells were also transduced using EGFP containing vectors.[28] Therefore, retroviral vectors containing EGFP genes can be used to transduce a variety of cells which can then be easily detected *in vitro* as well as *in vivo*.

To initiate an analysis directed at optimal vectors and transduction procedures, the MFG-EGFP retroviral vector produced by an amphotropic packaging cell line and the SF-EGFP vector pseudotyped for the GaLV envelope protein were used to transduce immature cell subsets in human UCB. The potential of these vector/packaging cell combinations for transduction of purified $CD34^+$ and $CD34^+CD38^-$ UCB subsets were compared by assessing the ability of transduced cells to produce EGFP-positive cobblestone areas in the CAFC assay and to contribute to multilineage human hematopoiesis in NOD/SCID mice.

Materials and methods

Human umbilical cord blood cells

UCB samples were obtained from placentas of full-term normal pregnancies after informed consent in conformity with legal regulations in The Netherlands. Mononucleated cells were isolated by Ficoll density gradient centrifugation (1.077 g/cm^2, Nycomed Pharma AS, Oslo, Norway), and were cryopreserved in 10% dimethylsulphoxide, 20% heat-inactivated fetal calf serum (FCS) and 70% Hanks Balanced Salt Solution (HBSS, Gibco, Breda, The Netherlands) at -196°C as described[41] before use. After thawing by stepwise dilution in HBSS containing 2% FCS, the cells were washed with HBSS containing 1% FCS and used for gene transduction experiments.

Viral vectors and packaging cell lines

The amphotropic retroviral producer cell line, MFG-EGFP, was obtained by a 20 hour incubation of GP+*env*Am12 under standard culture conditions with supernatants containing ecotropic retrovirus from the GP+E-86/MFG-EGFP cell line and hexadimethrine bromide at 4 μg/ml (Sigma, St. Louis, MO) as

described.[38] The pseudotyped retroviral producer cell line PG13/EGFP7 was developed by transducing the PG13 packaging cell line (kindly provided by D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) with 0.45 μm filtered supernatant from PA317/EGFP cell cultures.[28] EGFP expression was analysed by flow cytometry and bright single cells were sorted on 96-well plates by using an EPICS Elite ESP flow cytometer coupled to an autoclone device (both from Coulter, Miami, FL, USA). Single clones were cultured as previously described.[28] The sorted clones were additionally selected for high virus titer. The viral titer of both the amphotropic and the pseudotyped producer cell line was in the order of 10^6 infectious particles per ml as determined by supernatant titration on cultured murine NIH 3T3 cells and human HeLa cells, respectively. Absence of replication-competent virus was verified by failure to transfer GFP-expression from a transduced cell population to a secondary population. Additionally, for the SF-EGFP/PG13 vector/packaging cell combination pseudotransduction was tested on HeLa cells and found absent.

Subset purification

Purification of $CD34^+$ cells was performed by positive selection using Variomacs Immunomagnetic Separation System as described[42] (CLB, Amsterdam, The Netherlands). The percentage of $CD34^+$ cells in the unseparated population (low density UCB) and in the purified $CD34^+$ and $CD34^-$ fractions was determined by FACS-analysis. For isolation of $CD34^+CD38^-$ subsets, purified $CD34^+$ cells were stained with fluorescein isothiocyanate (FITC) and R-phycoerythrin (PE) conjugated antibodies against human CD34 and CD38 (CD34-FITC, CD38-PE, Becton Dickinson) for 30', on ice in HBBS, supplemented with 2% (vol/vol) bovine serum albumine (BSA; Sigma), 0.05% (wt/vol) sodium-azide (Merck, Darmstadt, Germany) and 2% (vol/vol) normal human serum (NHS). After incubation, the cells were washed twice, resuspended in HBSS and $CD34^+CD38^-$ cells, the window set at 5% of the $CD34^+$ population with the lowest CD38 expression levels (Figure 1) were sorted using a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA).

Retroviral transduction of UCB subsets

Supernatants containing recombinant retrovirus were generated by culturing approximately 80% confluent producer cells for 12 hours in culture medium

consisting of a serum-free enriched version of Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD).[3, 39, 43] Media for all cultures routinely included 100 U/ml of penicillin and 100 µg/ml of streptomycin. The cultures were maintained at 37°C with 10% CO_2 (measured every 15' with read-outs between 9.5% and 10%) in a humidified atmosphere. The culture supernatant was subsequently procured and passed through a 0.45 µm filter. To enhance the transfection efficiency, Falcon 1008 (35 mm) bacteriological culture dishes were coated with the recombinant fibronectin fragment CH-296 (Takara Shuzo, Otsu, Japan) at a concentration of 10 µg/cm^2 as described previously.[44] UCB subsets ($CD34^+$ or $CD34^+CD38^-$) were prestimulated for 2 days in either medium consisting of enriched Dulbecco's medium (Gibco, Gaithersburg, MD), or CellGro®SCGM (Boehringer Ingelheim, Heidelberg, Germany). Different combinations of human recombinant HGF were added to the culture medium; IL-3 (20 ng/ml; Gist-brocades NV, Delft, The Netherlands), IL-6 (100 ng/ml; Ares-Serono SA, Genève, Switzerland, thrombopoietin (TPO; 10 ng/ml, kindly provided by Genentech, South San Francisco, CA, USA), stem cell factor (SCF; 100 ng/ml and Flt3-L (50 ng/ml, the latter two kindly provided by Amgen, Thousand Oaks, CA, USA). The HGF combination Flt3-L, TPO, IL-6 and SCF was used during the transduction procedure; in some initial experiments, as indicated in the legend of the figures and tables, the IL-3, IL-6, SCF combination was used. Before adding purified cord blood subsets to the fibronectin-coated dishes, the CH-296 fibronectin fragment was preincubated with supernatant containing the amphotropic MFG-EGFP or the pseudotyped SP-EGFP vector for 1 hour at 37°C.[21, 45] Subsequently, nucleated cells were resuspended in the vector-containing supernatant supplemented with hematopoietic growth factors, and added to the dishes. Over a period of 2 days, culture supernatant was once replaced completely by resuspending nonadherent cells into fresh retrovirus supernatant and HGF. Finally the cells were harvested and used for FACS analysis, human granulocyte-macrophage colony forming units (GM-CFU) and erythroid burst-forming units (BFU-E) assays, CAFC assay, and transplantation into NOD/SCID mice.

Flow cytometry

Cell samples were analysed using a FACSCalibur flow cytometer (Becton Dickinson) as previously described.[38, 39] Immunophenotyping of EGFP-transduced cells was performed by staining with peridinin chlorophyll protein (PercP) labeled

anti-CD45 and cyanin-5-conjugated anti-CD34 (Cy_5; Amersham, Buckinghamshire, UK) or PE conjugated monoclonal antibodies against CD38, CD2, CD4, CD8, CD19, CD20, CD56, CD33 (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Mice were considered engrafted if the percentage $CD45^-$ cells exceeded 1%.

Transplantation of transduced UCB subsets in immunodeficient mice

Specific pathogen-free (SPF) NOD/LtSz-scid/scid (NOD/SCID) mice, 6 to 9 weeks of age, were bred and housed under SPF conditions in a laminar air flow unit and supplied with sterile food and acidified drinking water containing 100 mg/l ciprofloxacine (Bayer AG, Leverkusen, Germany) *ad libitum*. Housing, care and all animal experimentation were done in conformity with legal regulations in The Netherlands, which include approval by a local ethical committee. All mice received total body irradiation (TBI) at 3.5 Gy, delivered by a ^{137}Cs source adapted for the irradiation of mice (Gammacell, Atomic Energy of Canada, Ottawa, Canada), 2-4 hours before transplantation. The transplants were suspended in 200 µl HBSS containing 0.1% BSA and injected iv. into a lateral tail vein. Transplanted cell numbers were 10^5 $CD34^+$ cells and $5x10^3$ $CD34^+CD38^-$ cells. 35 Days after transplantation the mice were killed by CO_2 inhalation followed by cervical dislocation, both femurs isolated and BM cell suspensions prepared by flushing. After counting, the cells were cultured in colony assays and analysed by flow cytometry to determine the percentage of human $EGFP^+$ cells in the mouse BM.

In vitro colony assay

Purified UCB cells, EGFP-transduced cells and chimeric mouse BM samples were assayed for the presence of human GM-CFU and BFU-E by *in vitro* colony formation in viscous methylcellulose culture medium as previously described.[3, 43, 46, 47] The number of colonies was determined after 14 days of culture in a humidified atmosphere of 10% CO_2 at 37°C. $EGFP^+$ colonies were scored under excitation by ultraviolet light.

Stromal feeders and Cobblestone area forming cell (CAFC) assay

The contact inhibited FBMD-1 murine stromal cell line was used as described before.[6] After seven to ten days of culture at 33°C and 10% CO_2 the stromal layers

had reached confluence and were overlaid with non-transduced or transduced $CD34^+$ or $CD34^+CD38^-$ UCB cells within the subsequent week. Confluent stromal layers of FBMD-1 cells in flat-bottom 96-wells plates were overlaid with UCB cells in a limiting dilution set-up. Input values of the $CD34^-CD38^-$ population and the $CD34^+$ were 25 nucleated cells and 500 nucleated cells per well in the first dilution, respectively. Twelve two-fold serial dilutions were used for each sample with 15 replicate wells per dilution. The cells were cultured at 33°C and 10% CO_2 for six weeks with weekly half-medium changes. The percentage of wells with at least one phase-dark hematopoietic clone of at least five cells (i.e., a cobblestone area) beneath the stromal layer was determined weekly with an inverted microscope. Green fluorescent cobblestone areas were screened in the same way but with an UV-light excitation source. Frequencies of total and green-fluorescent CAFC were calculated by using Poisson statistics as described previously.[6] During the period of culture no transfer of the EGFP gene to the stromal underlayer has been observed.

Statistical analysis

Data are expressed as median (range). Statistical comparisons were performed according to Mann Whitney U-test. P values of <0.05, two tailed, were considered significant.

Results

Transduction efficiencies in purified cells with MFG-EGFP and SF-EGFP vectors.

Purified $CD34^+$ and $CD34^+CD38^-$ UCB cells (Figure 1.) were prestimulated for two days and subsequently transduced with either the MFG-EGFP/Am12 or the SF-EGFP/PG13 vector/packaging cell combination, during two days of exposure to virus-containing supernatants in fibronectin fragment-coated bacterial dishes. Transduction efficiencies obtained by infection using the amphotropic MFG-EGFP producer cell line were compared to those obtained with the pseudotyped SF-EGFP cell line.

Table 1. EGFP expression of UCB subsets

Vector/Packaging cell line	Purified $CD34^+$ cells	P-Value*	$CD34^+CD38^-$ population within purified $CD34^+$	P-Value^	Purified $CD34^+CD38^-$	P-Value#
MFG-EGFP/Am12	30 (8-51) (n=13)	>.05	25 (15-55) (n=9)	>.05	19 (8-21) (n=4)	0.02
SF-EGFP/PG13	75 (53-84) (n=7)	>.05	66 (58-81) (n=5)	>.05	62 (21-71) (n=4)	0.12
p-value$^{\Psi}$	0.0001		0.003		0.02	

Results are expressed as percentages of $EGFP^+$ cells and depicted as median (range).
For statistical analysis the Mann-Whitney U-test has been used.
* comparison of the median of purified $CD34^+$ cells and $CD34^+CD38^-$ subset within the purified $CD34^-$ population.
^ comparison of the median of $CD34^-CD38^-$ subset within the purified $CD34^-$ population and purified $CD34^+CD38^-$ cells.
# comparison of the median of purified $CD34^+$ cells and purified $CD34^+CD38^-$ cells.
$^{\Psi}$ comparison of MFG-EGFP and SF-EGFP transduced cells.

The percentage $EGFP^+$ cells was assessed by flow cytometry (Figure 2). The percentage $EGFP^+$ cells of the purified $CD34^+$ population transduced with the SF-EGFP/PG13 vector/packaging cell combination (median 75% $EGFP^+$) was more than 2-fold higher as compared to MFG-EGFP/Am12 transduced $CD34^+$ cells (median 30%) (Table 1). Sorted $CD34^+CD38^-$ cells were also transduced at a higher frequency using the SF-EGFP/PG13 combination (62%) than after transduction with the MFG-EGFP/Am12 combination (19%). On average, transduction frequencies were lower in the purified $CD34^+CD38^-$ cells than in the $CD34^+$ cell fraction, but only for the MFG-EGFP/Am12 transduced cells the difference was statistically significant. The level of transduction of the $CD34^+CD38^-$ subset within the purified $CD34^+$ population obtained with the SF-EGFP/PG13 vector/packaging cell combination was more than 2.5 fold higher than with the MFG-EGFP/Am12 combination. The differences in transduction

efficiency between the two vector/packaging cell combinations in these cell populations were significant ($p<0.025$).

Transduction efficiency of cobblestone area forming cell subsets.
The ability of transduced cells to form cobblestone areas was evaluated in long-term culture supported by FBMD-1 stromal cells. $EGFP^+$ cobblestone areas were identified by fluorescence microscopy (Figure 3). The absolute numbers of CAFC at different culture periods increased as a result of the transduction procedure without significant differences between the target cells and vector used (Table 2). The absolute number of CAFC week 2 in the MFG-EGFP/Am12 transduced $CD34^+$ UCB cells increased 5-fold, for the SF-EGFP/PG13 transduced $CD34^+$ UCB cells the increase was 7-fold. The CAFC week 6 expanded 10-fold and 5-fold, respectively. For the $CD34^+CD38^-$ UCB cells, similar results were obtained, 6-fold and 10-fold of CAFC week 6 after MFG-EGFP/Am12 and SF-EGFP/PG13 transduction, respectively. Consistent with the immaturity of the $CD34^+CD38^-$ cell population, CAFC week 2 could not be detected in the $CD34^+CD38^-$ cell fraction prior to transduction. These data show that the transduction protocol that has been used causes a modest expansion of both CAFC week 2 and week 6. The transduction efficiency of the CAFC week 2 in MFG-EGFP/Am12 transduced $CD34^+$ cells ranged between 23 and 30% with a median value of 26% and in SF-EGFP/PG13 transduced $CD34^+$ cells the median value was 60% (46-74%) (Table 2). The transduction efficiency of the CAFC week 6 in MFG-EGFP/Am12 transduced $CD34^+$ cells ranged between 0% and 11% with a median of 6% $EFGP^+$ cobblestone areas. CAFC week 6 in SF-EGFP/PG13 transduced $CD34^+$ cells showed as high as 27% transduction.
CAFC week 6 in SF-EGFP/PG13 transduced $CD34^+CD38^-$ cells showed a similar level of 25% transduction efficiency. Notably, highly purified $CD34^+CD38^-$ cells transduced with the amphotropic cell line did not produce $EGFP^-$ cobblestone areas week 6. These experiments clearly demonstrated the superiority of SF-EGFP/PG13 over MFG-EGFP/Am12 in transducing late appearing CAFC, in concordance with the results obtained in phenotypically identified immature $CD34^+$ subsets.

Repopulation of transduced subsets in NOD/SCID mice.
In parallel with analysis of cobblestone formation the ability of transduced cells to reconstitute hematopoiesis in vivo was examined by transplantation of the

equivalent of 10^5 non-cultured $CD34^+$ cells into sublethally irradiated NOD/SCID mice. After 35 days the level of chimerism and the percentage of $EGFP^+$ cells in mouse bone marrow were determined by flow cytometry (Table 3). Similar levels of engraftment were found in mice transplanted with non-cultured or cultured $CD34^+$ cells.

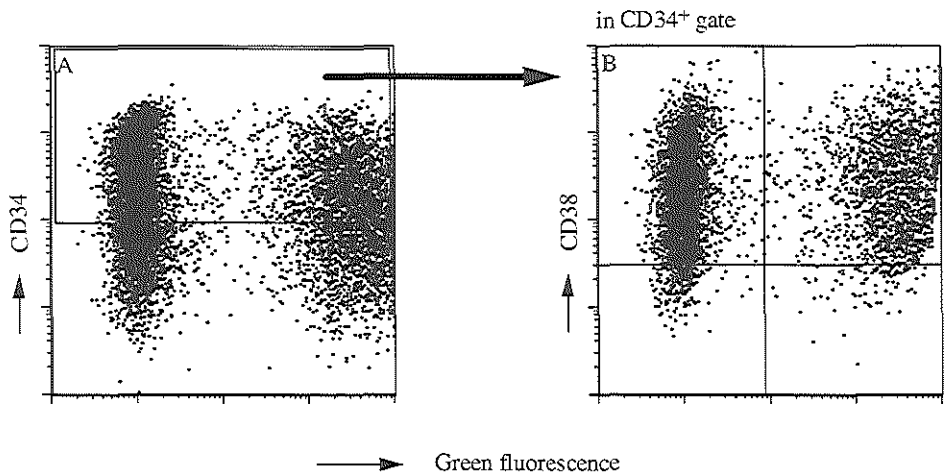

Figure 2. Flow cytometric analysis of a representative transfection of purified $CD34^+$ cells with the amphotropic MFG-EGFP retroviral vector after 2 days prestimulation and 2 days supernatant infection in the presence of IL-3, IL-6 and SCF. This particular transduction resulted in efficiencies of 30% within the $CD34^+$ population (A). In (B) $CD34^+$ cells were gated and the CD38 distribution of the EGFP-transduced cells was studied. Also $CD34^+CD38^-$ cells expressed the EGFP gene with efficiencies similar to the total $CD34^+$ population (30% $EGFP^+$).

Following transplantation of non-cultured $CD34^+$ cells human cells were detected in all mice (n=11) (median: 54% (6-64%) $CD45^+$ cells). $EGFP^+$ cells were found in 6 of 10 repopulated chimeric mice transplanted with MFG-EGFP/Am12-transduced $CD34^+$ cells with a median percentage of $EGFP^+$ cells of 2% (Table 3). $CD34^+$ cells transduced using the SF-EGFP/PG13 vector produced higher levels of $EGFP^+$ cells (median: 23%) in the human population in all 4 mice transplanted.

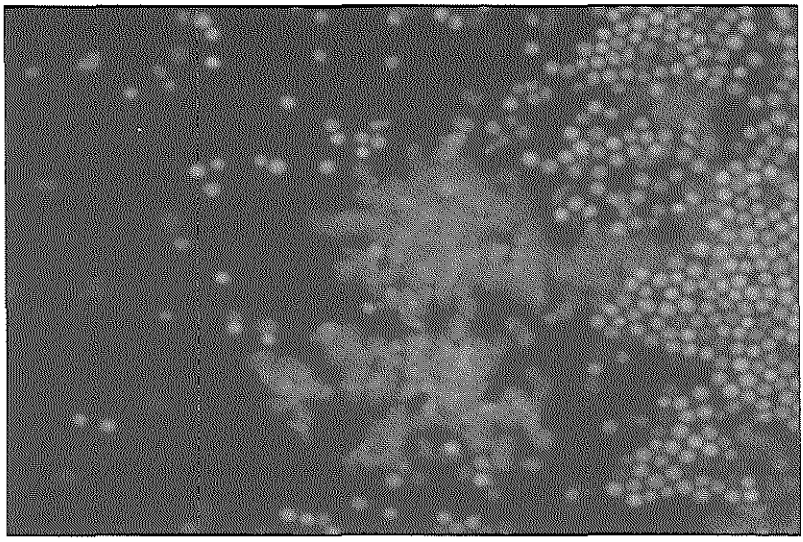

Figure 3. Fluorescence microscopic image of a representative $EGFP^+$ cobblestone area. The bright green cells are the mature cells on top of the stromal layer and the dim green cells represent the $EGFP^+$ cobblestone area.

These data demonstrated that the repopulating cells in the $CD34^+$ population can be transduced effectively and produce $EGFP^+$ progeny in transplanted NOD/SCID mice. In addition, SF-EGFP/PG13 was much more efficient in transducing the repopulating cells than MFG-EGFP/Am12.

Transplantation of non-cultured $CD34^+CD38^-$ cells and transduced $CD34^+CD38^-$ resulted in chimerism levels of median 10% (6-29%) for the non-cultured cells and 5% (1-24%) and 6% (4-9%) for the MFG-EGFP/Am12 or SF-EGFP/PG13 transduced cells, respectively. In contrast to the results with purified $CD34^+$ cells, $CD34^+CD38^-$ cells transduced with MFG-EGFP/Am12 were not able to repopulate mouse BM with EGFP-expressing cells, although all 4 mice engrafted with human cells (Table 2); this parallels the absence of EGFP expressing CAFC week 6 in $CD34^+CD38^-$ cells transduced with MFG-EGFP/Am12. Only 1 of 3 mice engrafted with SF-EGFP/PG13 transduced $CD34^+CD38^-$ cells. $EGFP^+$ could only be detected in 3% of the $CD45^+$ cells produced. This is in contrast to the results with the $CD34^+$ cells in that apparently most repopulating cells in the highly purified $CD34^+CD38^-$ subset were not transduced efficiently or the transduced cells displayed a significant reduction in their engraftment potential compared to the cells which

were not transduced during the procedure. Nevertheless, SF-EGFP/PG13 was also in these experiments apparently more efficient than MFG-EGFP/Am12.

Table 2. Absolute numbers of CAFC week 2 and week 6 and percentages of green fluorescent cobblestone areas after transduction of 10^6 selected UCB $CD34^+$ cells or $35x10^3$ $CD34^+CD38^-$ cells with the vectors MFG-EGFP or SF-EGFP.

	CAFC wk2				CAFC wk6			
	$CD34^+$	%	$CD34^+CD38^-$	%	$CD34^+$	%	$CD34^+CD38^-$	%
Non-transduced	$42x10^3$	-	nd	-	$4 x10^3$	-	$0.3 x10^3$	-
Am12/MFG-EGFP	$218 x10^3$*	26	$2 x10^{3\#}$	15	$41 x10^3$*	6	$2 x10^{3\#}$	nd
PG13/SF-EGFP	$315 x10^3$*	60	$2 x10^{3\#}$	24	$22 x10^3$*	27	$3 x10^{3\#}$	25

% percentage of green fluorescent cobblestone areas expressed as median.
nd not detectable.
* n=2
# n=1

Multilineage outgrowth of EGFP-transduced $CD34^+$ cells.
The composition of the $EGFP^+$ human cell population in 2 mice was assessed by flow cytometry using a panel of lineage specific markers (Figure 4). $EGFP^+$ cells of the myeloid lineage (CD33; 31-39%, $CD11b^+$; 20-25%, CD4; 30-45%), T lymphoid (CD2; 20-22%), B-lymphoid (CD20; 16-23%), and NK cells (CD56; 1%) were found in mice transplanted with EGFP-transduced $CD34^+$ cells. Also immature $EGFP^+CD34^+$ cells were present in the mouse BM (1.1-6.8%) (Figure 5). Transduced cells and chimeric mice BM were also cultured in standard methylcellulose medium under conditions which selectively favour the outgrowth of human monomyeloid and erythroid progenitors and fail to stimulate mouse progenitors. In both the graft and the chimeric mice BM, $EGFP^+$ GM-CFU (15/39 in the graft and 3/23 in the mouse BM) and BFU-E (23/40 in the graft and 5/25 in

the mouse BM) were identified by flow cytometry of isolated colonies or fluorescence microscopy of whole cultures.

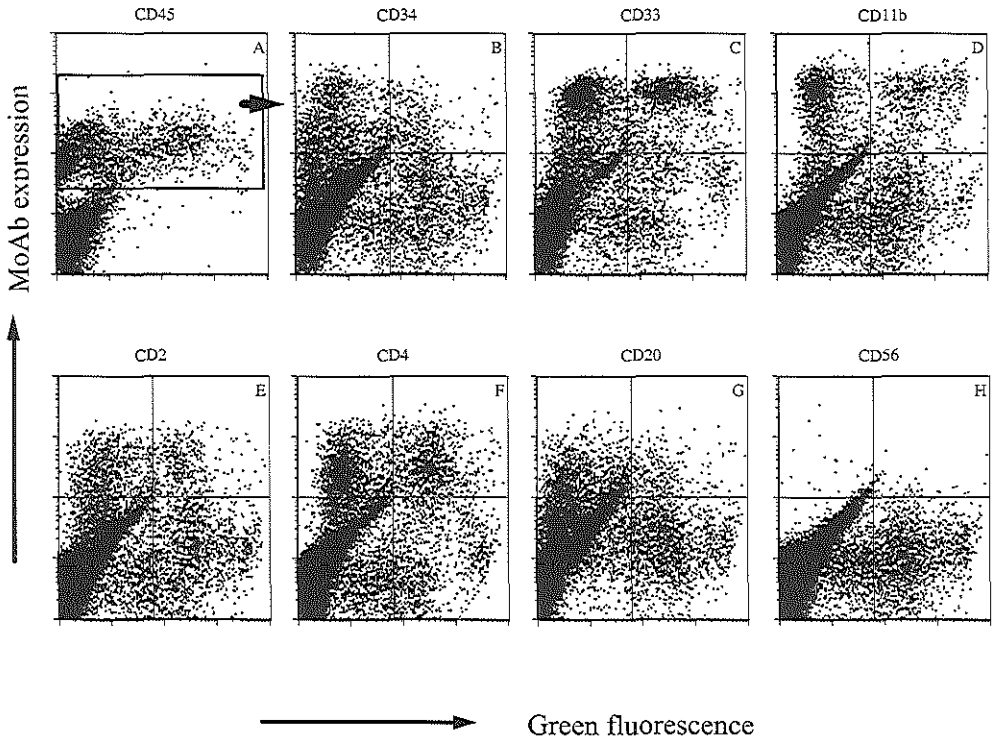

Figure 4. Representative immunophenotyping of chimeric NOD/SCID mouse BM 35 days after transplantation of MFG-EGFP/Am12 transduced, IL-3, IL-6, SCF-stimulated $CD34^+$ UCB cells. BM (>10% $CD45^+$) was stained with a panel of antibodies specific against different human blood cell lineages and CD45 as a marker for human cells. Figure A shows the bright green autofluorescence on the X-axes vs. CD45. The window represents all human, $CD45^+$ cells. The other dotplots shown, are gated cells in this $CD45^+$ window representing only human cells. Representative examples are shown for EGFP vs. CD34 (B), EGFP vs. CD33 (C) EGFP vs. CD11b, (D), EGFP vs. CD2 (E), EGFP vs. CD4 (F), EGFP vs. CD20 (G) and EGFP vs. CD56 (H).

Discussion

The versatile use of EGFP as a selectable marker of retroviral-mediated gene transfer in hematopoietic cells provides a basis to further optimise retroviral gene transfer to human repopulating stem cells and to evaluate the role of hematopoietic growth factors in activation and expansion of immature hematopoietic cells. This study focused on the development of optimal conditions for gene transfer to human $CD34^+$ and $CD34^+CD38^-$ UCB cells with the ability to reconstitute hematopoiesis in NOD/SCID mice and produce cobblestone areas for prolonged periods in stroma-supported long-term cultures.

Comparison of transduction frequencies of immunophenotypically characterised immature cells and those of SCID repopulating cells and CAFC may both demonstrate the relationship of these cell types as well as point to essential differences. In general, there was concordance between these assays, in that the GaLV-pseudotyped retroviral vector (SF-EGFP) transduction was much more efficient than the amphotropic retroviral vector (MFG-EGFP) transduction. Also, transduction frequencies of the immature $CD34^+CD38^-$ subset within the $CD34^+$ population related well to those obtained following transplantation of NOD/SCID mice and CAFC week 6. In addition, the study revealed that repopulating cells in the highly purified $CD34^+CD38^-$ cells were resistant to transduction in the absence of the $CD38^+$ subset, particularly notable for MFG-EGFP/Am12 as demonstrated by the finding that the EGFP transduced $CD34^+CD38^-$ subset in general failed to produce $EGFP^+$ progeny in NOD/SCID mice. One mouse transplanted with SF-EGFP/PG13 transduced sorted $CD34^+CD38^-$ cells displayed 3% human $EGFP^+$ cells, one order of magnitude less than the frequency of $EGFP^+$ CAFC week 6 in the same sample.

The more prominent transduction efficiency of the green fluorescent protein gene into purified and highly purified immature UCB cells with the GaLV-pseudotyped SF-EGFP compared to the MFG-EGFP/Am12 retroviral packaging cell combination, is consistent with earlier studies where transduction of human hematopoietic progenitors was more efficient with a retroviral vector that uses the GaLV receptor.[23-26] The lower transduction percentage obtained with the amphotropic vector may thus be primarily attributed to the low or absent expression of the amphotropic envelope-receptor on the target cells.[48, 49] This was particularly corroborated by the absence of EGFP expression in MFG-EGFP/Am12 transduced sorted $CD34^+CD38^-$ cells, both in the week 6 CAFC assay and

following transplantation into NOD/SCID mice. Alternatively, UCB cells may be more efficiently transduced by the SF-EGFP/PG13 vector/packaging cell combination due to the use of the SFFV/MESV hybrid promoter which has been designed to overcome transcriptional inefficiency and silencing associated with retroviral gene transfer into myeloid progenitors and hematopoietic stem cells.[50]

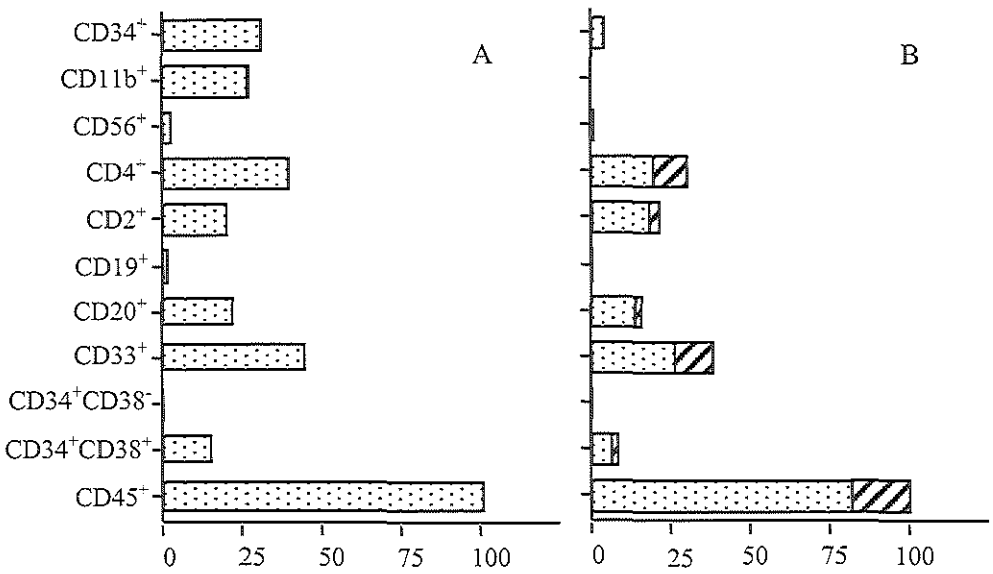

Figure 5. Representative chimerism (⊡) and EGFP expression levels (▨) in chimeric NOD/SCID mouse BM 35 days after transplantation of nontransduced (A) and transduced (B) $CD34^+$ UCB cells, relative to the numbers of human ($CD45^-$) cells found.

Other variables that obviously need to be further analysed include differences in titer and the ability and efficiency of the vectors to transduce EGFP in hematopoietic cells. The titers of the two vectors used were comparable, but tested in different assays. The colocalisation of vector and cells during transduction, using the CH-296 fibronectin fragment[21], makes it unlikely that differences in titer did heavily influence the results. This is the more so since preparative experiments (not shown) with the MFG-EGFP/AM12 retroviral vector revealed that additional charges of the virus supernatant in the transduction protocol did not result in higher

transduction frequencies, which indicated that the transduction system is sufficiently saturated with virus. Also Hanenberg *et al.* concluded that the amount of retroviral particles present in the supernatant was not a limiting factor for transduction of $CD34^+$ BM cells on CH-296-coated plates.[51] The higher efficiency of the SF-EGFP/PG13 combination when compared to the MFG-EGFP/AM12 combination should therefore not be considered as being due to supernatant virus titer differences.

Table 3. Repopulation of EGFP transduced UCB subsets in NOD/SCID mice and CAFC assay

Vector/Packaging cell line	UCB subset	Transduction efficiency %EGFP	$EGFP^+$/ chimeric mice*	Chimerism NOD/SCID %CD45	% $EGFP^+$ on $CD45^+$ cells	CAFC wk 6 %EGFP
Am12/MFG-EGFP	$CD34^+$	$^\phi$31 (29-51)	6/10	12 (2-65)	2 (0-18)	6 (0-11)
PG13/SF-EGFP	$CD34^+$	$^\phi$66	4/4	8 (3-12)	23 (2-41)	27 (26-27)
Pvalue		-	-	>.05	0.032	0.12
Am12/MFG-EGFP	$CD34^+CD38^-$	$^\phi$5	0/4	5 (1-24)	0	nd
PG13/SF-EGFP	$CD34^+CD38^-$	$^\phi$21	1/3	6 (4-9)	3	$^\phi$25
Pvalue		-	-	>.05	>.05	-

Results are depicted as median (range) of 2 or 3 experiments.
For statistical analysis the Mann-Whitney U-test has been used
nd not detectable.
$^\phi$ insufficient data to perform statistical analysis.
* all transplanted mice engrafted with >1% $CD45^+$ cells.

The observation that repopulating cells in the $CD34^+$ population can be transduced efficiently and produce transduced multilineage progeny in transplanted NOD/SCID mice, whereas repopulating cells in the highly purified $CD34^+CD38^-$ subset are either not transduced effectively or do not develop *in vivo* is of considerable interest for elucidation of mechanisms involved in successful transduction of immature hematopoietic cells. The transduction efficiency of the

$CD34^+CD38^-$ tended to be lower than that of the $CD34^+$ cells[2], and was significantly so for the MFG-EGFP/Am12 combination, which may be related to the low or absent expression of the amphotropic receptor. Since repopulating cells are exclusively present in the small $CD34^+CD38^-$ population, and $CD34^+CD38^+$ cells do not effectively engraft, the low levels of gene expression in the chimeric NOD/SCID BM after transplantation of transduced $CD34^+CD38^-$ cells may indicate that the growth factors used during prestimulation and virus infection were not sufficiently effective for activation and stable virus integration of the NOD/SCID repopulating cells. The much higher frequency of EGFP expressing cells in the BM of NOD/SCID mice after transplantation of transduced stem cells from the less pure $CD34^-$ fraction may indicate that stimuli provided by accessory $CD34^+$ cells were responsible for the more efficient transduction of repopulating $CD34^+CD38^-$ within the $CD34^+$ cell fraction. Alternatively, these accessory cells may be needed to maintain the repopulating ability of stem cells during the transduction procedure of 4 days, e.g. by preventing differentiation, or to promote the expansion and outgrowth of transduced stem cells after transplantation. We speculate that these accessory cells may be related to the accessory $CD34^+CD38^+$ cells which are involved in the maintenance and expansion of $CD34^+CD38^-$ cells in immunodeficient mice transplanted with non-transduced human UCB subsets.[3] Further identification of these accessory $CD34^+$ cells and elucidation of the active principle may therefore be both relevant for stem cell expansion physiology and for the design of successful gene transfer strategies for immature hematopoietic cells.
The absolute numbers of CAFC produced after week 2 and week 6 of culture show a modest increase after transduction with the MFG-EGFP or SF-EGFP vectors. The frequency of $EGFP^+$CAFC week 6 in SF-EGFP or MFG-EGFP transduced $CD34^+$ UCB cells was similar to levels of $EGFP^+CD45^+$ cells found in NOD/SCID mice. The reason for the 10-fold discrepancy between the levels of transduction of the CAFC week 6 and the very low numbers of $EGFP^+CD45^+$ in NOD/SCID BM after transplantation of the SF-EGFP/PG13 transduced $CD34^+CD38^-$ population is not clear. Studies with the murine ADA vector similarly yielded very low numbers of gene-marked human cells in the NOD/SCID mouse BM, in contrast to higher numbers of transduced LTC-IC and colony forming cells (CFC), which was interpreted as evidence that the latter cell types are functionally distinct from NOD/SCID repopulating cells.[1] However, this distinction might be artificial if effectively transduced $CD34^+CD38^-$ require the described $CD34^+$ accessory cells for *in vivo* maintenance and expansion but not for *in vitro* cobblestone area forming ability.

We conclude that retroviral mediated EGFP transduction in umbilical cord blood cells, in combination with functional assays for repopulating cells, is a rapid tool to study essential gene transfer variables such as vector tropism and transduction conditions. In addition, the use of the GaLV-pseudotyped retroviral vector SF-EGFP resulted in highly efficient gene transfer in both late CAFC and NOD/SCID repopulating cells, the latter presently the most immature subset of human $CD34^{+}CD38^{-}$ cells that can be approached by a functional assay. These results justify the expectation, that the imminent analysis of variables promoting genetic marking of primitive, transplantable hematopoietic cells, such as further optimised transduction conditions and vector constructs, lead to protocols for clinically relevant levels of therapeutic gene transfer.

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Chapter

V

Analysis of factors influencing retrovirus mediated gene transfer of human umbilical cord blood and rhesus monkey bone marrow $CD34^+$ hematopoietic cells

Co-authors: Kathelijn W. M. Peters, Trudi P. Visser, Albertus W. Wognum and Gerard Wagemaker

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Abstract

Retrovirus mediated gene transfer in hematopoietic stem cells of large outbred species including humans has met with considerable difficulty, with only low numbers of transplantable stem cells successfully transduced and a significant loss of repopulating capacity. We analysed the relative importance of variables determining the efficiency of gene transfer, using the gene encoding an enhanced version of the green fluorescent protein (EGFP) as a marker. Fibronectin fragment CH-296 supported supernatant transduction of $CD34^+$ umbilical cord blood (UCB) or rhesus monkey bone marrow (RhBM) cells in serum free cultures stimulated with Flt3 ligand (FL), stem cell factor (SCF) and thrombopoietin (TPO) was done with a virus pseudotyped for the gibbon ape leukemia virus (GALV). The EGFP gene was driven by the SF promoter, composed of elements of the spleen focus forming virus, SFFV, and the murine embryonic stem cell virus, MESV. The data revealed a substantial dependence of the CH-296 supported transduction on virus titer. Selection of high titer subclones of the virus producer cell line resulted in transduction efficiencies of more than 90% in $CD34^+$ RhBM cells and UCB cells. Prior cryopreservation of RhBM cells was essential for high transduction levels. Transplantation into NOD/SCID mice of UCB cells confirmed the validity of the optimal conditions for SCID repopulating cells (SRC's). The results revealed a threshold for transduction of SRC's relative to $CD34^+$ cells and demonstrated that transduction efficiencies of SRC's of ~80% can be obtained with retrovirus vectors under optimal conditions that transduce > 90% of the primary $CD34^+$ cells.

Introduction

Stable retrovirus mediated gene transfer into human hematopoietic $CD34^+$ cells with repopulating capacity has large potential for the treatment of a variety of

hereditary and acquired diseases and as an adjuvant in the treatment of tumors. The promise of clinical gene transfer has recently been highlighted by treatment of SCID-X1 disease, in which the transgene confers a selective advantage to transduced lymphoid progenitors[1], and in an initial study directed at transfer of the multidrug resistance 1 (MDR-1) gene, where a selective pressure can be exercised *in vivo*.[2] These successes were reached with relatively conventional retrovirus vectors and moderate transduction efficiency of the transplanted $CD34^+$ cells, which are amenable to further improvement required for many other clinical conditions that can potentially be cured by gene therapy. Despite these recent achievements, retrovirus mediated gene transfer is still problematic and its clinical application limited by low transduction frequencies in long term repopulating stem cells.[3]

Several improvements have been made recently which include new insights into the growth factors required to induce cell cycling of immature, transplantable stem cells[4-6], alternative viral receptors[7, 8], improved retroviruses[9-12], as well as methods to co-localise virus and cells, especially by the recombinant fibronectin fragment CH-296. In addition, the development of selectable reporter molecules such as the enhanced green fluorescent protein (EGFP) speeded up the analysis of transduction procedures, enabled viable selection of the transduced cells by flow cytometry, and provided a tool for rapid monitoring of the fate of the transduced cells and their progeny after transplantation.[13-17]

The low frequency of hematopoietic stem cells in most target populations and the inherent resistance to transduction due to their quiescent state likely are major limiting factors for transduction. Hence, the use of purified cell populations and induction of cell cycling without promoting excessive differentiation and, thereby, loss of repopulating capacity, are essential features of retrovirus mediated transduction procedures. Recently, the combination of Flt-3 ligand (FL), stem cell factor (SCF) and thrombopoietin has been identified as a minimum growth factor requirement to stimulate stem cells while preserving repopulating capacity.[18-21] Adding growth factors such as IL-3 or IL-11 to this combination resulted in a dramatic decline of repopulating capacity in short term serum free cultures with no difference being apparent in transduction efficiency[21], consistent with the detrimental effect of IL-3 on repopulating capacity reported earlier.[22]

Facilitation of retrovirus cell transductions by colocalisation of cells and virus using a human fibronectin fragment (CH-296)[23-25] increases the transduction of $CD34^+$ cells substantially, enabled efficient supernatant transduction procedures that replaced the cumbersome co-cultivation of virus producing cells and target

cells[23, 25], and was subsequently also shown to be effective for cells repopulating nonhuman primate bone marrow.[26]

Retrovirus vector design aims at viruses that are better suited to transduce hematopoietic stem cells than the classical Moloney virus derived vectors[27] and display increased gene expression and reduced silencing. Several improvements have been made, including the development of vectors based on the myeloproliferative sarcoma virus[11] and the Friend spleen focus-forming virus (SFFV).[9, 28] Combination of the U3 region of the SFFV and a permissive leader from the murine embryonic stem cell virus generates viruses that give superior gene expression in $CD34^{+}CD38^{-}$ cells.[12] Using such a GALV-pseudotyped SF virus and the EGFP reporter gene, we previously showed much higher transduction frequencies than those that could be achieved with the Moloney virus based amphotropic MFG-EGFP virus in UCB cells, able to reconstitute hematopoiesis in NOD/SCID mice and to produce cobblestone areas for prolonged periods in stroma-supported long-term cultures.[16] This was consistent with earlier studies which showed that replacing the conventional amphotropic packaging cell lines with the packaging cell line PG13, which generates virus, pseudotyped for the gibbon ape leukemia virus (GALV) envelope protein, results in higher transduction levels of transplantable immature hematopoietic cells,[9, 29-35] as well as with the characteristics of the SF virus used.[36] The comparatively low efficiency[37-42] of amphotropic retrovirus vectors is attributed to a lower expression level of the amphotropic retrovirus receptor (Pit-2) than the GALV receptor (GLVR-1, Pit-1) on immature hematopoietic.[16, 29, 30, 43-48] On the basis of these results we postulated that the further analysis of variables influencing transduction frequency should lead to much higher levels, bringing gene transfer within reach for disease entities in which a selective advantage of the transduced cells would be relatively small[49-52] or not be expected.

To meet this aim, the present study focuses on finding residual relevant variables to promote retrovirus mediated gene transfer in human umbilical cord blood and rhesus monkey immature hematopoietic cells. Briefly, we used the PG13 packaging cell line[30] for GALV pseudotyping and the SF-EGFP virus[43] in serum free cultures[53-55] supported by the fibronectin fragment CH-296.[23] The growth factors used were restricted to FL, SCF and TPO. Further variables studied included multiplicity of infection (i.e., virus titer *versus* cell concentration) and prior cryopreservation. Although the study was primarily aimed at rhesus monkey immature cells, the general applicability of the results was further verified in human UCB cells, using a rapid read-out for repopulating capacity of EGFP

transduced $CD34^+$ UCB cells by transplantation of $CD34^+$ cells into NOD/SCID mice[56, 57], to measure content of "SCID-repopulating cells" (SRC's)[58] and to assess the multilineage nature of the transduced cells.

Material and methods

Human umbilical cord blood and rhesus bone marrow cells

Human umbilical cord blood (UCB) samples were obtained after informed consent in conformity with legal regulations in The Netherlands from placentas of full-term normal pregnancies. Normal rhesus BM (RhBM) samples were obtained as described[59] from purpose-bred healthy rhesus monkeys (*Macaca mulatta*) housed in stainless steel cages in rooms equipped with a reverse filtered air barrier, provided with daylight rhythm, and conditioned to 20°C with a relative humidity of 70%. Animals were given free access to commercially available primate chow, fresh fruits, and acidified water. All animals were free of intestinal parasites and seronegative for herpes B, simian T-lymphotrophic viruses (STLV), and simian immunodeficiency virus (SIV). An ethics committee operating in conformity with legal regulations in The Netherlands approved housing, experiments and all other conditions. In a number of experiments, rhesus monkey BM was obtained following priming ("mobilisation") with 100 μg/kg G-CSF (Neupogen; Amgen Inc, Thousand Oaks, CA) for 4 consecutive days. UCB and RhBM mononucleated cells were isolated by Ficoll density gradient centrifugation (1.077 g/cm^2, Nycomed Pharma AS, Oslo, Norway), and were cryopreserved in 10% dimethylsulphoxide, 20% heat-inactivated fetal calf serum (FCS) and 70% Hepes-buffered Hanks Balanced Salt Solution (H+H, Gibco, Breda, The Netherlands) at -196°C as described.[60] After thawing by stepwise dilution in H+H containing 2% FCS, the cells were washed with H+H containing 1% FCS and subsequently suspended into the serum free transduction medium.

Subset purification

Purification of $CD34^+$ UCB cells was performed by positive selection using Variomacs Immunomagnetic Separation System[61] (CLB, Amsterdam, The Netherlands). RhBM $CD34^+$ cells were purified by positive selection using

Dynalbeads (Dynal, Oslo, Norway).[62] Briefly, low-density cells were incubated with an IgG2A antibody against CD34 (mAb 561; from G. Gaudernack and T. Egeland, Rikshospitalet, Oslo, Norway) that was covalently linked to rat anti-mouse IgG2A beads. $CD34^+$ cells devoid of the CD34-antibody were recovered using a polyclonal antibody against the Fab part of the CD34 antibody (Detachabead, Dynal). The percentage $CD34^+$ cells in the unseparated population and in the purified $CD34^+$ and $CD34^-$ fractions was determined by flow cytometric-analysis (FACS Calibur, Becton Dickinson, San José, USA)

Viral vector and packaging cell line

The pseudotyped retrovirus producer cell line PG13/SF-EGFP7 was kindly provided by J. Barquinero (Institut de Recerca Oncologica, Barcelona, Spain). The cell line was developed by transducing the PG13 packaging cell line[7] (kindly provided by D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) with 0.45 µm filtered supernatant from PA317/EGFP cell cultures.[43] Expression of the enhanced green fluorescent protein (EGFP) was analysed by flow cytometry and bright single cells were sorted on 96-well plates by using an EPICS Elite ESP flow cytometer coupled to an autoclone device (both from Coulter, Miami, FL, USA). Single clones were cultured as previously described.[43] The PG13/EGFP7 cell line was subsequently subcloned by diluting the cells to 1 cell per well of a 96-well plate. Single subclones were cultured in culture medium consisting of an enriched version of Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD)[14, 54] supplemented with 10% fetal calf serum (FCS) and kept at 37°C with 10% CO_2 in a humidified atmosphere. The subclones were analysed for transduction efficiency on rhesus BM and UCB $CD34^+$ cells. The viral titer of the cell lines used for further experiments (original and subclones) was in the order of $10^5 - 10^6$ infectious particles per ml as determined by supernatant titration on cultured human HeLa cells and Rat-2 cells. Absence of replication-competent virus was verified by failure to transfer EGFP-expression from transduced Rat-2 cells to a secondary Rat-2 cell population.

Retrovirus mediated transduction

Supernatants containing recombinant retrovirus were generated by culturing approximately 80% confluent producer cells for 12 hours in culture medium consisting of serum-free enriched version[53, 54] of Dulbecco's modified Eagle's

medium (DMEM, Gibco, Gaithersburg, MD), further modified with equimolar concentrations of cholesterol and linoleic acid.[55] Media for all cultures routinely included 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cultures were maintained at 37°C with 10% CO_2 (measured every 15' with read-outs between 9.5% and 10%) in a humidified atmosphere. The culture supernatant was subsequently produced and passed through a 0.45 μm filter. To enhance the transduction efficiency, Falcon 1008 (35 mm) bacteriological culture dishes were coated with the recombinant fibronectin fragment CH-296 (Takara Shuzo, Otsu, Japan) at a concentration of 10 μg/cm^2 as described previously.[25]

Table 1. Repopulation of EGFP-transduced CD34$^+$ UCB cells in NOD/SCID mice

Virus producer	Transduction efficiency %EGFP	EGFP$^+$/ chimeric mice	Chimerism in NOD/SCID %CD45	%EGFP$^+$ in CD45$^+$ cells ± SD (range)
PG13/SF-EGFP7	66	4/4	8 (3-12)	23 ± 17 (2-41)
PG13/SF-EGFP7.1	85	5/5	3 (1-6)	63 ± 17 (38-80)*

* $p<0.009$

CD34-selected UCB and normal RhBM cells were prestimulated for 2 days in enriched DMEM with the human recombinant growth factors (GF) fms-like tyrosine kinase-ligand (Flt3-L, 50 ng/ml, kindly provided by Immunex, Seattle, WA, USA), thrombopoietin (huTPO or RhTPO, 10 ng/ml, kindly provided by Genentech, South San Francisco, CA, USA) and stem cell factor (SCF, 100 ng/ml). Before adding purified subsets to the fibronectin-coated dishes, the CH-296 fibronectin fragment was preincubated with supernatant containing the pseudotyped vector for 1 hour at 37°C.[23, 25] Subsequently, nucleated cells were resuspended in the vector-containing supernatant supplemented with the GF cocktail, and added to the dishes. Over a period of 2 days, culture supernatant was replaced completely by resuspending non-adherent cells into fresh retrovirus supernatant and GFs. The standard cell number used in transduction experiments was 10^5 cells/ml. For cell titration experiments the cell concentration varied from $3x10^6$/ml to 10^3/ml. The producer cell line was routinely cultured in T75 cm^2 flasks

filled with 10 ml serum-free culture medium until 80% confluence. During the transduction, the virus supernatant was refreshed once. After 2 days of prestimulation and 2 days of supernatant infection in the presence of SCF, TPO and Flt3-L, the cells were harvested and the transduction efficiency was determined by visualising EGFP transduced cells by flow cytometry. The cells were counterstained with a monoclonal antibody against CD34 (566, G. Gaudernack and T. Egeland, Rikshospitalet, Oslo, Norway) that had been conjugated with the Cy-5 fluorochrome.

Transplantation of transduced UCB cells into NOD/SCID mice

Male, specified pathogen-free (SPF) NOD/LtSz-scid/scid mice (NOD/SCID) were bred and housed under SPF conditions and supplied with sterile food and drinking water containing 100 mg/l ciprofloxacine (Bayer AG, Leverkusen, Germany) *ad libitum*. Housing, care and all animal experimentation were done in conformity with legal regulations in The Netherlands, which include approval by a local ethical committee. All mice received total body irradiation (TBI) at 3.5 Gy, delivered by a ^{137}Cs source adapted for the irradiation of mice (Gammacell, Atomic Energy of Canada, Ottawa, Canada), 2-4 hours before transplantation. The transplants were suspended in 200 µl H+H and injected iv. into a lateral tail vein. Transplanted cell numbers were $2x10^5$ $CD34^+$ UCB cells. At day 35 after transplantation, the mice were killed by CO_2 inhalation followed by cervical dislocation, both femurs and the spleen were isolated and BM suspensions were prepared by flushing. After counting, BM cells were analysed by flow cytometry to determine the percentage human $EGFP^+$ cells in the mouse BM and cultured in colony assays as described[53-55, 57] to measure the transduction of colony forming units-granulocyte/monocyte (GM-CFU) and burst forming units-erythrocyte (BFU-E).

Statistics

Data were expressed as median (range). Statistical comparisons were performed according to Mann Whitney U-test. Two tailed P values of <0.05, were considered significant. Actual significance levels are indicated in the Tables and Figures.

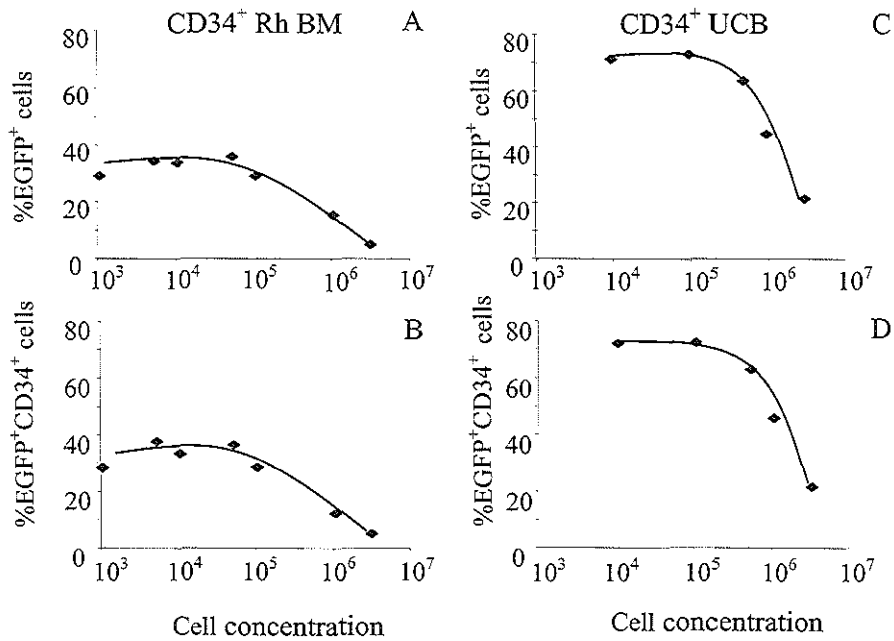

Figure 1. Titration of cryopreserved CD34-selected RhBM cells (panels A + B) and UCB (panels C + D) during transduction using the pseudotyped PG13/SF-EGFP7 packaging cell/vector combination.

Results

Decreasing cell numbers result in higher transduction efficiencies

The efficiency of transduction in cryopreserved purified $CD34^+$ cells using TPO, Flt3-L and SCF stimulation, appeared to be highly dependent on the number of target cells per culture (Figure 1). Cell numbers of $3x10^6$ CD34-selected rhesus monkey BM (RhBM)/ml resulted in very low levels of transduction of the $CD34^+$ cells, i.e., ~5% $EGFP^+$ cells. (Figure 1A+B). The transduction efficiency using the virus supernatant produced by the PG13/SF-EGFP7 cell line increased 3 to 6-fold with decreased cell numbers/ml. The maximum transduction levels were ~35% for RhBM cells at a concentration of 10^5 cells/ml. Decreasing the cell concentration to 10^3 cells/ml did not increase the transduction efficiency further. Transduction of

graded numbers of purified $CD34^+$ UCB cells (Figure 1C+D) showed the same effect. Low frequencies of $EGFP^+CD34^+$ cells (~20%) were found at cell numbers of $3x10^6$/ml, whereas frequencies of transduced cells increased to ~75% at target cell concentrations below $5x10^5$ cells/ml. The maximum transduction efficiency of UCB cells was more than 2-fold higher than the transduction efficiency of RhBM cells. Also, the apparent saturation occurred for RhBM at 5-fold lower concentrations than for UCB cells, suggesting a difference in GALV receptor density and/or affinity. All further experiments were performed using a cell concentration of 10^5 RhBM cells or $5x10^5$ UCB cells.

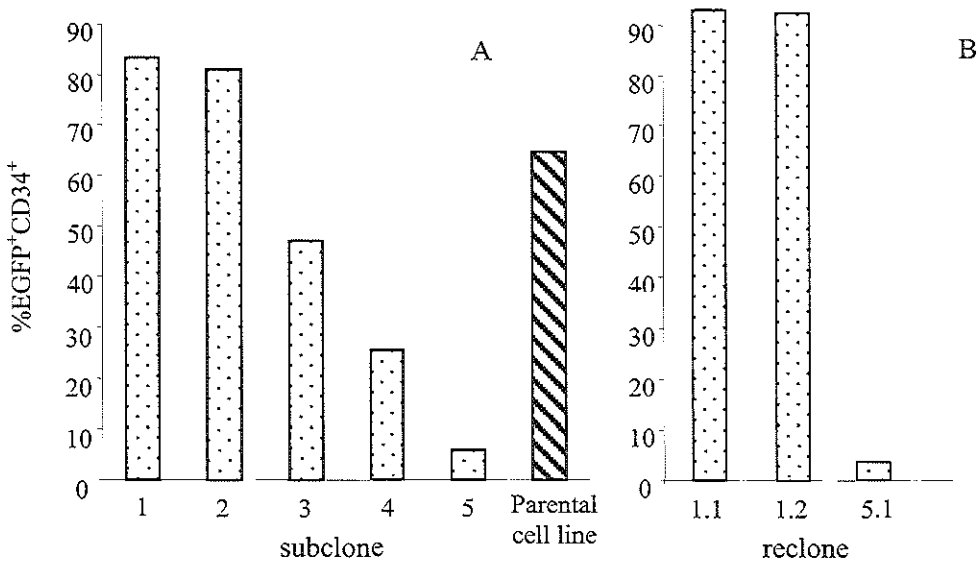

Figure 2. Transduction efficiency of RhBM $CD34^+$ cells of 5 subclones derived from the parental packaging cell/vector combination PG13/SF-EGFP7 (A). Transduction efficiencies of recloned producers 1, 2 and 5 are shown in B.

High titer clones of PG13/SF-EGFP resulted in high transduction efficiencies

Single cell culture of the PG13/SF-EGFP7 packaging cell/vector combination by limiting dilution resulted in five PG13/SF-EGFP subclones. The virus titers were determined by supernatant titration on human HeLa cells and Rat-2 cells. Subclone 1 (PG13/SF-EGFP7.1) showed the highest titer of $5x10^5$ infectious particles per ml, which was 2 times higher than the virus titer of the parental cell line (PG13/SF-EGFP7) (data not shown) whereas subclone 5 (PG13/SF-EGFP7.5) showed a titer

which was considerably lower than the parental PG13/SF-EGFP7 packaging cell/vector combination (10^4 infectious particles/ml). Therefore, the serial passage of the producer cell line had produced a significant heterogeneity, affecting virus titer.

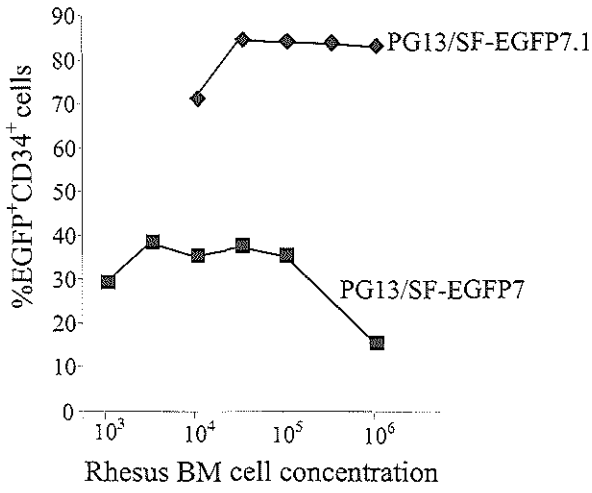

Figure 3. Titration of cryopreserved CD34-selected RhBM cells during transduction using the pseudotyped PG13/SF-EGFP7 packaging cell/vector combination and the subclone PG13/SF-EGFP7.1.

The transduction efficiency of the subclones was tested using cryopreserved CD34-selected RhBM cells (Figure 2). Clones with a higher virus titer (PG13/SF-EGFP7.1 and 7.2) resulted consistently in high transduction efficiencies in the order of magnitude of ~90% to be compared with the ~50% efficiency of the parental PG13/SF-EGFP7 cell line in the same experiment. The low titer subclone, PG13/SF-EGFP7.5, transduced the $CD34^+$ RhBM cells with a low efficiency of ~5%. Recloning the subclones 1, 2 and 5 transduced rhesus BM and UCB cells with the same efficiency as their parental subclones, demonstrating that these were homogeneous and stable (Figure 2B). Using the most efficient subclone 7.1, we examined the dependence of transduction efficiency on cell concentration in comparison to that obtained with the parental clone. Figure 3 demonstrates the difference in both the plateau level reached and the lack of dependence on cell concentration in the range studied when using the high titer subclone. This

demonstrated that the low level transduction of RhBM cells is not due to the presence of receptors on only a subset of $CD34^+$ cells[63] and that the maximum transduction can be achieved at higher cell concentrations by increasing the multiplicity of infection (m.o.i.). Consequently, there was a direct relationship between the virus titers and $CD34^+$ transduction efficiencies (Figure 4). The saturation level of the virus titer is $4x10^5$ particles/ml, corresponding to a m.o.i. ~4, above which the transduction levels did not increase further.

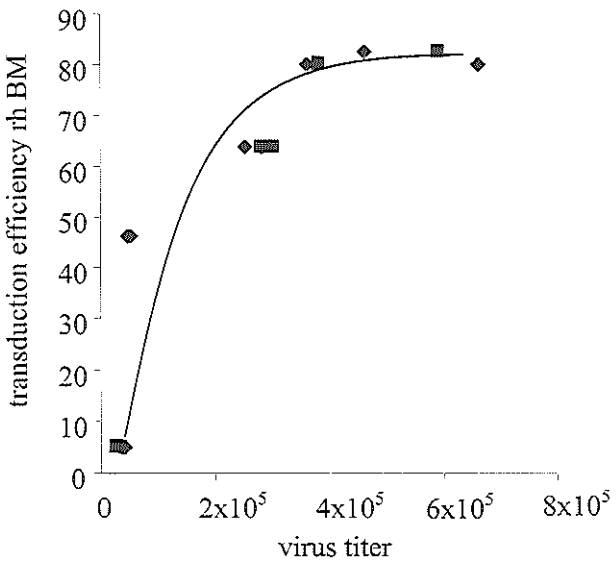

Figure 4. Virus titer related to efficiency of transduction of $CD34^+$ selected RhBM cells. The virus titer was determined on HeLa cells (◆) and on Rat-2 cells (■).

Transduction of cryopreserved vs fresh hematopoietic cells

Cryopreservation of normal or G-CSF primed rhesus BM resulted in 3-fold increased transduction levels compared to fresh cells (Figure 5). Transduction of fresh G-CSF primed RhBM with subclone 7.1 resulted in 30% ± 24% $EGFP^+$ cells (n=8), whereas frozen BM samples of G-CSF primed monkeys were transduced with significantly ($p<0.01$) higher numbers of 70% ± 10% $EGFP^+$ cells (n=4). A significant difference was not found between the transduction efficiency of frozen

G-CSF primed cells, frozen normal RhBM (70% ± 16% $EGFP^+$ cells, n=7) and frozen UCB cells (80% ± 12%, n=13).

Transduction efficiency of NOD/SCID repopulating cells (SRC's)

To determine the effect of the optimised transduction conditions on repopulating ability, transduced UCB $CD34^+$ cells were transplanted into NOD/SCID mice. Using the virus of the parental PG13/SF-EGFP cell line, 10^5 $CD34^+$ UCB cells were transduced with an efficiency of 66%. Injection of these transduced cells into 4 NOD/SCID mice resulted on average in 23% EGFP positive cells within the $CD45^+$ population.[16] Transplantation of $CD34^+$ UCB cells using the high titer virus producing subclone PG13/SF-EGFP7.1 (77% and 93% $EGFP^+$ cells, respectively in 2 experiments) resulted in 63 ± 17%, $EGFP^+CD45^+$ cells in the BM of engrafted mice (Table 1). The $\%EGFP^+$ cells in these mice was significantly higher than those obtained previously with 10^5 $CD34^+$ cells (66% $EGFP^+$) transduced with virus produced by the parental PG13/SF-EGFP virus producing cell line, i.e., 23% $CD45^+$ cells after transplantation with 10^5 $CD34^+$ cells ($p < 0.009$).

Table 2. Human CFU-GM and BFU-E in the chimeric NOD/SCID mouse BM 35 days after infusion

Graft	# $CD45^+$ cells/femur	# $CD34^+$ cells/femur	CFU-GM/ femur	CFU-GM/10^5 human cells	BFU-E/femur	BFU-E/10^5 human cells
	x10^6	x10^5				
Day 0*	4.2 ± 3.0	6.6 ± 5.4	8997 ± 9762	332 ± 316	10762 ± 14674	290 ± 275
Day 4*	0.5 ± 0.24	1.0 ± 1.0	1180 ± 1150	253 ± 135	1605 ± 2319	293 ± 294

* n=5

Immunophenotyping of the human cells that were present in the mouse BM revealed that high levels of EGFP were expressed in all human hematopoietic lineages assessed, i.e., $CD56^+$ NK cells, $CD20^+$ B-lymphocytes, $CD4^+$ or $CD8^+$ T-cells and $CD11b^+$ and $CD33^+$ monomyeloid cells, and immature $CD34^+$ cells

(Figure 6). The numbers of $CD45^+$ and $CD34^+$ cells as well as human progenitor cells, i.e., GM-CFU and BFU-E, present in the BM of mice transplanted with EGFP transduced $CD34^+$ cells were on average lower than those of mice injected with day 0 $CD34^+$ cells (Table 2), suggesting 0.5-1 log loss of SRC's under the conditions used, which is similar to data obtained in mice using similar transduction conditions and a quantitative transplantation assay.[21] Since these differences are not apparent in GM-CFU and BFU-E per 10^5 human cells (Table 2), the results point to an adequate preservation of repopulating capacity of the SRC's after transduction. Low numbers (0.4%) of human $CD45^+$ cells were found in the spleen of the mice, approximately 92% of these cells expressed the EGFP gene (data not shown).

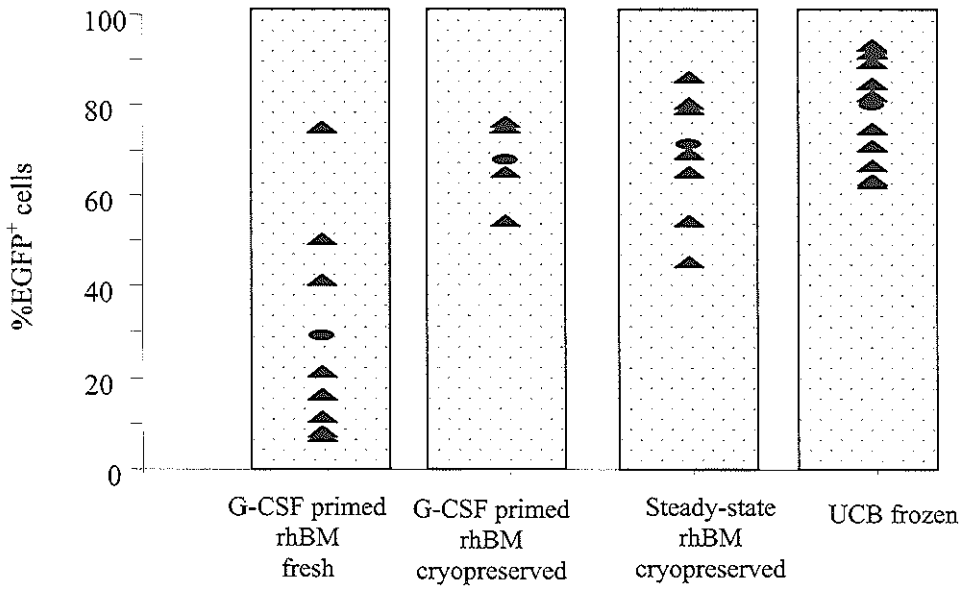

Figure 5. Cryopreservation of RhBM (stimulated with 4 days G-CSF treatment, or steady-state) and UCB cells before performing the transduction protocol results in a 2-fold increase of the efficiency as compared to the transduction efficiency of fresh G-CSF treated RhBM cells. The results of individual experiments (▲) are shown as well as the average % $EGFP^+$ cells (●) after transduction.

Since only a small subset of $CD34^+$ cells has the capacity to produce offspring after transplantation[57, 64], it was thought to be of interest to relate EGFP expression frequencies of $CD45^+$ cells in the engrafted NOD/SCID mice with the frequency of $EGFP^+$ cells in the transplanted $CD34^+$ cells (Figure 7). This analysis revealed a threshold of transduction of SRC's relative to the $CD34^+$ cells, which explains why substantial transduction frequencies in $CD34^+$ cells do not necessarily result in similar frequencies following transplantation, and also demonstrated that with the optimised procedure up to 80% of the SRC's should express the transgene.

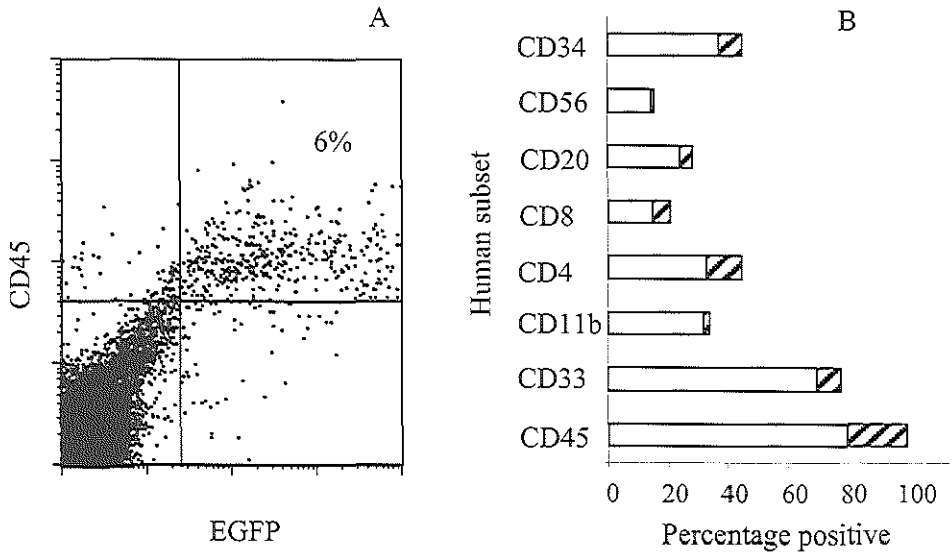

Figure 6. Chimerism and EGFP expression levels in a chimeric NOD/SCID mouse BM 35 days after transplantation of 10^5 $CD34^+$ UCB cells of which 93% expressed the EGFP gene. The bright green autofluorescence on the X-axes vs. CD45 on the Y-axes clearly shows that almost all human ($CD45^+$) cells (80%) express the EGFP. The right panel shows the distribution of $EGFP^+$ (❑) and $EGFP^-$ cells (◪) in all hematopoietic lineages assessed.

Discussion

In this study, the fibronectin-based retrovirus mediated transduction protocol was further optimised, which resulted in high level transductions of >90% in CD34-selected umbilical cord blood (UCB) and rhesus monkey adult BM cells. Transplantation of the transduced UCB cells into NOD/SCID mice resulted in up to 80% $EGFP^+$ human cells in the mouse BM 35 days after transplantation, demonstrating efficient transduction of cells capable of multilineage reconstitution following transplantation.

Initially surprising, the cell concentration of the $CD34^+$ RhBM or UCB cells appeared to be of critical importance for the efficiency of the fibronectin-supported transduction procedure at limiting virus titers. Decreasing numbers of human and rhesus monkey progenitor cells resulted in increased percentages of EGFP-gene transduction. Hanenberg *et al.*, demonstrated that the use of the fibronectin fragment during the transduction allowed large numbers of cells to be efficiently transduced on small surfaces coated with the CH-296 fragment and that the quantity of retroviral particles in the supernatant was not a limiting factor for the transduction of primary human clonogenic cells[24], using a virus titer of $>3x10^6$ cfu/ml corresponding to an m.o.i. of 4.8 in the highest concentration used. The virus titer of the parental virus producer cell line used in our studies was $\sim3x10^5$ /ml and the optimal concentration of $CD34^+$ UCB or RhBM cells used was $5x10^5$ and 10^5 cells/ml, respectively, resulting in a m.o.i. of ~1. This explains the negative effect of increasing cell concentration on the transduction efficiency and demonstrates that for optimal transduction efficiencies the cell concentration of the target cells should be carefully balanced with the virus titer.

The highest transduction levels in RhBM using the PG13/SF-EGFP7 combination was found at a cell concentration of 10^4 to $5x10^4$ cells/ml, which was 2 to 10-fold lower compared to UCB cells transduced under the same conditions of limiting virus titers with a maximum efficiency at cell numbers of 10^5 cells/ml. A likely explanation for this discrepancy between RhBM and UCB might be differences in virus receptor-expression of both cell types. This is corroborated by studies that compared the susceptibility of $CD34^+$ BM progenitors from four non-human primate species (pigtail macaques, rhesus monkeys, cynomolgus macaques and baboons) and humans to transduction with amphotropic retrovirus vectors, which showed differences in transduction efficiency.[65] Semiquantitative RT/PCR analysis

suggested these differences due to different expression levels of the amphotropic receptor, Pit-2 in the different species.

The pseudotyped PG13/SF-EGFP producer cell line transduced human UCB cells with NOD/SCID repopulating ability with higher efficiency than the amphotropic Am12/MFG-EGFP packaging cell line/vector combination.[16] After several transduction experiments using RhBM and UCB with variable results, variability in the results prompted an analysis of the producer cell line by flow cytometry. Although the PG13/SF-EGFP cell line was initially cloned by single cell sort[43], the expression of EGFP was heterogeneous, ranging from no expression to very high expression (up to 4 logs). The most likely explanation for the heterogeneity is silencing of expression of the EGFP gene and the packaging sequences during serial passage. Subcloning of the vector/packaging cell line by limiting dilution resulted in high titer clones (5 to 10-fold the titer of the parental PG13/SF-EGFP7 packaging cell line/vector combination) which were able to transduce UCB and RhBM cells more efficiently. Use of the selected high titer producer cells and transduction of graded numbers of CD34-selected RhBM or UCB cells did not reveal the dependence on cell concentration encountered with the PG13/SF-EGFP in the range of cell concentrations examined and resulted in expression levels of >90% of both UCB and RhBM $CD34^+$ cells.

Transplantation of the UCB cells into NOD/SCID mice resulted in high frequencies of $EGFP^+$ human cells 35 days. Compared to previous studies in which the parental clone was used to transduce NOD/SCID grafts[16], the subclone resulted in 2- to 4-fold higher engraftment levels of transduced SRC's, with a mean value of 63 ± 17%, similarly expressed in all hematopoietic lineages examined. This demonstrates, firstly, the validity of the optimisation for transplantable hematopoietic stem cells and, secondly, that those cells can be transduced under optimal conditions with frequencies up to 80%. Relating the frequency of EGFP expressing cells in NOD/SCID mice to that of the primary transplanted $CD34^+$ cells displayed a marked threshold of the SRC's relative to the $CD34^+$ cells, with virtually no engraftment of transduced cells at transduction frequencies of $CD34^+$ cells < ~30%. This might relate to a lower virus receptor expression at the small subset of repopulating cells and is a phenomenon to bear in mind for clinical gene therapy protocols necessarily utilising $CD34^+$ transduction frequencies for quality control. However, we do not exclude that the threshold is a purely numerical phenomenon attributable to the low seeding efficiency of human stem cells transplanted into the mouse environment.[16, 57, 66]

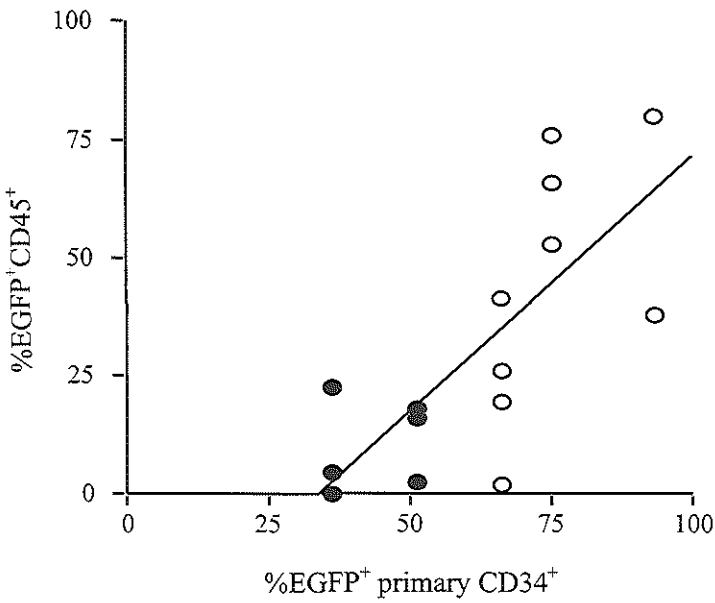

Figure 7. Percentage of EGFP positive $CD45^+$ cells in NOD/SCID mice 35 days after transplantation related to the percentage of primary EGFP positive $CD34^+$ cells transplanted. (●) AM12/MFG-EGFP transductions; (○) PG13/SF-EGFP transductions. The amphotropic data were taken from reference 16, the other data points represent the individual data summarised in Table 1. The regression line of the data without the amphotrophic transductions is identical to that shown of all data pooled.

When reanalysing data from all experiments, we noted that cryopreserved RhBM cells and UCB cells consistently gave higher transduction efficiencies when compared to fresh rhesus monkey BM used for transplantation purposes (manuscript in preparation), which prompted a systematic survey of freshly harvested versus cryopreserved RhBM cells. Cryopreservation of the target cells appeared to have an increasing effect on the transduction efficiency using the pseudotyped PG13/SF-EGFP packaging cell/vector combination. This has not been earlier described for this virus receptor type, but is consistent with the increase in mRNA expression of the amphotropic receptor found in cord blood samples after

cryopreservation.[67-69] The expression of the GALV receptor (Pit-1) might be similarly upregulated after cryopreservation. The exact mechanism involved is not clear yet. A recent study described that $CD34^+$ hematopoietic cells extend long, thin podia with adhesion receptors that are known to play important roles in blood-cell migration and adhesion, including CD34, L-selectin and CD45.[70] It is not unlikely that these podia also are the membrane location of other receptors and, due to their fragile nature, are damaged by cryopreservation, requiring upregulation of receptor expression after thawing. The expression of L-selectin, which is involved in migration and adhesion of primitive hematopoietic cells, is indeed decreased by the freezing procedure and restored after overnight incubation of the thawed cells in culture medium.[71, 72] All experiments with UCB cells were done after cryopreservation; initial experiments revealed no difference between fresh and cryopreserved UCB cells (data not shown), suggesting that receptor expression is already optimal in fresh UCB cells.
The high transduction levels achieved in repopulating cells likely result from the use of the fibronectin fragment mediated co-localisation of virus and cells, the selection of a minimum combination of hematopoietic growth factors capable of preserving repopulating capacity while efficiently inducing cell cycling, complex serum free media to support the cells during culture and the use of pseudotyped retrovirus to overcome the low expression levels of the previously used amphotropic receptor on immature repopulating cells.

The results shown in this study thus enable a reproducible, highly efficient retrovirus mediated method of gene transfer into hematopoietic cells with repopulating ability. We chose to test the repopulating capacity of the transduced cells by using UCB cells transplanted into NOD/SCID recipients, rather than the much more laborious and time-consuming rhesus monkey recipients. The ongoing preclinical nonhuman primate experiments are directed at its clinical development using further improved, high titer SF type retroviruses. We conclude that, taking these optimal conditions into account, clinically applicable retrovirus mediated gene transfer of therapeutic genes that do not necessarily confer a growth advantage to transplantable hematopoietic stem cells is now within short-term reach.

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CHAPTER

VI

Thrombopoietin is a major limiting factor for selective outgrowth of human umbilical cord blood cells in NOD/SCID recipient mice

Co-autors: Albertus W. Wognum, Gerard Wagemaker

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Abstract

A single dose of 0.3 μg recombinant human thrombopoietin (TPO) was injected into sublethally irradiated NOD/SCID mice immediately after transplantation of $1.5x10^5$ purified $CD34^+$ umbilical cord blood (UCB) cells. Bone marrow (BM) was analysed at day 35 for human cells by immunophenotyping and colony culture. TPO-treatment resulted in a 3 to 4-fold increase in the frequency and number of human $CD45^+$ cells; mouse type cells were not influenced. The lineage distributions among the human cells were essentially similar irrespective of TPO treatment, however, with a lower frequency of myeloid $CD33^+$ cells in the TPO treated mice and a prominent increase in the total number of erythroid and megakaryocytic cells. An impressive increase was observed in $CD71^+GpA^-$ cells, reflecting the proliferative stimulus provided by TPO administration. Also the frequency of immature $CD34^+$, $CD34^+CD38^-$ cells and of human GM-CFU and BFU-E in TPO-treated mice was similar to that of untreated mice, but their total number had increased proportional to the increase of human cells. The results demonstrate that human TPO is a major limiting factor for multilineage outgrowth of human UCB cells in NOD/SCID mice and can be conveniently supplemented by single dose treatment immediately after transplantation. The findings in this study make the NOD/SCID mouse a more efficient assay for human hematopoietic stem cells and provide a new basis for further studies on human stem cell regulation using immunodeficient mouse models. In addition, immunodeficient mice transplanted with human UCB cells yield a suitable assay for the development of novel human c-mpl agonists.

Introduction

Xenotransplantation into immunodeficient mice is a major tool to study the properties of normal and malignant hematopoietic stem cells[1-3], as well as the

development of *ex vivo* expansion[4] and gene transfer.[5] Following the development of the severe combined-immunodeficient (SCID) mouse[6], various crosses with other deficient mutants[7] have been explored, with more recently the non-obese diabetic (NOD)/SCID mouse[8] as a versatile recipient for these purposes.[9-11] Some obvious disadvantages are the limited mature end cell production and the low seeding efficiency of the human cells in the mouse environment[12], necessitating the use of large quantities of human cells, which could be partly attributed to accessory $CD34^{+}CD38^{+}$ cells.[13] Supplementation of human growth factors such as interleukin-3 (IL-3) and granulocyte/macrophage-colony stimulating factor (GM-CSF)[14], stem cell factor (SCF) and/or PIXY321 (a fusion of IL-3 and GM-SCF) and/or erythropoietin (EPO)[15, 16] or fms-like tyrosine kinase3-ligand (Flt3-L) and/or IL-7[17] has been used to augment the outgrowth of human cells in the mouse environment, or, more simply, addition of large quantities of irradiated human cells[16], of which the role is not fully clear. Recent novel crosses of mutants, such as the NOD/$Rag1^{null}$ or the NOD/SCID/β2$microglobulin^{null}$ mouse[18, 19], require lower numbers of human cells, suggesting that residual immune functions in the NOD/SCID mouse contribute to the relative inefficiency of the NOD/SCID mouse. Also, species barriers may exist for hematopoietic growth factors, murine growth factors not necessarily being optimal or suited to stimulate the human cells. Since thrombopoietin (TPO) is an essential physiologic factor for stem cell maintenance and proliferation[20], apart from its functions in platelet production and activation[21], in the present study we sought to explore the option of using human TPO to stimulate outgrowth of human cells in NOD/SCID murine recipients.

TPO is the ligand for the receptor c-mpl[22], which is expressed on megakaryocytes, platelets and their precursors and also on pluripotent hematopoietic progenitor cells. TPO was first isolated and cloned in 1994[23-25] and consists of a highly conserved amino-terminal domain that is related to EPO.[21] TPO is constitutively produced, mainly in the liver, and its levels are regulated by c-mpl-mediated clearance by platelets[26] and megakaryocytes.[27] As a consequence, exogenous TPO needs to saturate the c-mpl mediated clearance mechanism to be optimally active. Similar to many growth factors, TPO displays multiple biological activities and acts within the specific megakaryocytic lineage to produce platelets, has a role in platelet activation[21] and stimulates immature hematopoietic stem cells.[20] Therefore, TPO may play an important role in *in vivo* and *ex vivo* expansion of hematopoietic cells with repopulating ability.

Ex vivo culture of CD34-selected UCB cells for up to 10 weeks using stroma-free culture conditions containing 10% human plasma plus TPO and Flt3-L resulted in a

large amplification of $CD34^+$ cells, colony-forming cells, long-term culture-initiating cells (LTC-IC) and NOD/SCID repopulating cells.[4] Cultures of murine BM using TPO as a single exogenous GF could be maintained for 3 to 4 months without apparent loss of the primitive phenotype and with sustained high-level production of both megakaryocytic and non megakaryocytic cells able to reconstitute hematopoiesis in lethally irradiated mice.[28] Furthermore, knock-out mice lacking TPO or c-mpl expression are not only thrombocytopenic and show 80% to 90% reduction in megakaryocytes and platelets, are also seriously deficient in stem cell numbers, as was determined by serial transplantation experiments.[29] Further evidence for the stimulatory action of TPO on immature hematopoietic cells was provided by studies of *in vivo* hematopoietic recovery after BM suppression as a result of total body irradiation (TBI) and/or chemotherapy.[30, 31] Treatment of mice[32-35] or non-human primates without BM suppression[36-38] resulted usually only in a platelet response, whereas in myelosuppressed animals the response was convincingly multilineage in nature[39-44] Administration of TPO to animals after chemotherapy or radiation shortened the interval to platelet recovery.[31, 45] One single intravenous (iv.) dose of TPO in non-human primates shortly after intensive cytoreductive treatment was sufficient to significantly alleviate the course of thrombocytopenia[46], consistent with the results in mice,[47, 48] and, in addition, accelerated BM $CD34^+$ cell reconstitution, thereby potentiating the action of concomitant G-CSF or GM-CSF treatment. TPO treatment in myelosuppressed individuals did not result in adverse effects.[49]

This study focussed on the effect of a single dose of recombinant human TPO on the efficiency of engraftment of $CD34^+$ UCB cells in NOD/SCID mice. Previous studies in which the use of immunodeficient NOD/SCID mice for transplantation assays with purified human UCB subsets was demonstrated, provided evidence for differential regulation of the expansion of immature human hematopoietic stem cells[13], and opened an experimental approach to examine the effects of various GFs such as TPO. Briefly, $CD34^+$ cells, isolated from UCB samples, were iv. transplanted into sublethally irradiated NOD/SCID mice, immediately followed by a single intraperitoneal (ip.) injection of 0.3 μg TPO. The dose used is sufficiently high to overcome initial c-mpl mediated clearance[50], but found to be insufficient to stimulate the mouse type cells. After 5 weeks, the mice were sacrificed and the BM of the mice was analysed for human ($CD45^+$) cells, lineage specific phenotypes and human colony-forming cells.

Material and methods

Human umbilical cord blood cells

UCB samples were obtained from placentas of full-term pregnancies after informed consent in conformity with legal regulations in The Netherlands. Mononucleated cells were isolated by Ficoll density gradient centrifugation (1.077g/cm^2, Nycomed Pharma AS, Oslo, Norway), and were cryopreserved in 10% dimethylsulphoxide, 20% heat-inactivated foetal calf serum (FCS) and 70% Hanks Balanced Salt Solution (HBSS, Gibco, Breda, The Netherlands) at –196°C as described[51] before use. After thawing by stepwise dilution in HBSS containing 2% FCS, the cells were washed with HBSS containing 1% FCS and used for transplantation into NOD/SCID mice.

Subset purification

Purification of CD34$^+$ cells was performed by positive selection using Variomacs Immunomagnetic Separation System as described[52] (CLB, Amsterdam, The Netherlands). The percentage of CD34$^+$ cells in the unseparated population (low density UCB) and in the purified CD34$^+$ and CD34$^-$ fractions was determined by FACS-analysis. The CD34$^+$ subset used for transplantation contained 75% to 95% CD34$^+$ cells after purification.

Transplantation of UCB subsets in immunodeficient mice

Specific pathogen-free (SPF) NOD/LtSz-scid/scid (NOD/SCID) mice, 6 to 9 weeks of age, were bred (experiment 1) or obtained from Bomholtgard Breeding & Research Centre A/S, Denmark) (experiment 2) and The Jackson Laboratory (Bar Harbor, MA) (experiment 3), housed under SPF conditions in a micro-isolator and supplied with sterile food and acidified drinking water containing 100 mg/l ciprofloxacine (Bayer AG, Leverkusen, Germany) *ad libitum*. Housing, care and all animal experimentation were done in conformity with legal regulations in The Netherlands, which include approval by a local ethical committee. All mice received total body irradiation (TBI) at 3.5 Gy, delivered by a ^{137}Cs source adapted for the irradiation of mice (Gammacell, Atomic Energy of Canada, Ottawa, Canada), 2-4 hours before transplantation. The transplants were suspended in 200 µl HBSS containing 0.1% BSA and injected iv. into a lateral tail vein. Transplanted

cell numbers were $1.5x10^5$ $CD34^+$ cells. The group receiving human TPO was injected ip. with 0.3 μg full length recombinant human TPO (Genentech Inc., South San Francisco, CA) per mouse directly after transplantation. 35 Days after transplantation the mice were sacrificed by CO_2 inhalation followed by cervical dislocation, both femurs and spleen were isolated and cell suspensions prepared. After counting, the cells were cultured in colony assays and analysed by flow cytometry to determine the percentage and the distribution of human cells in the mouse BM. In total 32 mice were transplanted of which 16 received TPO injections. The survival of TPO-treated mice after transplantation was 11 (~70%), whereas only 7 mice of the 16 (~45%) transplanted without TPO treatment survived.

Flow cytometry

Cell samples were analysed using a FACSCalibur flow cytometer (Becton Dickinson) as previously described.[13] Immunophenotyping of engrafted cells was performed by staining with preidinin chlorophyll protein (PercP) labeled anti-CD45 and cyanin-5-conjugated anti-CD34 (Cy5, Amersham, Buckinghamshire, UK) or PE conjugated monoclonal antibodies against lineage markers CD38, CD4, CD8, CD19, CD20, CD56, CD33 (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Mice were considered engrafted if the percentage $CD45^+$ cells exceeded 1%.

In vitro colony assay

Purified UCB subsets and chimeric mouse BM samples were assayed for the presence of human GM-CFU and BFU-E by *in vitro* colony formation in viscous methylcellulose culture medium as previously described.[13, 53-55] The number of colonies was determined after 14 days of culture in a humidified atmosphere of 10% CO_2 at 37°C.

Statistical analysis

Data are expressed as median (range) or mean ± standard deviation (SD). Statistical comparisons were performed according to a two-tailed Mann-Whitney U-test. P values of <0.05 considered as statistically significant.

Table 1. Engraftment analysis of the BM after transplantation with $1.5x10^5$ $CD34^+$ UCB cells, mean values ± standard deviation (SD)

Exp	TPO	% CD45	# CD45 cells / femur	% CD45CD34	# CD34 cells/femur	% CD34CD38	# CD34CD38 /femur	# mouse cells/femur
			$x10^6$		$x10^5$		$x10^3$	$x10^6$
1	+ (n=4)	52 ± 19	6.5 ± 3.3	27 ± 9	20 ± 14	nd*	nd	5.5 ± 1.7
	- (n=3)	29 ± 18	2.4 ± 2.5	13 ± 8	4.3 ± 6.0	nd	nd	4.4 ± 1.0
2	+ (n=5)	25 ± 14	3.1 ± 2.4	11 ± 7	3.4 ± 3.0	1.2 ± 0.9	2.2 ± 1.3	7.9 ± 1.6
	- (n=2)	6-8	0.6 –1.0	7 (n=1)^	0.5 (n=1)	1.6 (n=1)	0.75 (n=1)	10 - 12
3	+ (n=2)	20-55	5.7 – 6.3	10 - 21	6.4 – 12	0.2 - 1.5	1.0 – 18.7	5.2 – 23
	- (n=2)	3-9	0.4 – 0.7	15 - 19	0.07 – 1.3	0.6 – 4.1	0.72 – 2.7	13

Exp: experiment, #: number, * nd: not done, ^ (n=1) as the cell number of one of the mice was insufficient for analysis

Results

Chimerism analysis

Purified $CD34^+$ UCB cells were transplanted into NOD/SCID mice with or without IP administration of 0.3 μg TPO. Chimerism levels were obtained 35 days later by staining the cells with the human pan-leukocyte antibody CD45. The results are shown in Figure 1A. The percentage $CD45^+$ cells obtained in the mouse BM without addition of TPO was on average 29% ± 18% (n=3) in the first experiment, 6% and 8% (n=2) in the second experiment, and 3% and 9% (n=2) in the third experiment. Injection of TPO resulted in a 2- to 6-fold higher level of engraftment; i.e., 52% ± 19% (n=3), 25% ± 14% (n=5), and 20-55 (n=2), respectively (Figure 1B, Table 1). These differences are statistically highly significantly ($p<0.001$) as shown in Table 2.

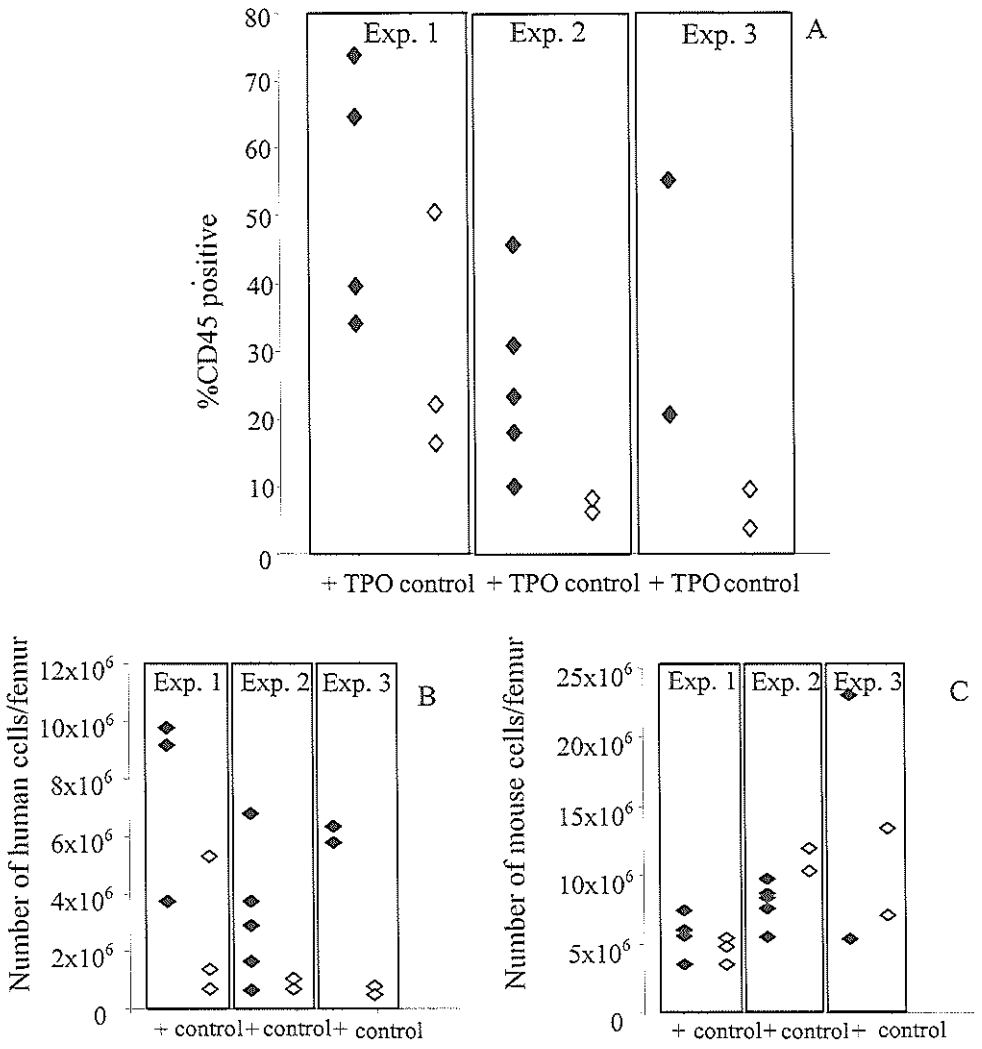

Figure 1. Levels of chimerism in NOD/SCID mice transplanted with $1.5x10^5$ $CD34^-$ UCB cells in 3 seperate experiments (A). In each experiment transplanted mice were either injected with 0.3 µg TPO (+, black symbol) or not (control, open symbol). Percentage CD45 was used as a measure for the level of chimerism. Numbers of $CD45^+$ cells per femur (B) were measured in individual mice that where injected with TPO (+) or no TPO (control). The number of mouse cells per femur in the NOD/SCID mouse BM is shown in Figure C.

The absolute numbers of human cells produced in the mouse BM after transplantation were 3 to 6-fold higher in TPO treated mice than those of the controls (Figure 1B), whereas the number of mouse cells in the BM was not influenced by the injection of TPO (Figure 1C, Table 2). Thus, the TPO administration had selectively expanded human cells and did not stimulate or negatively affect mouse hematopoietic cells during repopulation. Frequencies of $CD45^+CD34^+$ cells and $CD34^+CD38^-$ cells did not differ significantly with or without TPO treatment, but the absolute numbers of $CD34^+$ cells and $CD34^+CD38^-$ cells per femur were, proportional to the larger number of $CD45^+$ cells, higher in the TPO treated mice than in the controls (Table 1).

Multilineage outgrowth is similar with or without TPO injection

The lineage distribution among the human cells was assessed by flow cytometry using a panel of specific markers (Figure 2). TPO-treated mice produced human cells of the myeloid lineage ($CD33^+$; 24% ± 15%, $CD33^+CD15^-$; 11% ± 6%; $CD33^+CD15^+$; 14% ± 6%; $CD33^-CD15^+$; 6% ± 2%, $CD11b^+$; 21% ± 11%, $CD4^+$; 18% ± 14%), NK cells ($CD56^+$; 1%), B lymphoid ($CD20^+$; 10% ± 6%), T lymphoid ($CD8^+$; 0.1% ± 0.2%), and erythroid lineage ($CD71^+GpA^+$; 6% ± 2%, $CD71^{++}GpA^-$; 1% ± 1%, $CD71^-GPA^+$; 0.1% ± 0.02%). Also $CD34^+$ cells (16% ± 10%) and the highly immature $CD34^+CD38^-$ subset (1%) were present in the human cell population (Figure 2A). The control mice displayed an essentially similar pattern of phenotype frequencies. However, $CD71^+GpA^-$ cells were significantly more frequent in the TPO treated mice, whereas $CD20^+$, $CD33^+$ and $CD56^+$ cells were significantly less frequent. In absolute numbers of cells produced per femur (Figure 2B) the differences between TPO-treated and untreated mice were more profound. All phenotypes were increased in the TPO treated mice, most prominently so the $CD41^+$ and the $CD71^+GpA^+$ cells, reflecting the megakaryoctic and erythropoietic lineages, and the $CD71^+GPA^-$ cells, reflecting proliferating cells and thereby the proliferative stimulus provided by the TPO administration.

Chimeric BM was cultured in standard methylcellulose medium under conditions that selectively favor the outgrowth of human monomyeloid and erythroid progenitors and fail to stimulate mouse progenitors. In both TPO treated and untreated mice, GM-CFU and BFU-E were detectable at similar, approximately normal frequencies. The total number of colony-forming cells was considerably higher in the TPO treated mice than in the controls and proportional to the expansion of $CD45^+$ cells observed (Table 3).

Table 2. Statistical analysis of chimerism levels

Analysis	+ TPO	- TPO
Number of mice transplanted	16	16
Survival after transplantation	11	7
Number of $CD45^+$ cells / femur		
Mean	$4.9x10^6$	$1.4x10^6$
Median	$3.8x10^6$	$0.68x10^6$
Standard error	$0.88x10^6$	$0.65x10^6$
Standard deviation	$2.9x10^6$	$1.7x10^6$
P (two tailed)*	<0.01	
Number of mouse cells / femur		
Mean	$8.1x10^6$	$7.9x10^6$
Median	$7.3x10^6$	$6.9x10^6$
Standard error	$1.6x10^6$	$1.5x10^6$
Standard deviation	$5.2x10^6$	$3.8x10^6$
P(two tailed)*	<0.9	

* for statistical analysis the Mann-Withney U-test has been used

Discussion

The results presented, provide new insight into the factors limiting the outgrowth of human cells in the murine environment and emphasise the importance of TPO in the regulation of immature cells with multilineage reconstitution capacity. Since it has been previously established that the NOD/SCID repopulating cells reside exclusively in the $CD34^+CD38^-$ subset[13, 56], the data can be interpreted as evidence for a direct action of TPO on these immature cells. The single dose TPO treatment apparently resulted in a sustained effect in that all differentiation lineages examined had expanded grossly similar 5 weeks after administration.

Table 3. Colony-forming cells after transplantation into NOD/SCID mice

Exp	CFU-GM injected	BFU-E injected	TPO	CFU-GM/10^5 human cells in mouse BM	human* CFU-GM in mouse BM x10^4	BFU-E/10^5 human cells in mouse BM	human* BFU-E in mouse BM x10^4
1	$2.1x10^4$	$3.8x10^4$	+ (n=4)	129 ± 46	7.9 ± 1.4	50 ± 32	3.1 ± 1.4
			- (n=3)	117 ± 131	2.4 ± 1.2	62 ± 42	1.8 ± 0.4
2	$1.7x10^4$	$4.4x10^3$	+ (n=5)	123 ± 97	3.9 ± 3.9	55 ± 38	2.3 ± 0.3
			- (n=1)	255	1.9	138	1.0
3	$1.3x10^4$	$2.6x10^4$	+ (n=2)	272 – 910	21 – 65	234 – 1280	18 – 91
			- (n=2)	424 – 847	3.6 – 4.5	390 – 1075	3.3 – 5.6

* calculated on the assumption that 1 femur represents 8.5% of all BM cells[59]

TPO treatment did not essentially influence the lineage distribution among the human cells, although it cannot be entirely excluded that the slight, but significant decrease in frequency of myeloid $CD33^+$ cells is the result of preferential expansion of other cells types, notably erythroid and megakaryocytic cells. Even so, the action of TPO is remarkably neutral with respect to expansion of all major lineages of hematopoietic cells compared to the controls.

Administration of human growth factors, such as combinations of IL-3, GM-CSF[14], SCF, PIXY321, EPO[15, 16] or Flt3-L and IL-7[17] by multiple injections or osmotic pumps, has previously been attempted to augment the outgrowth of small numbers of human cells in transplantation assays using immunodeficient mice. Obviously, the effect of a single administration of TPO is much more prominent than that of each, or combinations of these growth factors. Therefore, this study identifies human TPO as a major limiting factor for the proliferation and outgrowth of human hematopoietic cells in a mouse environment.

The administration of human TPO did not noticeably influence the number of mouse cells per femur. This observation is consistent with the dose of TPO chosen, which in normal mice does not exert a stimulatory effect, and is remarkable in that the TPO induced expansion is not accompanied by a proportional reduction of

mouse cells. Apparently the irradiated mouse bone marrow provides sufficient space and support for both populations.

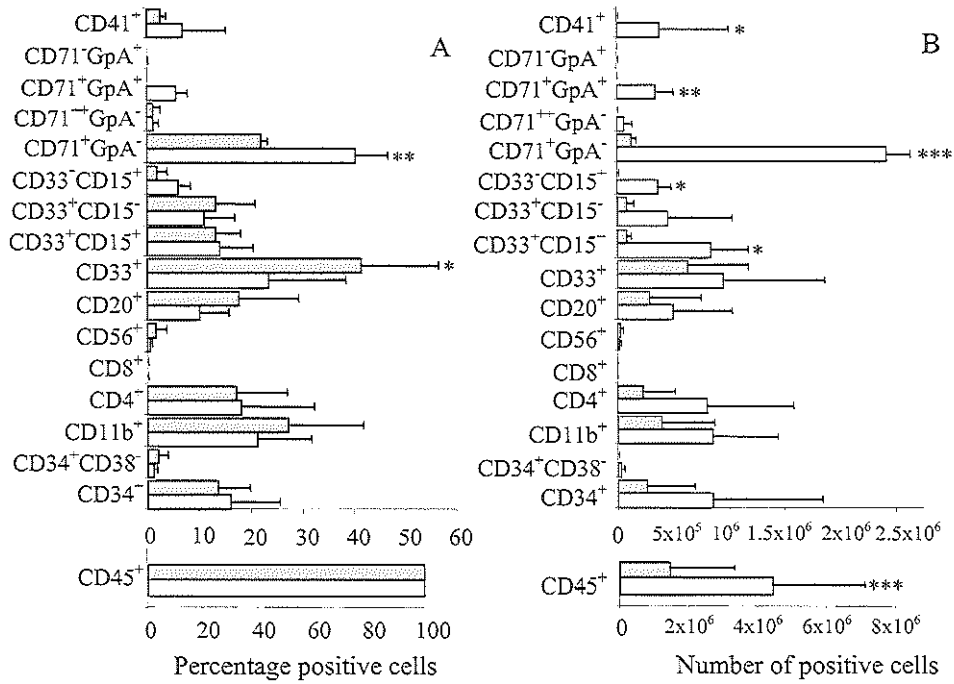

Figure 2. Immunophenotyping of chimeric NOD/SCID mouse BM 35 days after transplantation of CD34-selected UCB cells. BM was stained with a panel of antibodies specific for different human blood cell lineages using CD45 as a marker for human cells. Results for 6 mice that where not treated with TPO are shown in the dark grey columns, the 10 mice that were injected with TPO are shown in the light grey columns. The percentage of cells in each subset, relative to the %CD45$^+$ cells present in the BM is expressed as mean ± standard deviation (A). In figure B the number of lineage specific cells present per femur is calculated and expressed as mean ± standard deviation. Results of CD41 and CD71/GpA stainings are based on 3 mice treated with TPO and 3 untreated mice. Note the difference in X-axes scale used to express frequency and absolute numbers of the CD45 subset and other panels. The results of statistical analysis are represented as * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

Dose and dose schedule of TPO were derived from a previous study, in which it was shown that the dose of TPO should exceed saturation of the platelet c-mpl clearance mechanism to exert an optimal effect, and that there is a short critical time interval for optimal multilineage efficacy, delay of administration resulting in a reduced effect that was predominantly along the platelet lineage.[50] The critical time interval was related to increased survival and proliferation of multilineage repopulating cells at the expense of more immature precursor cells. The large effects presented here in the NOD/SCID recipients of human $CD34^+$ cells may suggest that these observations also apply here, which, however, needs to be explored further, while also the pharmacokinetics of human TPO in a mouse environment need to be assessed. Since a 10- to 30-fold higher dose of human TPO is fully capable of stimulating mouse platelet production (unpublished observation), advantage was taken of the affinity difference between mouse and human TPO for the mouse receptor to achieve selective outgrowth of human cells. This also implies that the 0.3 μg human TPO is sufficiently high to overcome the threshold of initial c-mpl mediated clearance.

The results are at variance with those obtained previously in lethally irradiated rhesus monkey recipients of limiting numbers of autologous bone marrow derived $CD34^+$ cells, where TPO was largely ineffective.[57, 58] In contrast, TPO was highly effective in 5 myelosuppressed monkeys after the midlethal dose of 5 Gy TBI[42], both models yielding an identical hematopoietic reconstitution rate in the placebo controls. So far, the possible reasons for this discrepancy (reviewed elsewhere[57]) have not been fully elucidated. The present study shows that it is not the transplant situation *per se* that makes cells unresponsive to exogenous TPO.

Although the frequencies of the various differentiation lineages were hardly influenced by TPO treatment, in absolute numbers a clear shift was noticeable in that megakaryocytic and erythroid cells were more prominently expanded in the TPO treated mice than in the controls, apparently at the relative expense of myeloid $CD33^+$ cells. This phenomenon is consistent with the known range of actions of TPO and with previous observations in myelosuppressed rhesus monkeys. Treatment of the latter with TPO alone demonstrated accelerated reconstitution along the platelet and red cell lineages, whereas the TPO potentiated expansion and terminal maturation of the neutrophil/monocyte lineage required additional stimulation with exogenous growth factors such as G-CSF or GM-CSF. Compared to the observed shift in favor of erythroid and megakaryocytic cells at the expense of other lineages, the TPO-induced expansion of immature $CD34^+CD38^+$ cells and $CD34^+$ cells is neutral and of the same order as the expansion of all human cells in

the NOD/SCID bone marrow. It remains to be explored as to whether TPO is capable to replace the accessory cells important for expansion of immature $CD34^+CD38^+$ cells described previously.[13]

The findings in this study clearly will make the NOD/SCID mouse a more efficient assay for human hematopoietic stem cell and provide a new basis for further studies on the effects of growth factors and other regulatory mechanisms involved in human stem cell regulation and differentiation. In addition, the NOD/SCID mouse transplanted with human UCB cells will provide a suitable assay for the development of novel genetically engineered, high-affinity c-mpl agonists and/or c-mpl ligand mimetic substances designed to circumvent the disadvantages of protein agonists.

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CHAPTER

VII

General Discussion

General discussion

Introduction

The hematopoietic system is derived from stem cells that have an extensive capacity to proliferate, differentiate and self-renew enabling repopulation of recipients after transplantation. The development of the hematopoietic system is tightly regulated by a combination of intrinsic factors and external stimuli such as hematopoietic growth factors (GFs) and contact with stroma in the microenvironment of the bone marrow. Disorders of the hematopoietic system such as leukemia, may result in dysregulation of hematopoiesis. Treatment of these diseases often involves chemotherapy or irradiation. Hematopoietic recovery after radiation exposure (therapeutic or accidental) or chemotherapy might be promoted by bone marrow transplantation (BMT), administration of GF or transplantation of *in vitro* stimulated stem cells. Understanding the cellular and molecular factors that regulate the development of normal stem cells and those that initiate proliferative diseases such as leukemia is, next to increasing the fundamental knowledge on hematopoiesis, essential for the design of therapeutic regimens, including gene therapy.

The conceptual framework of stem cell characterisation has changed in time. Forty years ago stem cells were quantitatively assayed using the spleen colony forming assay (CFU-S) and were defined as cells that are capable of homing to the spleen of irradiated mice 8-14 days after transplantation and form spleen colonies.[1] Since then, *in vivo* and *in vitro* assays have been developed to measure and characterise the small population of hematopoietic stem cells, all demonstrating the complexity and heterogeneity of this subset. Magli *et al.*[2] demonstrated that the CFU-S was more complicated than thought by showing that day 8 and day 12 spleen colonies originated from different cell types, which implied that the repopulating stem cell population is heterogeneous. In addition, heterogeneity of stem cells is observed in studies in which BM is serially passaged in reconstitute lethally irradiated mice.[3] Results indicate the presence of at least two phases of engraftment, which appear to be associated with progenitors at different stages of differentiation. Early

hematopoietic recovery is procured by an initial, unsustained engraftment phase produced by short-term repopulating cells, followed by a second sustained phase for which pluripotent stem cells are responsible. The presence of diverse subsets within the hematopoietic stem cell hierarchy is also expressed in the difference in radiosensitivity.[4, 5] Less primitive colony forming cells (CFU-C) and early cobblestone area forming cell (CAFC) are relatively radiation resistant, CFU-S day 7 and CAFC day 10-14 became more sensitive, whereas with increasing primitiveness of the hematopoietic stem cell subsets (CFU-S day 12 and marrow repopulating ability (MRA)) the radioresistance is greater.[5]

Apart from the difference in long and short term repopulating ability and in radiosensitivity, the distribution of phenotypic markers and growth factor receptors may also illustrate stem cell heterogeneity. The $CD34^+CD38^-$ immunophenotype defines a primitive subpopulation of human progenitor cells which is present in foetal liver, foetal and adult bone marrow (BM) and umbilical cord blood (UCB).[6-9] Although these cells all express CD34 without coexpression of CD38, at least 2 functionally distinct subpopulations exist within this population, i.e., those able to produce distinct colonies in semi-solid medium (CFU-C) within the standard long-term culture initiating cells (LTC-IC) assay period and a more primitive, rare subset that begins to proliferate in culture after 8 weeks.[9] $CD34^+$ cells expressing Thy-1[10] or c-kit (SCF-receptor)[11] in the absence of lineage markers, HLA-DR[12], transferrin receptors[13] and with a low uptake of rhodamine[123] are categorised as primitive hematopoietic stem cells.[14] Recently, a novel marker, vascular endothelial growth factor receptor-2 (VEGFR-2, also known as KDR) was found to be a positive, functional marker defining stem cells and distinguishing them from progenitors.[15] KDR is present on 0.1 to 0.5% of the $CD34^+$ cells that are positive for early progenitor and stem cell markers, such as $CD38^-$, Thy-1^+, and kit^{low}.

Growth factor receptors expressed on $CD34^+$ cells are receptors for early acting growth factors and include IL-3-R, IL-6-R, GM-CSF, c-kit (SCF-R) and c-mpl (TPO-R).[16-19] Receptors for late acting GF such as EPO, G-CSF and M-CSF are not expressed on immature cells, but exclusively on specific, committed subsets.

Novel techniques such as cell separation and selection methods and the (further) development of *in vitro* assays such as the LTC-IC assay[20, 21] and surrogate *in vivo* transplantation assays such as transplantation of purified human hematopoietic cells into immunodeficient mice[22, 23] or the use of non-human primates[24-29] have helped to identify a small subset of pluripotent stem cells that is exclusively capable of repopulating the bone marrow (BM) of a recipient and to proliferate and differentiate into all hematopoietic cells.

The use of human umbilical cord blood (UCB) as an additional stem cell source in hematopoietic stem cell transplantation has been a successful alternative for allogeneic BM transplantation. It has been shown in early studies that both term and preterm cord blood contains a significantly higher number of early and committed progenitor cells than adult peripheral blood.[30] Furthermore, transplantation with UCB cells results in a decreased incidence of graft versus host disease (GVHD) compared to BM.[31-33] Although it has been demonstrated that UCB contains sufficient numbers of hematopoietic progenitor cells to engraft larger size children and adults[34], others have suggested that to gain sufficient immature cells for transplanting large adults, *ex vivo* manipulation of cells might be necessary.[35] For grafts containing suboptimal numbers of stem cells such as the average cord blood sample, expansion may result in an earlier engraftment following transplantation and in a reduction of the transplant-related morbidity. However, the composition of GF receptors and the response pattern to GF on repopulating stem cells is as yet insufficiently known. Thus the presently available GFs may either not stimulate stem cells or stimulate them to differentiate rather than to self-renew. The immunophenotypic and functional characterisation and determination of the cell cycle and growth characteristics of these most primitive hematopoietic progenitors are essential steps toward the identification of hematopoietic stem cells which are important target cells for gene transfer. Since only a small population of stem cells is responsible for the long-life formation of all blood cell lineages, hereditary and acquired hematopoietic diseases may be treated with gene transfer to hematopoietic stem cells. Furthermore, as peripheral blood cells traverse the entire organism, transduced hematopoietic cells can act as a delivery system for the treatment of non-hematopoietic diseases such as enzyme deficiencies. To reach clinically relevant levels, the transduction of the target cells should be highly efficient. Transduction efficiencies of 10% to 20% can be sufficient to cure some genetic diseases[36], especially those in which expression levels are not of critically importance, such as enzyme-deficiencies. However, most diseases require transduction of long-term repopulating cells with higher levels.

The studies presented in this thesis focus on the quantitative use of severe combined immunodeficient (SCID) mice[37] and non obese diabetic (NOD)/SCID mice[38] as recipients of human normal progenitor cells ($CD34^+$ and $CD34^+CD38^-$) isolated from UCB and malignant ($CD34^+$) cells isolated from the peripheral blood of chronic myeloid leukemia (CML) patients to get insight into the characteristics of cells capable of multilineage *in vivo* repopulation (chapters 2 and 3). Second, by retroviral transduction of human stem/progenitor cells using the fluorescent

enhanced green fluorescent protein (EGFP) marker gene, human repopulating cells were marked. With this procedure, the quality of the transduced cells can be rapidly assessed and the cells can be selected after transduction and their fate and progeny tracked after transplantation (chapter 4). For quantitative analysis, stable and high levels of integration of the EGFP gene in the DNA of the target cell is required. Therefore, variables influencing the transduction efficiency were analysed and the transduction protocol optimised (chapter 5) to reach high-level transduction efficiencies of hematopoietic stem cells. To analyse stimulating or inhibiting factors, such as GFs or other agonists which possibly influence the repopulation of normal progenitor cells, the NOD/SCID mouse assay can be effectively used. In chapter 6 the effect of the GF thrombopoietin (TPO) on the engraftment of normal $CD34^+$ UCB cells in NOD/SCID mice is studied.

Requirement of accessory cells

The study described in chapter 2 of this thesis focuses on quantitative analysis of the maintenance and differentiation of distinct UCB subsets with repopulating potential after transplantation in the (NOD) / SCID mouse model. These studies have provided essential information that can be used to design and test conditions for *ex vivo* activation of immature hematopoietic cells, and of various experimental purposes such as the effect of various GFs on the self-renewal of immature hematopoietic cells as well as the development of efficient gene transfer protocols. Transplantation of 10^5 purified $CD34^+$ human UCB cells into sublethally irradiated, macrophage-depleted SCID mice or irradiated NOD/SCID mice resulted in high levels of multilineage chimerism, measured by flow cytometry. An equivalent number ($5x10^3$) of purified $CD34^+CD38^-$ cells also engrafted efficiently with chimerism levels similar to those observed in studies in which the NOD/SCID mice were cotransplanted with irradiated non-repopulating $CD34^-$ cells and/or GF supported NOD/SCID mice.[39] The human cell population in the SCID mouse BM consisted of prominent numbers of B-lymphoid cells (25% to 50% of the human population), but also high levels of monocytes, granulocytes and immature myelomonocytic cells, erythroblasts and immature $CD34^+$ cells were present at 5 weeks after transplantation. Purified $CD34^+CD38^+$ cells and $CD34^-$ UCB cells did not engraft, indicating that the $CD34^+CD38^-$ subset is exclusively able to reconstitute the BM of a recipient after transplantation. Studies analysing the kinetics of the engrafted cells showed that the most primitive human cell populations reached maximum levels at 5 weeks posttransplantation, after which

they declined.[40] At 10 weeks, most of the human cells present in the BM were mature (mostly $CD34^-CD19^+$) cells and at the end of the experiment at 16 weeks, all subsets of human cells had declined significantly.[40]

Transplantation of graded numbers of $CD34^+$ UCB cells into SCID mice showed a linear relation between the cell number transplanted and the percentage of $CD45^+$ cells produced. The percentage of $CD34^+$ cells within the human cell population also increased proportionally with cell dose, in contrast to the production of $CD34^-CD38^-$ cells that showed a steeper dependence on the number of $CD34^+$ cells transplanted. This, and the fact that no $CD34^+CD38^-$ cells and 10-fold lower numbers of $CD34^+$ cells were produced after transplantation of purified $CD34^+CD38^-$ UCB cells showed that $CD34^+CD38^-$ cells can only be maintained with support of accessory cells among the $CD34^+CD38^-$ population. This feature was directly demonstrated by cotransplantation of $CD34^+CD38^-$ cells with $CD34^+CD38^+$ cells, a cell subset that itself was not capable of engrafting the mouse BM, but restored the propagation of $CD34^-CD38^-$ cells in repopulated mice. The function of these $CD34^+CD38^+$ cells during the repopulation of long-term engrafting cells remains unclear. One possible function could be that the maturing cells prevent elimination of the small numbers of $CD34^+CD38^-$ cells by residual immune reactivity of the NOD/SCID mouse. Another, more likely explanation could involve growth factor production as has been shown for $CD34^+$ cells which can produce various GF such as IL-3 and G-CSF.[41] Also other stimuli may be assessed by the $CD34^+CD38^+$ accessory cells which are needed for self-renewal of the $CD34^+CD38^-$ subset and are not provided by the mouse microenvironment.

Transplantation of graded numbers $CD34^+$ G-CSF mobilised PB cells into NOD/SCID mice resulted in a dose-dependent increase in engraftment in BM and spleen.[42] Compared to $CD34^+$ UCB, higher cell numbers were needed to result in similar engraftment levels, probably caused by an intrinsic difference in SCID-repopulating cell (SRC) frequency or by a difference in the biological properties of $CD34^+$ cells derived from foetal or adult hematopoietic tissue. In contrast to UCB and mobilised PB cells, transplantation of human BM cells into NOD/SCID mice requires the addition of human growth factors to establish high-level human cell engraftment, suggesting that neonatal cells either respond differently to the murine microenvironment or provide their own cytokines in a paracrine fashion.[43, 44]

Stem cell frequency

Ideally, all stem cells that are present in a graft home to the BM of the recipient and proliferate and differentiate there to fully reconstitute the BM. In reality, syngeneic mouse studies showed that 18-20% of the injected progenitor cells home to the BM and 1-10% could be found in the spleen.[45-47] Because BM and spleen comprises 5 to 10% of the mouse body weight[48], and the graft is injected into the blood vein, in theory it is likely that 5 to 10% of a graft reaches the BM and the spleen. The other 90-95% of the graft is then captured in other organs and eliminated. However, homing to spleen and BM is 30% instead of 5 to 10% which indicates specific homing mechanisms for hematopoietic cells to go to these 2 hematopoietic organs.[45, 47, 49] The seeding of human progenitor cells in the BM and the spleen of NOD/SCID mice is less efficient (1-7% to the BM and 0.2-0.9% to the spleen)[50] which should be taken into account when calculating the frequency of human cells with NOD/SCID repopulating activity. The frequency of $CD34^+CD38^-$ cells that can maintain or expand after infusion into NOD/SCID mice was estimated using Poisson statistics and was found to be 1 in 70,000 $CD34^+$ cells. This was in the same order of magnitude as the frequency that was found in other studies, which was 1 in 3,500 $CD34^+CD38^-$ cells.[50] Taking the low seeding efficiency into account, the frequency of human SCID-repopulating cells is certainly underestimated. In addition, other factors such as the efficacy of the stimuli of the mouse microenvironment and the role of accessory cells might be involved in the efficiency of homing and proliferation of repopulating cells.

Apart from the quality of the donor cells, recipient properties and residual immunity influence the efficiency of engraftment. Early studies in which human hematopoietic cells were transplanted into immunodeficient animals used the C.B.-17-*scid/scid* (SCID) mouse which was highly radiosensitive, had a short life span due to the formation of lymphoma and showed low levels of engraftment partly due to residual immune reactivity.[37] When NOD/LtSz-scid/scid (NOD/SCID) mice were used, 5 to 10-fold higher engraftment levels were obtained.[38] However, NOD/SCID mice are very sensitive to radiation, which limits effective conditioning, have a short life span and become leaky with age.[51-53] Although the seeding efficiency is low, conditions prevailing within the microenvironment of the (NOD/SCID) mouse BM are able to sustain many aspects of human hematopoiesis.[23, 54-58] Novel mouse strains, such as NOD/LtSz-scid/scid $\beta 2m^{null}$ ($\beta 2m^{null}$ NOD/SCID) mice engraft with 10-fold higher levels but have a shorter life span compared to NOD/SCID mice.[59, 60] The efficiency of homing and proliferation of transplanted cells is much higher in this mouse strain than any

other immunodeficient mouse available. Engraftment of human cells in these mice requires 10-times less cell numbers, which obviously is useful for studies on rare cell populations as hematopoietic stem cells. The most recent immunodeficient hybrid developed is the NOD/LtSz-Rag1^{null} mouse, which has a normal sensitivity to radiation and does not become leaky with age. Transplantation of human hematopoietic cells in these mice results in similar levels of engraftment as the NOD/SCID mice.[61] This strain would seem to be particularly useful to study engraftment during longer time intervals.

Leukemic and normal engraftment of CML cells in immunodeficient mice

A useful model for chronic phase CML should support and maintain the outgrowth of both normal and malignant progenitors, as CML is clinically characterised by a mosaicism of Philadelphia-chromosome positive (Ph^+) and Ph^- progenitors. Only models in which stable mosaicism can be reached that reflects the relative frequencies in the patients' blood and BM may allow the evaluation of treatment strategies aimed at selective outgrowth of normal cells. Early attempts to establish an *in vivo* transplantation model of chronic phase CML using SCID mice as recipients were not successful [49, 62] unless large numbers of cells were injected.[63] Evaluation of the engraftment of normal and leukemic cells after transplantation of 10^7 unfractionated or $2x10^5$ purified $CD34^+$ CML cells in NOD/SCID mice (Chapter 3) was performed using fluorescent *in situ* hybridisation (FISH) analysis on sorted subsets of patient samples and harvested BM of engrafted mice. Both Ph^- as Ph^+ cells proliferate and differentiate in the mouse BM with the same distribution as the cell subsets in the original graft which makes this assay useful to study the effect of (*ex vivo*) manipulations on the survival and proliferation capability of normal vs. malignant cells.

Cells from CML patients in blast phase engrafted SCID mice at much higher levels than cells from chronic phase CML, resembling that of leukemic blasts from AML patients.[64-66] As both cells from chronic phase CML and blast crisis CML engraft NOD/SCID mice with different characteristics, this assay could also be exploited in examining the processes involved in acceleration from chronic phase CML to blast crisis CML and to test treatment procedures that can prevent this development.

Ex vivo expansion

The integration of transgenes by retrovirus mediated gene transfer requires cell cycling.[67] To accomplish this, target cells are cultured *in vitro* for the duration of the transduction procedure. In addition, expansion of hematopoietic pluripotent progenitor cells in *ex vivo* culture may have important implications in transplantation, stem cell marking and gene therapy, as it may allow the use of stem cell sources which are available in limited quantities, such as UCB. It is of importance to choose the culture conditions and the use of GF or GF combinations in such a way that stem cells be induced to proliferate, but not differentiate, as the stem cells most likely loose their repopulating capability.

In initial gene transfer experiments, hematopoietic cells were prestimulated and transduced in serum-free culture medium in the presence of IL-3, IL-6 and SCF with or without stromal support.[68, 69] Moderate transduction efficiencies were reached but the target cells differentiated into the myeloid lineage, resulting in loss of the repopulating ability of the stem cells. The availability of newly cloned GF such as Flt3-L[70] and TPO[71-74] in combination with SCF resulted in higher transduction efficiencies and maintenance of cells with an immature phenotype.

Although extensive amplification of primitive progenitor cells assayed as colony-forming cells (CFC) or cobblestone area-forming cells (CAFC) / long-term culture initiating cells (LTC-IC) has been described[75-77], the repopulating activity of the expanded cells can only be assayed in transplantation models. During *ex vivo* culture, phenotypes of hematopoietic cells may alter, such as the downregulation of CD38 on maturing $CD34^+CD38^+$ cells, resulting in increasing numbers of $CD34^+CD38^-$ cells which have the same phenotype as the rare $CD34^+CD38^-$ population that is exclusively capable of repopulating the BM of a recipient after transplantation. Injection of these cultured $CD34^+CD38^-$ cells into NOD/SCID mice shows a clear dissociation between the $CD34^+CD38^-$ cell surface phenotype and the NOD/SCID repopulating ability.[78]

Retrovirus mediated gene transfer

Stable integration of high levels of the EGFP marker gene into the DNA of immature hematopoietic cells allows tracking of the transduced cells and their progeny after transplantation in a recipient. This facilitates the study of the mechanisms that are involved in *in vivo* repopulation of the BM but also the effects of different agents such as GFs or (inhibitory) cytokines on the process of BM reconstitution. Studying the optimal conditions by which genes are integrated into

the DNA of the target cells with high efficiency is essential for the eventual goal of gene therapy. Hematopoietic stem cells are obvious targets to use in the treatment of hereditary hematological and non-hematological diseases. Although the quality of the virus particles produced by retroviral producer cell lines is not always stable, the integration of the vectors into the target cell DNA is. Other gene transfer systems such as adenovirus and liposome-based methods do not integrate genes stably at high frequencies. Furthermore, adenoviral vectors tend to be toxic at high concentrations and can be highly immunogenic.[79, 80] Although lentivirus mediated gene transfer can transduce non-dividing cells with high efficiency, the possibility of mutations or recombinations which may lead to replication-competent virus with pathogenic potential complicates, at this time point, their choice for clinical applications.[81, 82] Novel lentivirus vectors are being developed with a reduced likelihood of recombination and pathogenesis. These vectors contain, for instance, genetic elements from three different viruses (simian immunodeficiency virus; SIV, human immunodeficiency virus-1; HIV-1, and vesicular stomatitis virus; VSV) which have low nucleotide homology reducing the possible recombination between vector elements.[83] The use of self-inactivating (SIV-based) vectors that contain minimal virus sequences also reduces the risk both of emergence of replication-competent recombinants and of mobilisation of the vector in target cells.[84] Once these safety concerns are overcome, the potential for modifications of the human genome for therapeutic purposes using lentiviral vectors is promising.[81] However, the present way of production of the lentiviruses makes it difficult to ensure quality control under GMP conditions, which makes these viruses, apart from the risks, less likely to be developed rapidly for clinical application.

Gene marking in the studies described in this thesis (chapter 4) has been mediated by retroviral gene transfer, using either an amphotropic virus producing cell line based on the Moloney murine leukemia virus (MoMLV) or a gibbon ape leukemia virus (GaLV) pseudotyped virus producer. Both cell lines were transfected with an EGFP containing vector, the amphotropic cell line with MFG-EGFP (Am12/MFG-EGFP)[85, 86], and the pseudotyped cell line with the SF-EGFP vector (PG13/SF-EGFP).[87] Consistent with earlier studies in which amphotropic and pseudotyped virus producing cell lines were compared[88-91] the pseudotyped PG13/SF-EGFP packaging cell/vector combination transduced purified $CD34^+$ and $CD34^+CD38^-$ UCB cells with a higher efficiency than the amphotropic Am12/MFG-EGFP possibly caused by the low or absent expression of the amphotropic envelope-receptor (GLVR-2 or Pit-2) on the target cells.[92, 93] Transplantation of the EGFP-

marked $CD34^+$ and $CD34^+CD38^-$ UCB cells in NOD/SCID mice revealed that cells, transduced with the Am12/MFG-EGFP combination, engrafted either with low levels of efficiency or did not engraft at all. Injection of $CD34^+$ and $CD34^+CD38^-$ cells transduced with the pseudotyped virus producing cell line into NOD/SCID mice resulted, 35 days after transplantation, in higher levels of transduced human cells in the mouse BM. Periodic analysis of BM samples from transplanted NOD/SCID mice revealed a sustained engraftment up to 90-120 days post transplantation of human hematopoietic cells expressing the transgene.[94]

Although hematopoietic progenitor/stem cells were efficiently transduced, the transduction of NOD/SCID repopulating cells was low in efficiency and the variability between experiments was large. The retroviral transduction procedure appeared to be suboptimal and had to be optimised and the quality of the virus containing supernatant had to be stabilised (chapter 5). The gene transfer efficiency into immature $CD34^+$ hematopoietic cells has been significantly improved by the use of the human fibronectin fragment CH-296[95], the use of GALV-pseudotyped vectors rather than amphotropic virus vectors, novel stem cell purification techniques and the use of novel growth factors (GF) such as thrombopoietin (TPO)[71-74] and fms-like tyrosine kinase ligand (Flt3-L).[70] In addition, the development of fluorescent markers, such as EGFP significantly speeded up the time required to analyse variables in the transduction procedure.[96]

Despite the many difficulties in therapeutic gene transfer, Cavazzana-Calvo *et al* have successfully treated two infants (for at least up to 10 months) suffering from severe combined imuunodeficiency (SCID) using retroviral mediated gene transfer to $CD34^+$ cells.[97] The superior results of this study, as compared to previous gene therapy trails that treated ADA-deficient SCID patients[98-101], are partly due to the optimisation of the transduction conditions during the last decade. The presence of Flt3-L in the growth factor cocktail during the transduction procedure, the use of human fibronectin-coated culture dishes and the choice of the retroviral vector has resulted in higher levels of gene transduction sufficient to successfully treat two SCID-children. In addition, the choice of X-linked SCID as a target for gene therapy has added to the success because this model did not need a strict control of expression of the gene. Second and more important, in this disease the transduced cells acquire a significant selective advantage over non-transduced cells as the product of the transduced gene delivers a proliferating signal to cells that express the gene. A third reason for the successful treatment is that transduced T cells (differentiated from purified $CD34^+$ cells) are long-lived and may be present for several years.[102]

Despite this singular therapeutic success in SCID-patients, further optimisation of the transduction conditions to reach high levels of transduction and stable integration is necessary for long-term multilineage integration of the foreign gene. Viral titers of retrovirus vectors (Mo-MLV-based) are relatively low (10^5 to 10^6/ml), and cannot infect some human cells efficiently.[103, 104] Attempts to concentrate retroviral vectors by physical methods such as filtration or ultracentrifugation have generally resulted in massive loss of infectious virus, presumably due to instability of the retroviral envelope protein, which is essential for the integration of virions with the cell surface receptor and for their entry into the cells.[105] Pseudotyping the Mo-MLV-derived retroviral vectors with the G-protein of the vesicular stomatitis virus (VSV-G, member of the rhabdovirus family) results in a vector that can be concentrated to high titers (10^8-10^9/ml) by ultracentrifugation.[105] In addition, pseudotyping the Mo-MLV vector with gibbon ape leukemia virus (GALV) increases the transduction of purified hematopoietic[88, 89, 106] probably due to the different distribution of the viral receptors on the cell surface.[89, 90, 106] Apart from high titer production, lentivirus vectors have the advantage of being able to infect non-dividing cells which makes these vectors, in spite of the safety concerns, superior for transducing quiescent hematopoietic stem cells. Studies using the lentivirus based gene transfer system have lead to stable transduction of large fragments of the human β-globin gene to mouse BM cells. In addition, transplantation of syngeneic BM transduced with the human β-globin gene into mice, resulted in the production of clinically relevant levels of human β-globin in the peripheral blood of the transplanted mice.[107]

In addition to the use of fibronectin coated dishes, the most promising GF combination at this time point (SCF, Flt3-L and TPO), and pseudotyped retrovirus vectors, the ratio virus titer – target cell concentration is of critical importance to get sufficient high levels of transduction (>90%). This ratio is variable between different target cell populations. CD34-selected UCB cells transduce highly efficient using a lower titer – target cell ration as compared to $CD34^+$ rhesus BM cells supposedly because of receptor differences between the species. Transplantation of $CD34^+$ cells of which >90% expressed EGFP into sublethally irradiated mice resulted in high levels (40-80%) $EGFP^+$ human cells in all lineages assessed.

Growth factor stimulated in vivo expansion of human cells transplanted in NOD/SCID mice

Some disadvantages of human cells transplanted into NOD/SCID mice are the limited mature end cell production and the low seeding efficiency of the human cells in the mouse environment[50], necessitating the use of large quantities of human cells, which could be partly attributed to the described accessory $CD34^+CD38^+$ cells.[108] Supplementation of human GFs such as IL-3 and GM-CSF[23], SCF and/or PIXY321 (a fusion of IL-3 and GM-SCF) and/or erythropoietin (EPO)[40, 54] or Flt3-L and/or IL-7[109] has been used to augment the outgrowth of human cells in the mouse environment, or, more simply, addition of large quantities of irradiated human cells[40], of which the role is not fully clear. Recent novel crosses of mutants, such as the NOD/Rag1^{null} or the NOD/SCID/β2microglobulin^{null} mouse[60, 61], require lower numbers of human cells, suggesting that residual immune functions in the NOD/SCID mouse contribute to the relative inefficiency of the NOD/SCID mouse. Also, species barriers may exist for hematopoietic growth factors, murine growth factors not necessarily being optimal or suited to stimulate the human cells. Since thrombopoietin (TPO) is an essential physiologic factor for stem cell maintenance and proliferation[110], apart from its functions in platelet production and activation[72], in the study described in chapter 6 we sought to explore the option of using human TPO to stimulate outgrowth of human cells in NOD/SCID murine recipients. A single dose of human TPO was injected into sublethally irradiated NOD/SCID mice immediately after transplantation of purified $CD34^+$ UCB cells. TPO-treatment resulted in a 3 to 4-fold increase in the frequency and number of human $CD45^+$ cells in the mouse bone marrow, assessed 5 weeks after transplantation, while mouse type cells were not influenced. The lineage distributions among the human cells were essentially similar irrespective of TPO treatment, however, with a slightly lower frequency of myeloid $CD33^+$ cells in the TPO treated mice and a prominent increase in the total number of erythroid and megakaryocytic cells. An impressive increase was observed in $CD71^+GpA^-$ cells, reflecting the proliferative stimulus provided by TPO administration. Also the frequency of immature $CD34^+$, $CD34^+CD38^-$ cells and of human GM-CFU and BFU-E in TPO-treated mice was similar to that of untreated mice, the increase in their total number being proportional to the increase of human cells. The results demonstrate that human TPO is a major limiting factor for selective multilineage outgrowth of human UCB cells in NOD/SCID mice and can be conveniently supplemented by single dose treatment immediately after transplantation. Also, the

study emphasises the importance of TPO for immature hematopoietic cell maintenance and proliferation, in accordance with studies in myelosuppressed animals, which demonstrated that TPO not only accelerates platelet and red cell reconstitution, but also that of immature $CD34^-$ cells, thereby potentiating the action of growth factors such as G- and GM-CSF and alleviating neutropenia.[111-113] The findings make the NOD/SCID mouse a more efficient assay for human hematopoietic stem cells and provide a new basis for further studies on human stem cell regulation using immunodeficient mouse models. In particular, synergism and/or antagonism of TPO and other growth factors on human stem cells can now be studied in the transplant setting, while also analysis of the described accessory cells[108] can be approached under more optimal growth factor stimulation. In addition, immunodeficient mice transplanted with human UCB cells yield a suitable assay for the development of novel human c-mpl agonists, in particular non-protein substances[114], that have an easier pharmacology, can potentially be orally given and avoid the problem of antibody formation that has jeopardised the pharmaceutical development of TPO.[115]

Final remarks

Much of our understanding of the organisation of the cells that comprise the hematopoietic system and the cellular and molecular mechanisms that regulate their development is derived from mouse models. Studies of the human hematopoietic system have long been hampered by the absence of *in vivo* assays that measure their repopulation capacity. The development of methods to transplant normal and leukemic human hematopoietic cells into immune-deficient (NOD)/SCID mice provides the foundation for human stem cell assays and are essential to further understand and define the characteristics of repopulating human stem cells. Hence, the NOD/SCID mouse assay is well known in studies of proliferation and differentiation of selected primitive hematopoietic cells and the influence of, for instance, *in vitro* culture conditions or GF treatment on their growth. However, the limitation of being able to study short-term repopulation only and the need for high cell numbers to be transplanted makes this assay less attractive. Newly developed mouse assays using the NOD/SCID β2 microglobulin knockout (β2MKO) mice or the NOD/LtSz-$Rag1^{null}$ mouse are superior to NOD/SCID mice in engraftment efficiency and life span, respectively and will be used in the future for human cell transplantation studies.

Umbilical cord blood has, since the first transplant in 1989[116], been used to successfully transplant many people of which most were children. Although UCB may be an enriched source of hematopoietic stem cells, the quantity of a UCB sample is usually too low to transplant a large adult patient with.[34, 117] Expansion of these cells is therefore essential and subject of many studies. Clinical trials in which the use of expanded/cultured UCB cells are studied are planned. However, as the use of certain GF, such as SCF, causes, next to a large expansion of the target cells, unacceptable adverse effects in patients, the step from bench to bed is not easy. To overcome this problem, there are plans to obtain BM stromal cells from the patient before the UCB transplant, expand the UCB cells on the BM stroma and harvest these together for the transplant.[118] Due to the high proliferative potential of hematopoietic cells, stable gene transfer is a key requirement for stem cell characterisation as well as for clinically efficacious stem/progenitor cell-based gene therapy. Retroviral vectors are most commonly used for gene transduction of hematopoietic cells, but recently, also lentiviral vectors have been used to transduce progenitor/stem cells with high efficiency and are even found to be superior to retroviral vectors.[119-121]

The studies described in this thesis reveal that efficient retroviral mediated gene transfer involves complex interactions between target cells and virus particles. Therefor, stabilisation of the virus production and quality of infectious, replication-incompetent virus particles is one of the main issues to work on. Transplantation of transduced cells into NOD/SCID mice resulted in multilineage engraftment of cells expressing the transduced EGFP gene suggesting efficient transduction of pluripotent progenitor cells. However, as the NOD/SCID mouse assay ends at 5 weeks after transplantation (which is too early to conclude about the transduction of long-term repopulating cells), it is of importance to measure repopulating activity of transduced cells with long-term repopulating ability in a preclinical setting, for instance in rhesus monkeys. Although progress made in the optimisation of the transduction protocol is considerable and results in high levels of transduced cells, transplantation of these cells into rhesus monkeys did not result in long-term engraftment of $EGFP^+$ cells in the BM and PB of the animals. This could partly be due to the insufficient stimulation with GFs during the transduction procedure and/or *in vivo* to mobilise stem cells to the peripheral blood, by which stem cells are not properly stimulated or differentiate and loose their repopulating ability. Combining the right GFs and searching for novel GFs that influence stem cell activity without causing differentiation remains an issue to be studied.

Furthermore, virus titers could be insufficiently high to transduce enough stem cells. Retrovirus based transductions are most commonly used in transduction protocols for their stable integration into the cell's DNA, but show several major shortcomings, of which the most critical is the relatively low virus titer that can be achieved and the need for cell division for stable integration in the target cell DNA. Lentiviral based vectors produce very high titers of virus particles and only need low reverse transcriptase activity for infection. During the last few years, the possible hazards of using HIV-related viruses have been decreased by the reduction of the possibility of production of replication-competent viruses, which makes lentiviral vectors more attractive for the use in hematopoietic stem cell research.

Suggestions for the near future include the development of systems for regulated expression that would allow on/off switching of the integrated gene when needed and the development of vectors which utilise specific, safe integration sites to avoid the possible hazard of malignancy of the transduced cells. Also targeting specific cell subsets for instance $CD34^+$, or leukemic cells could help the further development of safe efficient tools for gene therapy.

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Summary

The stem cell subset is a rare population of cells mostly present in the BM and is able to repopulate the bone marrow (BM) of patients who have undergone BM depletion, for instance due to total body irradiation and/or chemotherapy. All blood cell types descent from these so-called totipotent stem cells which have unique properties that allow useful physical and biochemical characterisation and quantification. Many enrichment procedures such as density gradient centrifugation and selection methods such as immunomagnetic subset selection or fluorescent activated cell sorting (FACS) have been developed to isolate primitive hematopoietic subsets to study its features using *in vitro* and *in vivo* assays. *In vitro* experiments are used to detect hematopoietic precursor cells in different stages of development. However, the repopulating activity of (isolated) precursor/stem cells can only be studied in transplantation experiments.
Hematopoietic stem cells are among the main targets for gene therapy as these cells can proliferate and differentiate into all blood cells after repopulation of the BM. This is essential for the treatment of hereditary or acquired diseases of the hematolymphoid system. Furthermore, gene transfer into stem cells makes it possible to mark cells with specific genes to be able to select the transduced cells and/or track their progeny after transplantation into a recipient, thereby gaining insight into the biology of stem cells. Although several gene delivery systems are known, up till now, retrovirus mediated gene transfer is mostly used for stable integration of genes into hematopoietic cells.

The work described in this thesis focuses on the self-renewal and differentiation characteristics of isolated human progenitor/stem cells of normal origin (isolated from human umbilical cord blood) and malignant cells (isolated from patients with chronic myeloid leukemia, CML) by performing transplantation assays into immunodeficient mice (non-obese diabetic / severe combined immunodeficient; NOD/SCID mice). Using retrovirus mediated gene transfer, repopulating cells were marked by integration of a gene coding for the green fluorescent protein. Five

weeks after transplantation into NOD/SCID mice (transduced) human cells can be traced and analysed in the BM of the mice by flow cytometry.

The study reported in chapter 2 focuses on the *in vivo* expansion and multilineage outgrowth of immature cell subsets from UCB after transplantation into macrophage depleted SCID mice. As measured by immunophenotyping, transplantation of equivalent numbers of unfractionated cells, purified immature $CD34^+$ and $CD34^+CD38^-$ cells resulted in similar levels of human $CD45^+$ cells in the SCID mouse BM 5 weeks after infusion of the cells. However, the number of immature $CD34^+CD38^-$ cells produced *in vivo* was not proportionally to $CD34^+$ graft size, but showed a second-order relation. In addition, mice engrafted with purified $CD34^+CD38^-$ cells produced 10-fold fewer $CD34^+$ cells without detectable $CD34^+CD38^-$ cells. Addition of $CD34^+CD38^+$ cells to a $CD34^+CD38^-$ graft restored the maintenance and expansion of the immature subset. These results indicated that SCID repopulating $CD34^+CD38^-$ cells require $CD34^+CD38^+$ accessory cells for survival and expansion of immature cells, but not for the production of mature multilineage progeny in the SCID mouse BM.

As the development of mouse assays went on, a newly developed, more immunodeficient mouse strain, NOD/SCID mice became available for transplantation experiments. Compared to irradiated, macrophage-depleted SCID mice, the engraftment of cells from chronic phase CML patients, but not of UCB cells, in irradiated NOD/SCID mice was 10-fold higher. Chapter 3 discusses the ability of malignant and normal progenitors in the peripheral blood and the BM of CML patients in chronic phase to proliferate and produce mature progeny after transplantation into NOD/SCID mice. As demonstrated by fluorescent *in situ* hybridisation (FISH) analysis, purified human myeloid, B-lymphoid, erythroid and $CD34^+$ cells that were isolated from chimeric mouse BM contained Philadelphia-chromosome (Ph)-positive cells (characteristic for CML) and Ph^- cells in similar frequencies as primary cells from the CML patients. This demonstrates that the production of normal as well as malignant cells of multiple lineages were supported in the mouse assay with similar efficiency which is essential for a reliable transplantation assay. The NOD/SCID mouse assay for CML can facilitate the examination of differential effects of growth factors, inhibitory cytokines and cytotoxic drugs on the survival of normal and malignant stem cells. Furthermore, as cells isolated from CML blast crisis patients grew with high efficiency in the NOD/SCID mouse BM, this assay can also be useful to study the progression of chronic phase CML towards blast crisis.

Hematopoietic stem cells are targets for gene transfer because of their ability to permanently reconstitute the hematopoietic system after transplantation. Gene marking of cells with repopulating ability using genes that code for a fluorescent protein such as the green fluorescent protein (GFP) gene, facilitates easy analysis of transduced cells and their progeny after transplantation in real time without additional steps. In chapter 4 the transduction efficiency of the recombinant variant of the Moloney leukemia virus (MoMLV), (Am12/MFG-EGFP) was compared to the gibbon ape leukemia virus (GALV) pseudotyped PG13/SF-EGFP combination. Purified $CD34^+$ and $CD34^+CD38^-$ UCB cells were efficient transduced using either cell line after a 2-day growth factor prestimulation, followed by a 2-day fibronectin fragment (CH-296)-supported transduction. Transduction efficiencies using the PG13/SF-EGFP were consistently 2-fold higher as compared to the Am12/MFG-EGFP combination. Transplantation of the EGFP transduced $CD34^+$ or $CD34^+CD38^-$ UCB cells into NOD/SCID mice resulted in EGFP levels of 2% and 23% of the engrafted cells, transduced with the Am12/MFG-EGFP and PG13/SF-EGFP, respectively.

Although EGFP-transduced cells repopulated NOD/SCID mice BM, the transduction protocol used needed to be optimised to gain stable high transduction levels with clinical relevance in hematopoietic stem cells. In chapter 5, the steep correlation between transduction efficiency and cell concentration (RhBM and UCB cells) using the PG13/SF-EGFP packaging cell/vector combination is described. Subcloning of this virus producer resulted in 2 high titer clones with transduction efficiencies in hematopoietic progenitor cells up to 90%. In addition, cryopreservation of the target cells resulted in a 2-fold higher efficiency of transduction. NOD/SCID mouse transplantation of $CD34^+$ UCB cells transduced with the fibronectin-based supernatant infection method using the high level clone at the optimal cell concentration using GF combinations including SCF, TPO and Flt3-L, resulted in high levels of 40% to 80% $EGFP^+$ human cells in all hematopoietic lineages assessed.

The use of GFs to stimulate immature cells into cell cycle and/or to expand cell samples that are too small to use for transplantation has been extensively studied by several groups. Several GF combinations are known to stimulate the differentiation of stem cells (combinations including IL-3), maintenance and proliferation of immature cells, however, is much more difficult to achieve. Newly cloned GFs such as TPO and Flt3-L are known, at least *in vitro*, to stimulate the proliferation of immature cells in order to expand the cells with an immature phenotype. *In vivo* administration of GFs such as G-CSF, is usually done to

mobilise hematopoietic stem cells into the peripheral blood to use as graft. Expansion of hematopoietic stem cells *in vivo* following transplantation of limited numbers of BM cells or of highly purified stem cells might be efficienty accomplished by administration of GFs directly after transplantation. Injection of one single dose of rHuTPO after transplantation of purified $CD34^+$ UCB cells into irradiated NOD/SCID mice resulted in an accelerated engraftment and higher percentages and numbers of human cells in the mouse BM. (chapter 6)

Samenvatting (summary in Dutch)

De hematopoietische stamcelpopulatie is een zeldzame populatie cellen die voornamelijk aanwezig is in het beenmerg (BM) waar ze (in volwassen zoogdieren) gedurende het hele leven voortdurend verantwoordelijk is voor de vorming van alle typen gedifferentieerde bloedcellen. Dit proces wordt hematopoiese genoemd. Naast differentiatie zijn stamcellen in staat dochtercellen te produceren die alle eigenschappen van de stamcellen behouden (self-renewal) wat de continuïteit van de hematopoiese waarborgt. Stamcellen kunnen het BM van ontvangers repopuleren na BM depletie ten gevolge van bestraling en/of chemotherapie. Alle celtypen die aanwezig zijn in het bloed stammen af van deze zogenaamde totipotente stamcellen die, door hun unieke eigenschappen, fysisch en biochemisch te karakteriseren en te kwantificeren zijn. Om de mechanismen van stamcelproliferatie en -differentiatie naar mature bloedcellen te kunnen bestuderen, is het noodzakelijk deze immature cellen te isoleren. Hiervoor zijn een aantal verrijkingsprocedures ontwikkeld zoals dichtheidsgradiënt centrifugatie en selectiemethoden waaronder immunomagnetische selectie of flow cytometrie (FACS). Met *in vitro* experimenten kan de aanwezigheid van hematopoietische voorlopercellen in diverse stadia van ontwikkeling worden aangetoond. Echter, alleen door het uitvoeren van transplantatie experimenten wordt inzicht verkregen in de repopulerende activiteit van de (geïsoleerde) voorloper/stamcellen.
Omdat hematopoietische stamcellen kunnen uitrijpen in alle celtypen aanwezig in het bloed, zijn deze cellen uiterst geschikt voor het transduceren van genen met als doel onder meer gentherapie voor de behandeling van erfelijke of verkregen ziekten. Daarnaast kan gentransfer een middel zijn waarmee cellen gemarkeerd kunnen worden met een specifiek marker gen, bijvoorbeeld een fluorescerend gen, die vervolgens na transplantatie in een ontvanger gevolgd kunnen worden. Hoewel een aantal systemen bekend is waarmee genen kunnen worden geïntroduceerd in een cel, wordt transductie middels retrovirussen het meest gebruikt. Retrovirale gentransfer geeft de mogelijkheid van stabiele integratie van genen in hematopoietische cellen.

In het hier beschreven onderzoek zijn experimenten uitgevoerd met het doel meer inzicht te krijgen in de karakteristieken van hematopoietische stam- en voorlopercellen. Hiervoor zijn normale en maligne $CD34^+$ voorlopercellen geïsoleerd uit humaan navelstrengbloed en uit beenmerg van patiënten met chronische myeloide leukemie (CML) en vervolgens getransplanteerd in immuundeficiente muizen (non-obese diabetic / severe combined immunodeficient, NOD/SCID muizen). Door de afwezigheid van specifieke afweer cellen (T en B-lymfocyten) in de muis wordt het transplantaat met menselijke cellen niet afgestoten maar kan het prolifereren en differentieren in alle bloedceltypen inclusief de immature populatie in het beenmerg. Met retrovirale gentransductie zijn cellen gemarkeerd met een gen dat codeert voor het groen fluorescerende eiwit (EGFP). Na transplantatie van getransduceerde cellen in NOD/SCID muizen is, met behulp van flow cytometrie of fluorescentie microscopie op eenvoudige wijze, de uitgroei van menselijke, $EGFP^+$ cellen in het muizenbeenmerg te analyseren.
De studie die besproken wordt in hoofdstuk 2 belicht de *in vivo* expansie en de multilineage uitgroei van immature sub-populaties die geïsoleerd zijn uit navelstrengbloed na transplantatie in macrofaag-gedepleteerde SCID muizen. Cellen die afkomstig zijn van het menselijk navelstrengbloed kunnen worden onderscheiden van muizencellen door het beenmerg te isoleren uit de muis en de cellen te kleuren met een CD45 antilichaam gekoppeld aan een fluorchroom. CD45 is een specifiek antigeen dat aanwezig is op alle menselijke leukocyten, maar niet op muiscellen. Transplantatie van equivalente aantallen ongefractioneerde cellen, $CD34^-$ of $CD34^+CD38^-$ cellen resulteerde in identieke percentages humane cellen 5 weken na transplantatie. Echter, in tegenstelling tot transplantatie van $CD34^+$ cellen, nam het aantal immature $CD34^+CD38^-$ cellen dat geproduceerd werd *in vivo* niet proportioneel toe met de grootte van het $CD34^+CD38^-$ transplantaat, maar verliep volgens een tweede-orde relatie. Transplantatie van geïsoleerde $CD34^+CD38^-$ cellen resulteert in 10-voudig lagere aantallen $CD34^+$ cellen, waaronder geen $CD34^+CD38^-$ cellen te detecteren waren in het muizenbeenmerg. Het toevoegen van $CD34^+CD38^+$ cellen aan het $CD34^+CD38^-$- transplantaat herstelde het behoud en de expansie van de immature sub-populatie wat impliceert dat SCID repopulerende $CD34^+CD38^-$ cellen $CD34^+CD38^+$ accessoire cellen nodig hebben voor overleving en expansie van deze immature populatie.
Met de ontwikkeling van nieuwe muisstammen kwam de NOD/SCID muis tot onze beschikking. Deze muisstam heeft, naast de specifieke afweer ook een deficiënte a-specifieke afweer en is daarom nog beter toepasbaar voor stamcel onderzoek. Hoofdstuk 3 beschrijft het gebruik van NOD/SCID muizen in het onderzoek naar

de uitgroei van normale en kwaadaardige voorlopercellen afkomstig van patiënten met chronische myeloide leukemie (CML). Met fluorescente *in situ* hybridisatie (FISH) analyse is aangetoond dat uit chimeer muizenbeenmerg gezuiverde myeloide, B-lymfoide, erythroide en $CD34^+$ cellen het voor CML karakteristieke Philadelphia (Ph) chromosoom bezitten in dezelfde frequenties als de primaire cellen van de CML patiënten. Dit geeft aan dat de productie van normale als ook maligne cellen voorkomt met dezelfde efficiëntie in het NOD/SCID muis beenmerg. Met deze proefopzet voor CML is het mogelijk de effecten van verschillende groeifactoren, remmende cytokines en cytotoxische stoffen op de overleving en uitgroei van normale en maligne cellen te onderzoeken. Omdat cellen die geïsoleerd zijn uit patiënten met CML blasten crisis met hoge efficiëntie uitgroeien in het NOD/SCID muis beenmerg, kan deze test ook gebruikt worden om de progressie van chronische fase CML naar blasten crisis te bestuderen.
Hematopoietische cellen zijn evidente doelcellen voor gentransfer dankzij de mogelijkheid na transplantatie het hematopoietisch systeem volledig te repopuleren. Het markeren van cellen met repopulerende activiteit met genen die coderen voor bijvoorbeeld het groen fluorescente eiwit (GFP) gen, maakt eenvoudige, snelle analyse van de getransduceerde cellen en hun dochtercellen na transplantatie mogelijk. In hoofdstuk 4 wordt de efficiëntie waarmee gezuiverde sub-populaties navelsrengbloed cellen getransduceerd worden met een 'verbeterd' (enhanced) GFP (EGFP) gen gemeten. Daarbij is gebruik gemaakt van twee virus producerende cellijnen met daarin twee verschillende vectoren die het EGFP gen bevatten, Am12/MFG-EGFP en PG13/SF-EGFP, respectievelijk. De Am12 cellijn is een amfotrope cellijn die aan de Pit-2 receptor ofwel gibbon ape leukemia virus-receptor-2 (GLVR-2) kan binden. De PG13 cellijn is gepseudotypeerd voor het gibbon ape leukemia virus (GALV) en bindt aan een andere receptor, GLVR-1 ofwel Pit-1. Gezuiverde $CD34^+$ en $CD34^+CD38^-$ navelstrengbloed populaties worden efficiënt getransduceerd met beide cellijnen na een twee dagen durende groeifactor stimulatie met TPO, SCF en Flt3-L gevolgd door een twee dagen durende periode met virus bevattend supernatant. Hierbij wordt gebruik gemaakt van een methode waarbij een humaan fibronectine fragment (CH-296) coating zorgt voor een efficiëntere transductie. De percentages EGFP na transductie met de gepseudotypeerde PG13/SF-EGFP cellijn waren hoger vergeleken met de amphotrope Am12/MFG-EGFP cellijn. Transplantatie van getransduceerde, $CD34^+$ geselecteerde navelstrengbloed cellen in NOD/SCID muizen resulteerde in percentages $EGFP^+$ cellen van 23% en 2%, respectievelijk.

Hoewel EGFP-getransduceerde cellen het BM van de NOD/SCID muizen repopuleren, bleek het noodzakelijk het transductie protocol te optimaliseren om zo een stabiele, klinisch relevante expressie van het getransduceerde gen te krijgen. Hoofdstuk 5 beschrijft ondermeer de relatie tussen transductie efficiëntie en cel concentratie (rhesus aap BM en navelstrengbloed cellen) bij gebruik van de PG13/SF-EGFP virus producerende cellijn. Subcloneren van deze cellijn resulteerde in subclones met een hogere virus titer die in staat waren de transductie percentages te verhogen naar 90%. Daarnaast bleek cryopreservatie van RhBM cellen de transductie efficiëntie te verhogen. Omdat uitsluitend transplantatie experimenten inzicht kunnen geven in de mate van transductie in repopulerende cellen, zijn getransduceerde voorlopercellen uit navelstrengbloed in NOD/SCID muizen getransplanteerd. Dit resulteerde in 2 tot 4-voudig hogere percentages van gerepopuleerde $EGFP^+$ cellen dan na transductie onder suboptimale condities.
Hematopoietische groeifactoren worden gebruikt om immature cellen te stimuleren en/of om BM of bloed afnames die te klein zijn voor transplantatie te expanderen. Het effect van diverse combinaties groeifactoren op de groei van BM en bloed in kweek is uitgebreid bestudeerd *in vitro*. Van bepaalde combinaties is het bijvoorbeeld bekend dat ze de differentiatie van de cellen stimuleren (combinaties met IL-3), het behoud en proliferatie van immature cellen echter, is moeilijker te realiseren. Recent gecloneerde groeifactoren als thrombopoietine (TPO) en fms-like tyrosine kinase3 ligand (Flt3-L), stimuleren de proliferatie van immature cellen in elk geval *in vitro*, waardoor deze groeifactoren veelbelovend zijn in de expansie van stam cellen. *In vivo* toediening van groeifactoren (bijvoorbeeld granulocyte colony-stimulating factor (G-CSF)) wordt vaak toegepast om stamcellen uit het beenmerg naar het perifere bloed te mobiliseren, waarna grote hoeveelheden eenvoudig kunnen worden verzameld. Expansie van hematopoietische stamcellen *in vivo* na transplantatie van gelimiteerde hoeveelheden beenmergcellen of gezuiverde stamcellen kan wellicht efficiënt gestimuleerd worden door middel van het toedienen van groeifactoren direct na transplantatie. Injectie van een enkele dosis rHuTPO direct na transplantatie van gezuiverde $CD34^+$ cellen uit navelstrengbloed in bestraalde NOD/SCID muizen resulteerde in een hoger percentage en absoluut celaantal van humane cellen in het muizenbeenmerg. (Hoofdstuk 6).

List of abbreviations

7-AAD	:	7-aminoactinomycin D
AAV	:	adeno-associated virus
ADA	:	adenosine deaminase
AML	:	acute myeloid leukemia
ALL	:	acute lymphoblastic leukemia
BFU-E	:	erythroid burst-forming unit
BMT	:	bone marrow transplantation
BSA	:	bovin serum albumin
CAFC	:	cobblestone area forming cell
CFU-S	:	spleen colony forming unit
CL_2MDP	:	di-chloromethylene di-phosphonate
CML	:	chronic myeloid leukemia
EGFP	:	enhanced green fluorescent protein
Epo	:	erythropoietin
FACS	:	fluorescent activated cell sorter
FCS	:	fetal calf serum
FISH	:	fluorescent in situ hybridisation
FITC	:	fluorescein iso thio cyanate
Flt3-L	:	fms-like tyrosin kinase-3 ligand
GALV	:	gibbon ape leukemia virus
GF	:	growth factor
GLVR-1	:	gibbon ape leukemia virus receptor
G(M)-CSF	:	granulocyte (macrophage)-colony stimulating factor
GM-CFU	:	granulocyte-macrophage colony forming unit
HBSS	:	Hanks'balanced salt solution
HGF	:	hemopoietic growth factor
HIV	:	human immunedeficiency virus
HPP-CFC	:	high proliferative potential colony-forming cell

IFN-α	:	interferon-α
IL	:	interleukin
LTBMC	:	long-term bone marrow cultures
LTC-IC	:	long-term culture initiating cells
LTR	:	long terminal repeat
LTRA	:	long-term repopulating ability
M-CSF	:	monocyte-colony stimulating factor
MESV	:	murine embryonic stem cell virus
MIP-1a	:	macrophage inflammatory protein-1a
MoMLV	:	Moloney murine leukemia virus
MRA	:	marrow-repopulating ability
NFD-milk	:	non-fat dry milk
NK-cell	:	natural killer cell
NOD/SCID	:	non-obese diabetic/severe combined immunodeficient
PCR	:	polymerase chain reaction
PDGF	:	platelet-derived growth factor
PE	:	phycoerythrin
Ph-chromosome	:	Philadelphia chromosome
PI	:	propidium iodide
Ram-1	:	receptor for murine retrovirus-1
SCF	:	stem cell factor
SCID	:	severe combined immunodeficient
SFFV	:	spleen focus forming virus
SPF	:	specific pathogen free
STRA	:	short term repopulating ability
TBI	:	total body irradiation
TPO	:	thrombopoietin
UCB	:	umbilical cord blood

Curriculum Vitae

Monique Maria Andrea Verstegen werd op 3 augustus 1970 geboren in Roermond. In 1987 haalde zij het H.A.V.O. examen aan de Rijks Scholen Gemeenschap in Roermond. In dat zelfde jaar begon ze met de Hogere Laboratorium Opleiding (H.L.O.) aan de Hogeschool Heerlen te Sittard, waar het propeadeutisch examen werd behaald in 1989. De H.L.O. opleiding werd voortgezet aan de Hogeschool Rotterdam en Omstreken te Delft met als afstudeerrichting Zoölogie/Medische Biologie. Van september 1991 tot mei 1992 was ze werkzaam als H.L.O. stagaire bij de vakgroep Celbiologie en Genetica van de Erasmus Universiteit Rotterdam, begeleid door Prof. dr J. F. Jongkind. Het onderwerp van deze afstudeerstage was: Bindingskarakteristieken van geïsoleerde monocyten aan humaan veneus navelstreng endotheel. Na het behalen van het ingenieurs diploma werd in 1992 de studie biologie (verkort, voltijd) voortgezet aan de Universiteit Utrecht. De afstudeerstage aldaar werd begeleid door Prof. dr D. J. van der Horst en dr J. H. B. Diederen van de vakgroep Experimentele Dierkunde, projectgroep Stofwisselingsfysiologie en het afstudeerverslag was getiteld : Formation and function of the ergastoplasmatic granules in the corpus cardiacum of the African migratory locust, *Locusta migratoria*. Ook de literatuurscriptie werd binnen deze projectgroep voltooid; Protein kinase C, a central enzyme in signal transduction. De studie biologie werd afgerond in mei 1995 en vanaf september 1995 was ze werkzaam als assistent in opleiding (A.I.O.) in het Instituut Hematologie van de Erasmus Universiteit Rotterdam onder leiding van dr G. Wagemaker. Nu werkt ze als postdoc in de projectgroep van dr G. Wagemaker.

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